Chapter 4

Establishment of an *in vitro* model of ATMs

Numerous studies have indicated that the majority of the macrophage population in AT, despite having pro-inflammatory characteristics, differs from the classical M1 subtype. Kratz *et al*., demonstrated that macrophages exposed to a combination of palmitate, glucose and insulin, conditions akin to metabolic syndrome, undergo a distinctive phenotypic transformation referred to as "metabolically activated" or "MMe" (Kratz et al., 2014). This MMe phenotype exhibits unique features, resembling characteristics observed in ATMs found in both mice and humans thereby extending beyond the traditional M1/M2 paradigm. Most of the studies uses animals or biopsies to understand the ATMs. This makes it difficult to fully understand the characteristics of ATMs. For this we require a simpler and reliable model. In our lab, we utilised THP-1, a monocytic cell line, to establish an *in vitro* model for MMe.

4.1 Morphological distinctions among M1, M2 and MMe

Elevated FFA (mostly palmitate) is found in AT during obesity. AT in obesity associated diabetes often presents with hyperglycemia and hyperinsulinemia. Thus, metabolic stimulations are important in defining the characters of ATMs. To study features of these ATMs, we used THP-1 cells to establish *in vitro* models for M1 (classically activated), M2 (homeostatic), and MMe. These were initially induced to transit into resting Macrophages (M0) by treating them with phorbol 12-myristate 13-acetate (PMA). Subsequently, these M0 cells were then stimulated with a combination of palmitate, insulin, and glucose to generate the MMe phenotype. For comparative purposes, we also generated M1 and M2 macrophages. M0 cells were subjected to stimulation with LPS and IFNγ to mimic the M1 phenotype, while IL4 was used to drive their differentiation into M2 macrophages.

Distinct morphological differences were observed among the three subtypes. MMe displayed a rounded appearance and exhibited a notable accumulation of lipid droplets within the cytoplasm, displacing the nucleus. It was validated by oil red-O staining (Fig 4.1 A). The accumulation of lipids in MMe (Fig 4.1 A) was also observed as increase in side scatter indicating increased granularity (Fig 4.1 A, E, G). In contrast, M1 macrophages showed no lipid accumulation but displayed cytoplasmic extensions. These were elongated and had irregular amoeboid shape (Fig 4.1 A). M2 macrophages, on the other hand, appeared flattened, expanded and presented very few small lipid droplets primarily at the periphery (Fig 4.1 A).

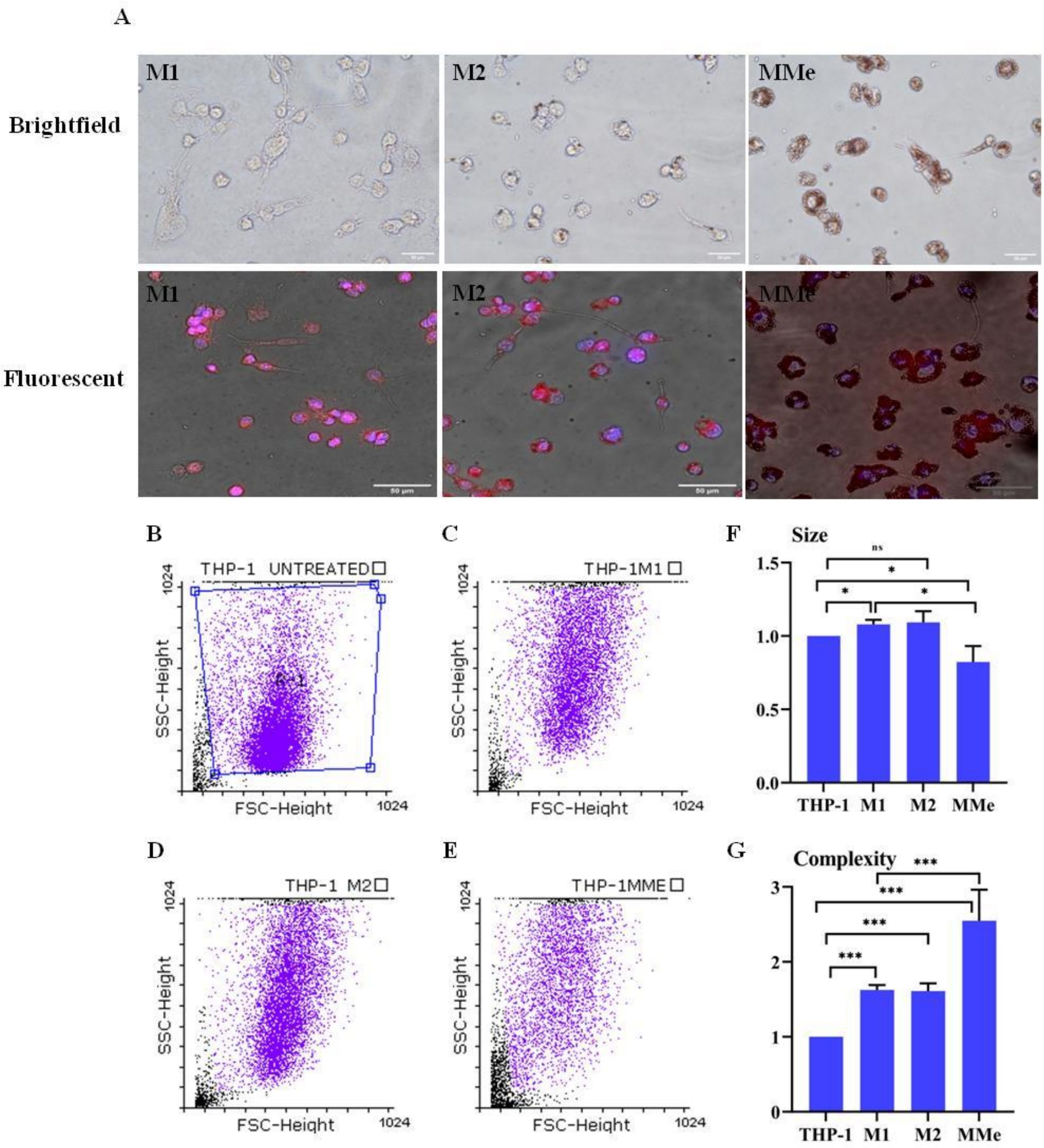

Figure 4.1 Morphological characterization of THP-1 derived macrophages differentiated into M1, M2 and MMe: (A) oil-red -o staining of M1, M2 and MMe macrophages visualised under brightfield and fluorescence microscopy. (Nuclear staining-DAPI, blue) Scale bar = 50 µm, (B-E) Flow cytometry scatter plots of side scatter (SSC) vs forward scatter for THP-1 (B), M1 (C), M2 (D) and MMe (E). The distribution of the cell population indicates differences in size and complexity among subtypes. (F-G) Quantitative analysis of FSC and SSC data representing relative cell size and complexity. Data represented as Mean±SD (n=3), $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

4.2 MMe displays differentiation markers similar to ATMs:

Zeyda *et al*. proposed that ATMs exhibit M2 characteristics by expressing markers like CD163 and MRC1 (Zeyda et al., 2007). However, these ATMs are highly capable of producing elevated levels of cytokines. In a similar vein, Kratz *et al*. demonstrated that ATMs do not express M1 markers such as CD38, CD274, and CD319, while ABCA1 and CD36 showed correlations with BMI (Kratz et al., 2014). We also looked at surface marker expressions. We found upregulation of CD38 in M1 macrophages (Fig 4.2 A). CD38, a robustly expressed marker for classically activated macrophages, promotes cytokine secretion (Amici et al., 2018). Additionally, CD319 was found to be upregulated in M1 macrophages (Fig 4.2 B). CD319, also known as SLAMF7, is a key molecule in inflammation regulated by IFNγ (Simmons et al., 2022). CD274, or PD-L1, was also increased in M1 in comparison to M2 and MMe (Fig 4.2 H). CD274 regulates inflammatory programs induced by ROS through NF-κb (Cao et al., 2024). Interestingly, CD319 and CD38 showed negligible expressions in MMe and M2.

Several research outcomes indicate that macrophages from adipose tissue of obese humans, which although are pro-inflammatory, are characterized by their high lipid content and the expression of CD9, CD36, PLIN2, ABCA1 and FABP4 (Jaitin et al., 2019; Kratz et al., 2014). Consistent with these studies, MMe demonstrates distinctive markers such as CD36, ABCA1, PLIN2 and FABP4 (Fig 4.2 D-F, J-K). CD36, a class B scavenger receptor expressed on various cell types, including macrophages, plays a role in chronic inflammation and contributes to the insulin resistance commonly observed in obesity and dyslipidemia (Kennedy et al., 2011). PLIN2 promotes the formation of lipid droplets, leading to lipid accumulation, and its over-expression has been observed in monocytes of children with overweight/obesity (Pisano et al., 2021). The ABCA1 transporter plays a crucial role in maintaining cellular cholesterol homeostasis by participating in reverse cholesterol transport. FABP4 in macrophages promote IL1β (Yang et al., 2023). Therefore, these MMe markers indicate signatures associated with lipid metabolism. These results may be linked to the release of free fatty acids, among other factors, from dead adipocytes, potentially playing a key role in promoting these molecules and the inflammation marked in ATMs during obesity.

Additionally, CD209 has been identified as an M2 macrophage marker. CD209, also known as DC-SIGN (Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing

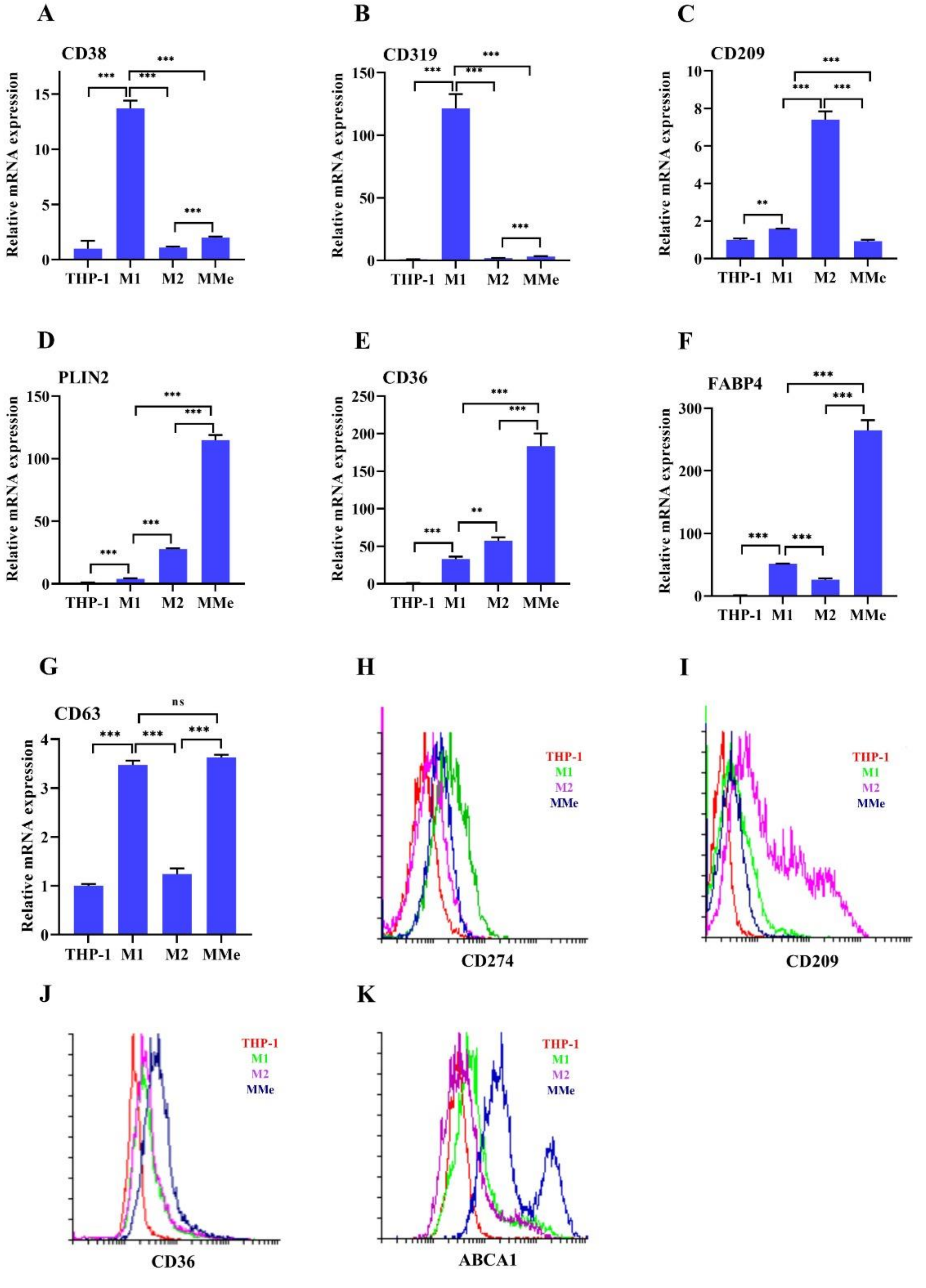

Figure 4.2 Differential expression of markers in THP-1-derived macrophages differentiated into M1, M2, and MMe subtypes: (A-G) Relative mRNA expression levels of key surface markers and lipid-related genes in THP-1 monocytes, M1 macrophages, M2 macrophages, and MMe macrophages. Markers analyzed include CD38 (A), CD319 (B),

CD209 (C), PLIN2 (D), CD36 (E), FABP4 (F), and CD63 (G). Data are normalized to untreated THP-1 monocytes and presented as Mean±SD (n=3), *p<0.05, **p<0.01, *******p<0.001 (H-K) Flow cytometry histograms illustrating protein expression of CD274 (H), CD209 (I), CD36 (J), and ABCA1 (K) across THP-1 monocytes and macrophage subtypes. Each colour represents a specific cell population: red (THP-1), green (M1), purple (M2), and blue (MMe). Histograms depict fluorescence intensity profiles of each markers for macrophage subtypes.

Non-integrin), is a C-type lectin receptor present on the surface of diverse immune cells, such as macrophages and dendritic cells. Its expression is primarily induced by IL-4 and is downregulated by LPS and IFN-γ (Relloso et al., 2002). CD209 is highly expressed in M2 (Fig 4.2 C, I). We observed negligible expressions of CD209 in M1 and MMe macrophages, making them distinguishing from M2 macrophages.

CD63 is also upregulated in M1 and MMe equally (Fig 4.3 G). CD63 belongs to tetraspanin family of proteins and is a marker of macrophage activation and differentiation. CD63 is upregulated in inflammatory macrophages and important in exosome release. This coordinates with autophagy and promotes macrophage recruitment (Xia et al., 2022).

4.3 Distinct inflammatory pattern in MMe

It is widely recognized that M1 macrophages, release proinflammatory cytokines, while in contrast, M2 macrophages, generate anti-inflammatory cytokines. However, metabolic stress and dysfunction in adipose tissue establish a close connection between obesity and inflammation. Importantly, macrophages associated with adipose tissue in obese individuals serve as a significant source of inflammatory mediators. Our observations revealed that both M1 macrophages and MMe exhibit increased expressions of pro-inflammatory cytokines, including TNFα, IL1β, IFNγ, and IL6 albeit at varying levels (Fig 4.3 A, B, C,D). Further, the secreted levels of TNFα are equally well coordinated with its expressions (Fig 4.3 E). We see that STAT1 is highly upregulated in M1, while at lower levels in MMe (Fig 4.3 F). STAT1 phosphorylation is important for pro-inflammatory response (Kovarik, 1998). Palmitic acid, akin to LPS, is thought to trigger a TLR4 response, activating NF-κB and leading to inflammation (Shi et al., 2006). However, Lancaster *et al.*, 2018, proposed that palmitate is not an agonist for TLR4 but rather primes and alters gene expressions and metabolism, contributing to inflammation (Lancaster et al., 2018). Consequently, MMe demonstrates a distinctive inflammatory pattern separate from that of M1. It clearly indicates that MMe are associated with low grade chronic inflammation while M1 exhibit heightened inflammation.

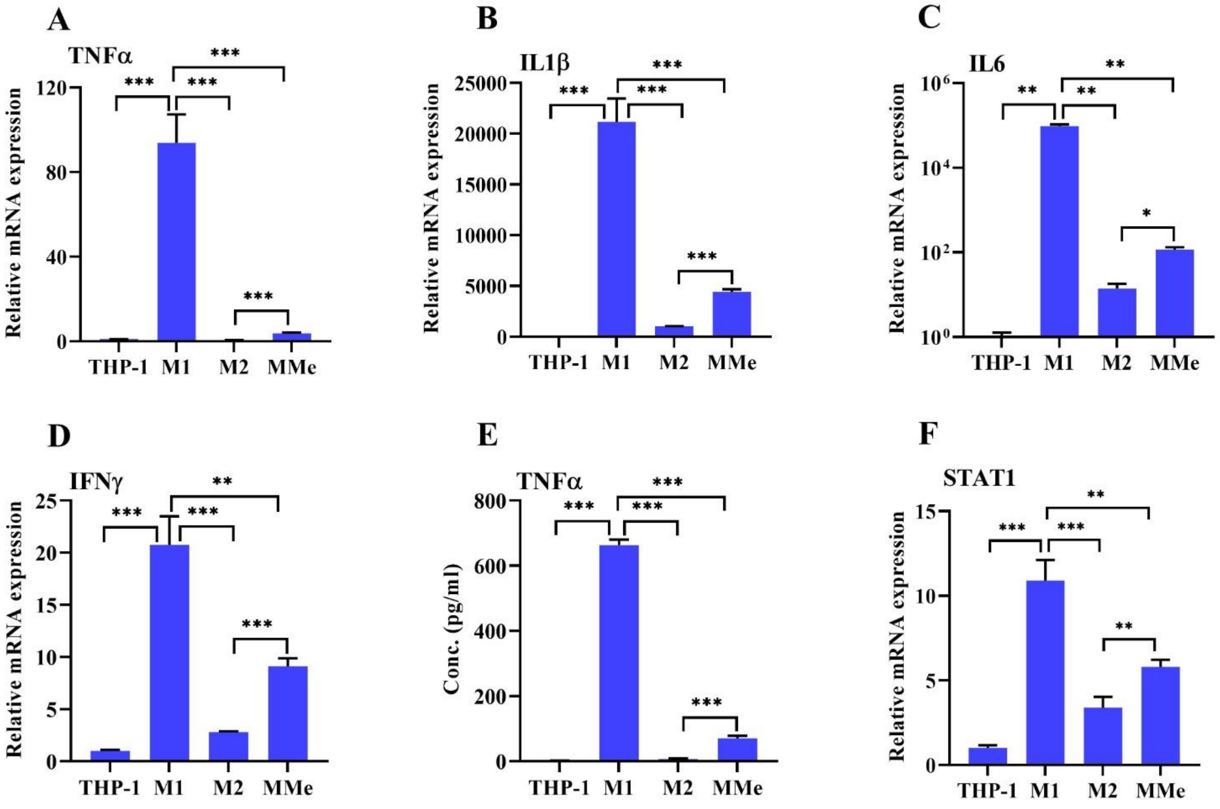

Figure 4.3 Inflammatory markers in THP-1 derived macrophages polarized into M1, M2 and MMe: (A-D, F) Relative mRNA expression levels of pro-inflammatory cytokines TNFα (A), IL1β (B), IL6 (C), IFNγ (D), and transcription factor STAT1 (F) across THP-1 monocytes, M1 macrophages, M2 macrophages, and MMe macrophages. Data are normalized to untreated THP-1 monocytes. TNFα secretion measured in the supernatants of differentiated THP-1 monocytes (THP-1, M1, M2, and MMe) by ELISA (E). Cytokine concentration is expressed in pg/mL. Data presented as Mean±SD (n=3), $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

4.4 MMe exhibit ER stress due to metabolic activation

Nutritional abundance induces alterations in AT homeostasis, marked by a chronic low-grade inflammatory state. This condition triggers ER dysfunction, promoting the unfolded protein response (UPR). ER stress markers induced in human AT during obesity have been found to be associated with BMI (Sharma et al., 2008). ER stress is intricately linked with inflammation, forming a bidirectional relationship. ER stress contribute to the inflammatory response through NF-κB and JNK signaling pathways. XBP1 activation via the IRE1α pathway regulates the expression of cytokines (S. Kim et al., 2015).

In our study of macrophage subtypes, we also looked at the expressions of ER stress markers. Our findings highlight a significant association between inflammation and ER stress. It was noted that both M1 and MMe exhibited elevated expressions of ER stress markers, whereas these markers were absent in M2 macrophages. Intriguingly, MMe demonstrated a higher expression of XBP1s compared to M1, suggesting its involvement in a prolonged adaptive phase (Fig 4.4 A). Conversely, M1 displayed increased expressions of CHOP, GADD34, ATF4, and ATF3 when compared to MMe (Fig 4.4 B,C).

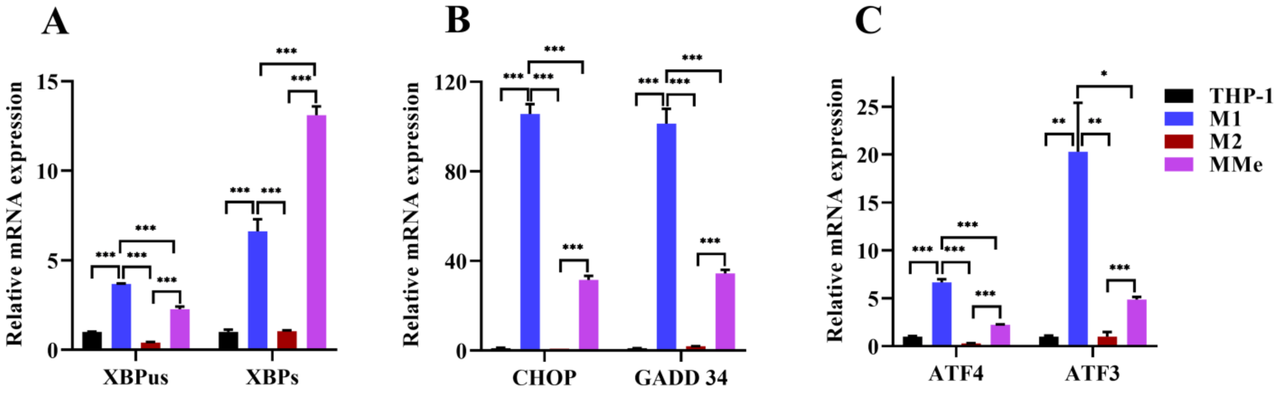

Figure 4.4 ER Stress in macrophages: Relative mRNA expressions of ER stress markers (A) XBPus, XBPs (B) CHOP, GADD34 (C) ATF4 and ATF3. Data are normalized to untreated THP-1 monocytes and presented as Mean±SD (n=3), *p<0.05, **p<0.01, ***p<0.001.

These markers are indicative of pathways leading to apoptosis under conditions of persistent ER stress (Park et al., 2021). The distinct ER stress marker profiles observed in MMe cells compared to M1 cells strongly indicate that MMe cells exhibit a unique signature of ER stress. This observation emphasizes the diverse and complex nature of ER stress responses in different macrophage subtypes, shedding light on potential implications for their functions in inflammatory and adaptive processes.

4.5 Nutritional stress activates autophagy in MMe

Lipotoxic stress alters autophagy by blocking the degradation of autophagic cargo through several distinct mechanisms (Namkoong et al., 2018). We noted increase in expression of LAMP1 in MMe and LAMP2 is non-significant increase in M1 and MMe cells. This indicates lysosomal biogenesis in MMe as a result of lipid stress (Fig 4.5 A, B). Additionally, we observed upregulation of key autophagy markers ATG5 and ATG7 in M1 and MMe (Fig 4.5 C, D). Accumulation of LC3-II and p62 is observed in MMe (Fig 4.5 E, F). These changes suggest that while the biogenesis of lysosomes may be enhanced, their late processing seems to be impaired in MMe cells. More experiments would be required to understand the changes in autophagic pathway clearly.

Discussion:

Macrophage recruitment is associated with chronic low-grade inflammation, termed metainflammation, which is characterized by the upregulation of pro-inflammatory molecules in adipose tissue. This amplifies inflammation, leading to metabolic dysregulation (Hotamisligil, 2017; Lumeng & Saltiel, 2011). In our THP-1 based *in vitro* model, we also observed that MMe accumulated lipids droplets and have a different inflammatory cytokine profile distinguishing it from M1 and M2. Further, MMe also expresses genes related to fatty acid metabolism CD36, ABCA1, FABP4 and PLIN2 on their surface. The crosstalk between ER stress and inflammation has significant implications for metabolic health. In obesity, for instance, excess nutrient intake can lead to ER stress in AT, promoting the release of proinflammatory cytokines and aggravating IR (Gregor & Hotamisligil, 2007). Similarly, metabolic activation of IRE1α suppresses M2 polarization. Myeloid-specific ablation of IRE1α increases energy expenditure, improves metabolic dysfunction of brown adipose tissue, and corrects the M1-M2 imbalance in adipose tissue (Shan et al., 2017).

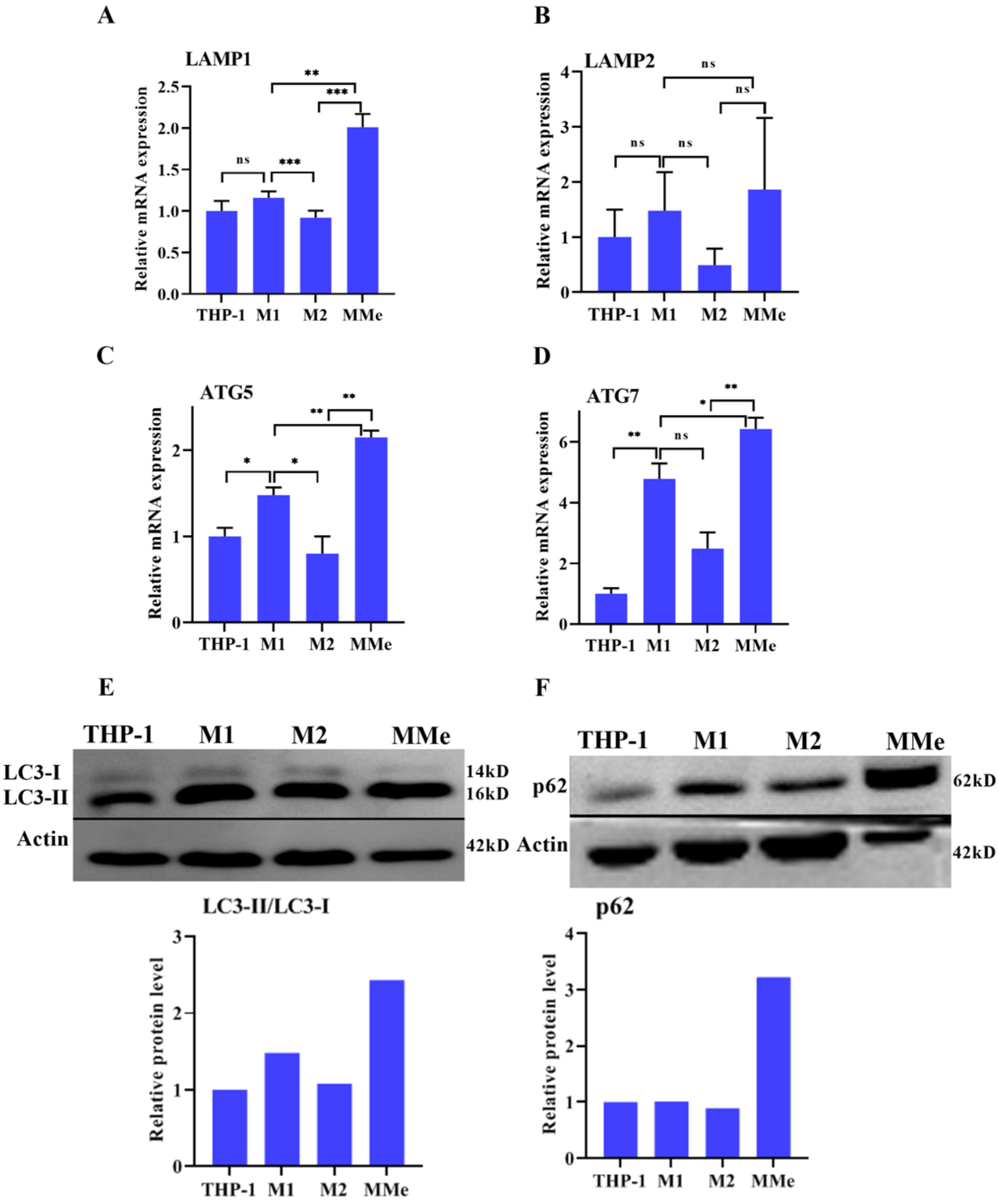

Figure 4.5 Autophagy-related gene and protein expression in THP-1-derived macrophage subtypes: (A-D) Relative mRNA expression levels of autophagy-related genes LAMP1 (A), LAMP2 (B), ATG5 (C), and ATG7 (D) across THP-1 monocytes, M1, M2, and MMe macrophages. Data are normalized to untreated THP-1 monocytes. Immunoblot analysis of LC3-I and LC3-II protein levels (E) and p62 (F) in THP-1, M1, M2, and MMe macrophages. Actin was used as a loading control. Data presented as Mean±SD (n=3), *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

Lipid accumulation alters lysosomal activity in ATMs and modulates lipid flux in AT (Xu et al., 2013). We observed that MMe exhibited lysosomal biogenesis and activation of autophagy. LC3 and p62 accumulation suggest that the autophagic flux might be impaired at the later stage as a result of prolonged stress. Although this should be confirmed by inhibiting the flux. Other than this, markers for the lysosomal metabolism should be tracked to fully understand the role of lysosomes in MMe phenotype.