Chapter 5

Macrophage polarization and metabolism

Macrophages, essential players in the immune system, exhibit remarkable plasticity and can adopt distinct functional states in response to signals from microenvironment. The two well-characterized polarization states of macrophages M1 and M2, intricately linked with specific metabolic profiles, reflect the dynamic adaptation of macrophages to different physiological demands. The intricate connection between macrophage polarization and metabolism underscores the versatility of these immune cells. However, ATMs display a distinct metabolic activation characterized by an augmentation in OXPHOS and glycolysis (Boutens et al., 2018). This metabolic signature aligns with similar observations in macrophages isolated from the adipose tissue of obese subjects with type 2 diabetes. Understanding these relationships provides insights into the regulation of immune responses and opens avenues for therapeutic interventions in diseases characterized by macrophage dysfunction.

5.1 Metabolic characters of MMe:

The processes of macrophage polarization and metabolism are intricately connected (Jha et al., 2015). In response to inflammatory signals such as LPS and IFNγ, the generation of M1 macrophages induces a metabolic shift that favours glycolysis and fatty acid synthesis while compromising fatty acid oxidation to supports the production of pro-inflammatory cytokines and ROS (Lee et al., 2019). In contrast, IL4-stimulated macrophages, known as M2, predominantly rely on oxidative phosphorylation and FAO. IL4 activation leads to the stimulation of STAT6 and PGC1β, facilitating the process of FAO in M2 macrophages (Biswas, 2015). Xu *et al*. (2013) observed lipid droplet accumulation, enhanced lysosomal biogenesis, and increased Lipa (lipase A) activity in mice ATMs along with expressions of Lamp2, CD36, and Plin2, emphasizing the involvement of lipid-related proteins in ATM functions (Xu et al., 2013). Likewise, investigations on obese humans demonstrated elevated expressions of PPARγ, an important regulator of lipid metabolism, in ATMs (Jaitin et al., 2019; Kratz et al., 2014; Vijay et al., 2020). These observations collectively highlight the intricate involvement of lipid-related processes in the functions of ATMs in both murine and human adipose tissue.

In our study, we assessed various metabolic markers to understand the metabolic state of macrophage subpopulations. We observed an increase in the expression of glycolytic genes such as PFKFB3, PKM2 and PDK1 upon stimulation with LPS and IFNγ (Fig 5.1 A, B, C). IFNγ influences glycolysis by inducing the expression of the glycolytic activator PFKFB3, needed for antiviral activity. PFKFB3-mediated glycolysis, promotes M1 polarization via

JAK2/STAT1 signalling pathway (Chen et al., 2022). Knocking down PDK1 results in reduced aerobic glycolysis and, consequently, a dampened inflammatory response while an increase in mitochondrial respiration in M1 macrophages (Tan et al., 2015). PDK1 is important in maintaining M1 state. Inhibiting PKM2 increases IL10 production and thus affects cytokine production, and direct M2 polarization (Na et al., 2020).

Additionally, M1 macrophages exhibited increased expression of GLUT1 (Fig 5.1 D), a glucose transporter crucial for glucose uptake, a process essential for maintaining an inflammatory state (Kelly & O'Neill, 2015). Furthermore, Fatty Acid Synthase (FAS) was found to be upregulated in M1 macrophages (Fig 5.1 E). This observation aligns with its role in supporting prostaglandin synthesis through citrate accumulation (Palmieri et al., 2015; Williams & O'Neill, 2018). These findings shed light on the metabolic adaptations occurring in M1 macrophages, emphasizing their reliance on glycolysis and fatty acid synthesis to sustain an inflammatory phenotype. M2 macrophages exhibit a preference for lipid metabolism, as indicated by the increased expressions of PPARγ and PGC1α (Fig 5.1 F and G). In contrast, MMe display lipid droplet accumulation in their cytoplasm and increased transcription of PPARγ and PGC1α compared to M2, suggesting an elevated engagement in lipid metabolism. Furthermore, in comparison to M2, MMe displays elevated expression levels of PDK1 and PKM2 (Fig 5.1 B and C). Although the levels of glycolytic markers are lower than M1. This suggests that MMe not only tends towards oxidative phosphorylation (OXPHOS) but also maintains persistent glycolysis, even in the presence of lipid accumulation. MMe macrophages demonstrated elevated transcript levels of ACACA1 and Acox1 (Fig 5.1 H and I) indicating a potential augmentation in peroxisomal fatty acid metabolism in MMe. Evidence suggests an upregulation in peroxisomal fatty acid oxidation (in macrophages) during obesity, highlighting its potential role in addressing altered lipid homeostasis characteristic of this condition (Reddy & Mannaerts, 1994). The intricate link between peroxisomal activity and lipid metabolism is important, in obesity-associated inflammation that potentially influences peroxisomal function (Schrader & Fahimi, 2008). As a response to nutrient excess, common in obesity, peroxisomes may undergo adaptive changes to maintain cellular homeostasis (Lodhi & Semenkovich, 2014). Dysregulation of peroxisomal activity has implications for metabolic disorders associated with obesity, such as insulin resistance (Novikoff & Novikoff, 1982).

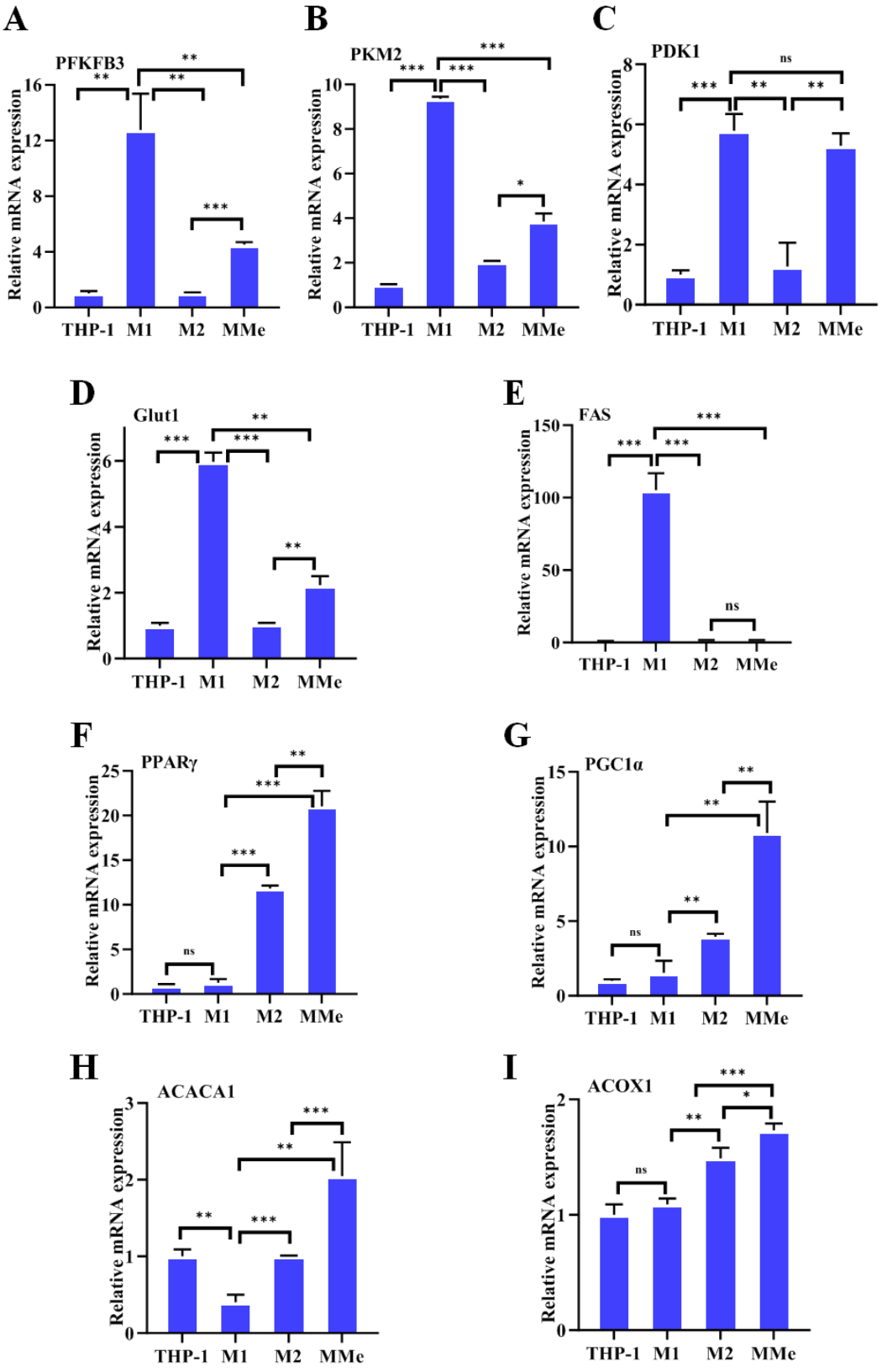

Figure 5.1 Differential expressions of metabolic pathway markers in THP-1-derived macrophage subtypes: (A-D) Relative mRNA expression of glycolytic markers: PFKFB3 (A), PKM2 (B), PDK1(C) and Glut1 (D) were analyzed in THP-1 monocytes, M1

macrophages, M2 macrophages, and MMe macrophages. (E-G) Fatty acid (FA) metabolism genes: mRNA expression of FAS (E), PPARγ (F) and PGC1α (G). (I-J) Peroxisomal metabolism genes: mRNA levels of ACACA1 (I) and ACOX1 (J). Data are normalized to untreated THP-1 monocytes and presented as Mean±SD (n=3), *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

5.2 Metabolic interventions affect macrophage polarization

Interfering with cellular metabolism has been shown to influence the differentiation of macrophages. Numerous studies have emphasized the impact of LPS/IFNγ stimulation on metabolic reprogramming, directing macrophage metabolism towards glycolysis (Mills et al., 2016; F. Wang et al., 2018). Notably, inhibition of glycolysis with 2-deoxy-D glucose (2-DG) has been shown to diminish IL-1β production through the reduction of STAT1 phosphorylation, concurrently affecting ATP production (F. Wang et al., 2018). Similarly, investigations have also highlighted the crucial role of glycolysis in M2 activation (S. C.-C. Huang et al., 2016). Manipulating metabolic pathways in ATMs of obese mice has been demonstrated to influence cytokine release (Boutens et al., 2018).

5.2.1 Glycolysis Inhibition: In our study, we aimed to understand the impact of metabolic interventions on the characteristics of MMe. We first inhibited the glucose metabolism by 2-DG during differentiation. 2-DG inhibited cytokine production in M1 as inferred from a significant reduction in TNFα and IL1β expressions (Fig 5.2 A). Remarkably, MMe exhibited an upregulation of inflammatory markers upon 2-DG treatment, suggesting a distinctive response compared to M1 (Fig 5.2 B). Furthermore, the anti-inflammatory characteristics of M2 were augmented by 2-DG treatment as indicated by further reduction in TNFα and IL1β expressions which were anyway much lower compared to M1 and MMe (Fig 5.2 C).

Lipid metabolism: Similar to glucose metabolism lipid metabolism also impacts the macrophage phenotype. The crucial role of lipogenesis in the synthesis of inflammatory molecules in M1 macrophages indicates the significance of metabolic pathways in immune responses. Lipid biosynthesis, regulated by FAS, is key in mediating inflammation. In M1 macrophages, upregulation of glycolysis contributes to the generation of acetyl CoA from citrate through the tricarboxylic acid (TCA) cycle, providing a substrate for lipid

biosynthesis. In our study, interference with glycolysis was found to have a notable impact on FAS levels across different macrophage subtypes M1 and MMe but not in M2 (Fig 5.2 A, B, C).

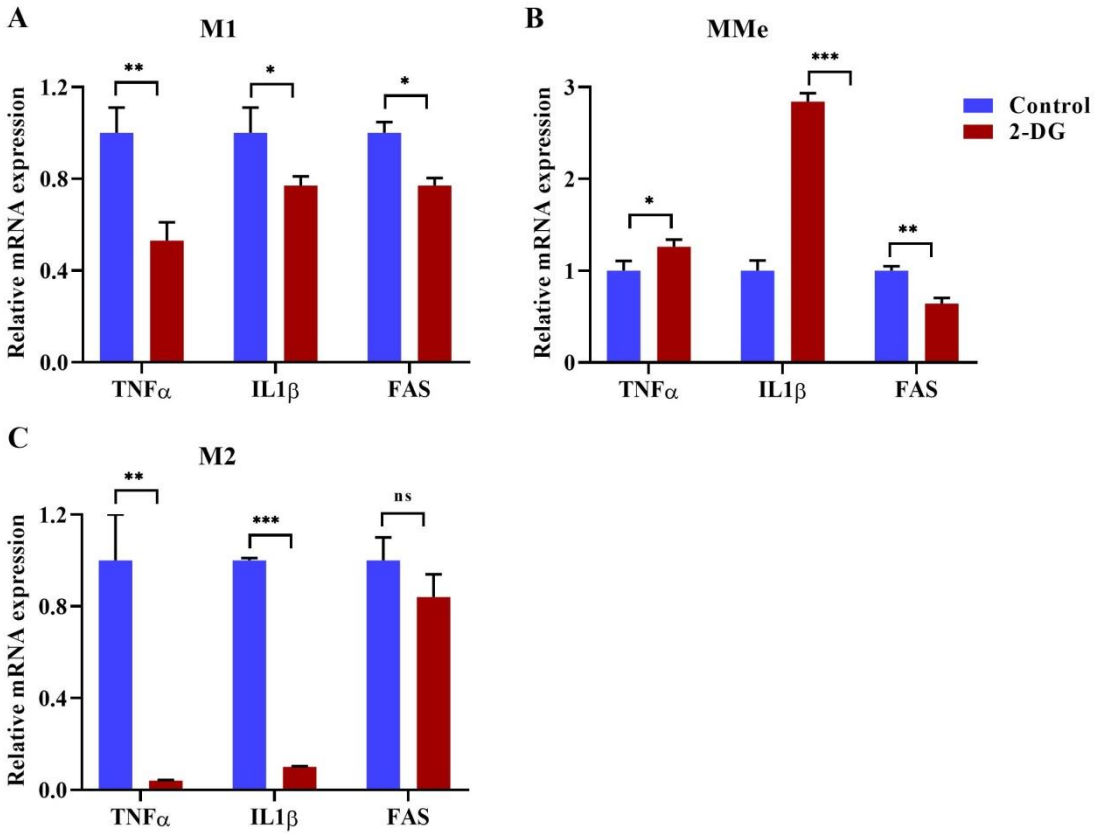

Figure 5.2 Effect of glycolytic inhibition using 2-deoxy-D-glucose (2-DG) on macrophage polarization: (A-C) Relative mRNA expression of TNFα, IL1β, and FAS in (A) M1 macrophages, (B) MMe macrophages, and (C) M2 macrophages following 24 hours treatment with 2-DG. THP-1 monocyte-derived macrophages were polarized into M1, MMe, and M2 subtypes, and glycolysis was inhibited using 25 mM 2-DG for M1 and M2 macrophages, and 30 mM 2-DG for MMe macrophages. 2-DG treatment was performed alongside specific stimulations for 24 hours. Data represented Mean±SD (n=3), *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

5.2.2 FAO Inhibition: The reliance on FAO for macrophage activation is critical for their function. Unlike M1 macrophages, which require both glycolysis and fatty acid synthesis, M2 macrophages shift their metabolism towards OXPHOS through an intact TCA cycle and

enhanced FAO. In a study, Feilong Wang *et al.*(2018), have proposed that glycolysis is not essential for M2 polarization as long as OXPHOS remains intact, while disrupting OXPHOS can impact M2 differentiation (Wang et al., 2018). However, other research suggests that FAO is dispensable for M2 polarization (Namgaladze & Brüne, 2014). In our investigation into the importance of fatty acid metabolism in MMe, we employed etomoxir, an inhibitor of Cpt1α responsible for controlling the entry of fatty acids into mitochondria. Treatment with etomoxir resulted in reduced expressions of TNFα and IL1β in M1 macrophages (Fig 5.3 A). Interestingly, in MMe, etomoxir selectively affected IL1β, while TNFα mRNA levels remained unaffected (Fig 5.3 B). Similarly, in M2, only TNFα levels were reduced little, while IL1β remain unaffected (Fig 5.3 C). Further we observed the significant increase in CD36 among all macrophage subtypes (Fig 5.3 A, B & C). Macrophages derived from CPT1a and CPT2 M-KO mice have increased expression of the CD36 scavenger receptor and impaired FAO (Nomura et al., 2019). These findings indicate the subtle regulation of fatty acid metabolism in different macrophage polarization states.

5.2.3 PPARγ Inhibition: PPARs (Peroxisome Proliferator-Activated Receptors) play a crucial role in transcriptionally regulating macrophage activation, extending beyond their well-established function in adipogenesis and fatty acid storage in adipocytes. While PPARγ is primarily associated with adipogenesis, it also holds significance in macrophage activation and function (Rosen et al., 1999). Pharmacological activation of PPARγ has been shown to mitigate inflammatory programs in macrophages, promoting an M2 phenotype with anti-inflammatory properties. PPARγ influences lipid uptake and mitochondrial biogenesis through transcriptional control of target genes, such as PGC1α. Deletion of PPARγ in macrophages impairs their ability to induce oxidative metabolism (Yu et al., 2023).

In our study, we explored the role of PPARγ in ATMs using an *in vitro* MMe model. We employed the PPARγ antagonist GW9662 (Seargent et al., 2004). TNFα levels were significantly reduced in both M1 and MMe, despite PPARγ's known role in suppressing inflammation. Although, a significant increase in TNFα expression was observed in M2 macrophages (Fig 5.4 A). For confirmation, we kept dexamethasone as a control which is known for its anti-inflammatory property (Fig 5.4 B). However, PPARγ antagonists modulate expression of inflammatory genes through PPARγ-independent mechanisms.

PPARγ inhibition by GW9662 promotes M2c like differentiation which secretes low amounts of TNFα and IL10 via MerTK/Gas6 axis (Zizzo & Cohen, 2015).

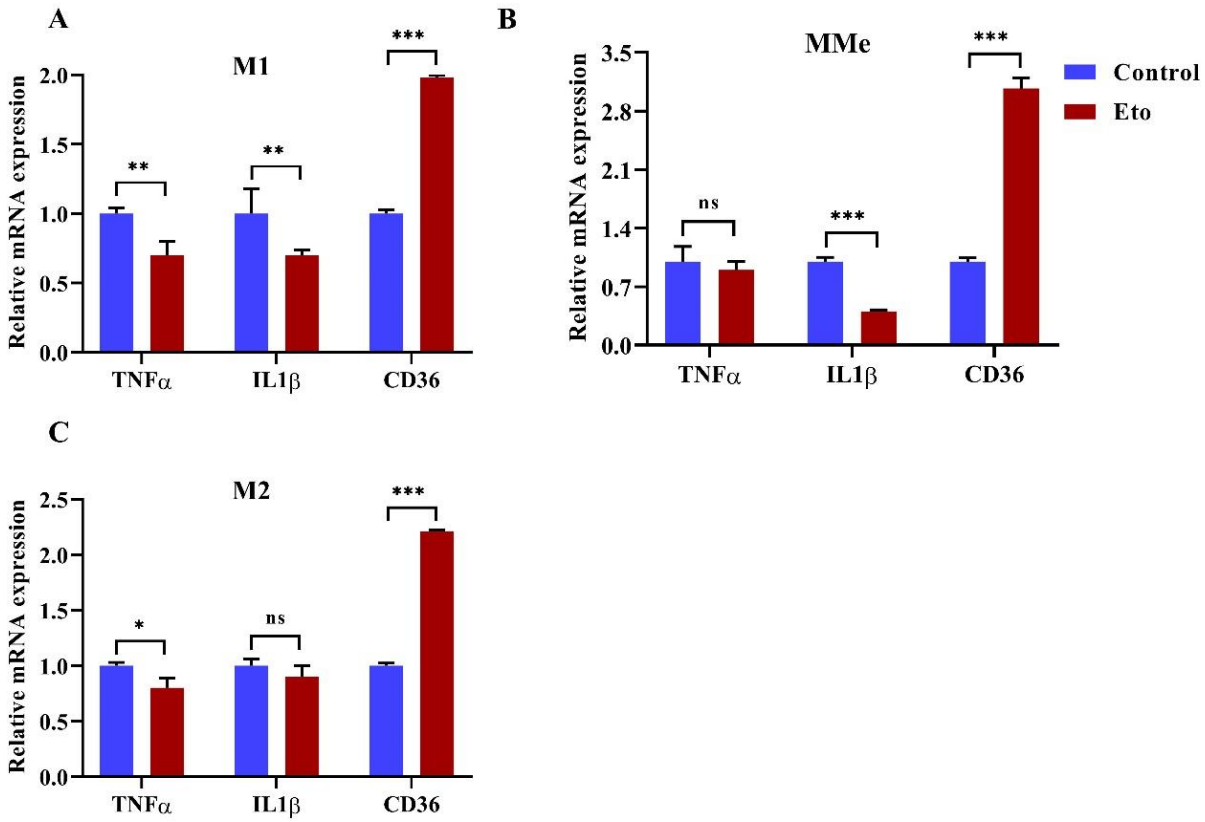

Figure 5.3 Effect of fatty acid (FA) uptake inhibition using Etomoxir (Eto) on macrophage polarization: (A-C) Relative mRNA expression of TNFα, IL1β, and CD36 in (A) M1 macrophages, (B) MMe macrophages, and (C**)** M2 macrophages following 24 hours treatment with Eto. THP-1 monocyte-derived macrophages were polarized into M1, MMe, and M2 subtypes, and FA uptake was inhibited using 100 μM Eto alongside specific stimulations for 24 hours. Data represented Mean±SD (n=3), *p<0.05, **p<0.01, ***p<0.001, ns= not significant

Moreover, PPARγ upregulates CD36 expression, facilitating lipid accumulation, and increases ABCA1 expression, which is pertinent in diseases associated with lipid-filled macrophages. We observed that inhibiting PPARγ using GW9662 led to increased levels of CD36 and PLIN2 in M1, M2, except MMe macrophages (Fig 5.4 C, D and E). In MMe,

PLIN2 expression was significantly increased, while CD36 remained unchanged, indicating a partial modulation of lipid metabolism. Notably, previous studies have demonstrated that GW9662 upregulates PLIN2 and CD36 expressions in a dose-dependent manner, promoting lipogenesis in macrophages by activating PPARδ (Schubert et al., 2020). PPARδ promotes STAT6 while suppresses inflammation (Adhikary et al., 2015). Activation of PPARδ may be the reason for downregulation of inflammatory cytokines even when PPARγ is inhibited. Overall, PPARγ inhibition is suppressing M1 type phenotype. Moreover, further studies would be important to confirm this.

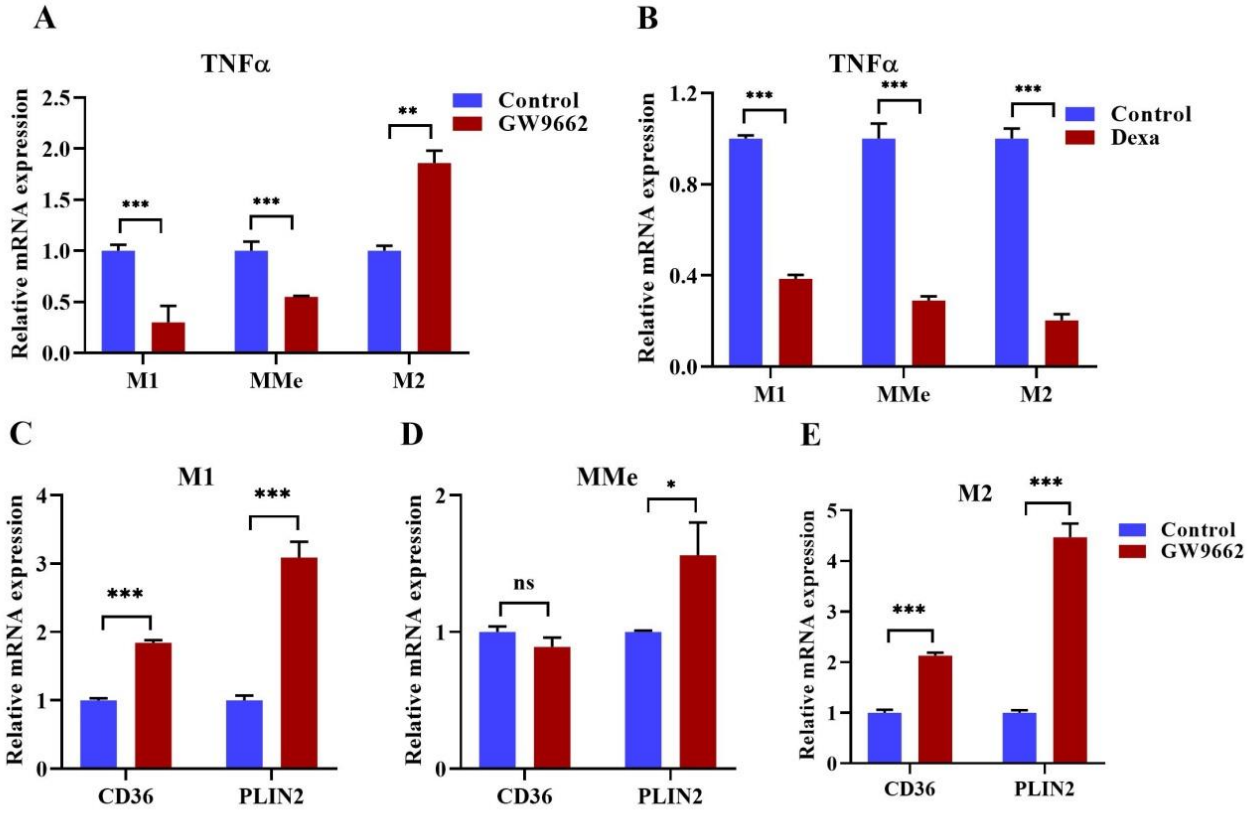

Figure 5.4 Effect of PPARγ inhibition using GW9662 on macrophage differentiation: (A) Relative mRNA expressions of TNFα in M1, M2 and MMe with and without GW9662, (B)Relative mRNA expressions of TNFα in M1, M2 and MMe with and without Dexamethasone, (C-E) Relative mRNA expressions of lipid metabolism gene CD36 and PLIN2 in M1 (C), M2 (D) and MMe (E). THP-1 monocyte-derived macrophages were polarized into M1, MMe, and M2 subtypes. Cells were treated with GW9662 (10 μM) or dexamethasone (10 μM) (Dexa, control) along with respective stimulations. Data represented Mean±SD (n=3), *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

Discussion:

Metabolic rewiring highlights the adaptation of macrophages to different environmental cues and their functional roles in immune response. With well-known binary classification, M1 and M2 is tightly linked to their specific metabolic profiles. M1 macrophages are often associated with increased glycolysis, which provides energy for the production of pro-inflammatory cytokines. M2 macrophages tend to rely on FAO and OXPHOS. LPS stimulation induces GLUT1 which further promotes increased glucose utilisation as well as inflammation (Freemerman et al., 2014). We observed that M1 macrophages demonstrated upregulation of GLUT1 as well as key glycolytic markers PFKFB3, PKM2 and PDK1, while M2 were skewed towards FAO. On the other hand, MMe demonstrated both glycolytic and oxidative capacity along with lipid accumulation. Macrophages in adipose tissue from obese mice exhibit distinctive gene pathways associated with both glycolysis and oxidative phosphorylation, distinct from LPS-activated macrophages in other contexts (Boutens et al., 2018).

As mentioned above, manipulating metabolism can significantly influence macrophage polarization, shaping their functional characteristics and immune responses. M1 macrophages, associated with upregulated glycolysis, demonstrate a regulation for producing pro-inflammatory cytokines. Inhibition of glycolysis using 2DG has been proven to diminish M1 polarization and the production of pro-inflammatory cytokines, such as IL1β (Tannahill et al., 2013). We observed that 2DG reduced the inflammation in M1 while increased in MMe. Dietary administration of 2DG has been shown to reduce inflammation and oxidative stress induced by endotoxemia (Pandey et al., 2023). On the other hand, persistent elevation of glucose uptake by adipose tissue, results in increased lactate production, has been observed to improve insulin sensitivity in mice, instead of promoting obesity (Muñoz et al., 2010). Further, we observed that inhibiting the fatty acid uptake also reduced the inflammation in MMe. Similarly, inhibiting PPARγ resulted in elevated levels of CD36 and PLIN2. GW9662 promotes lipogenesis in macrophages by activating PPARδ (Schubert et al., 2020). The PPARδ plays a selective role in promoting lipid accumulation in macrophages and regulates lipid uptake through the CD36 receptor. In contrast, PPARγ is known to be involved not only in lipid uptake but also in lipid efflux processes (Chawla et al., 2001). Unlike M1, inflammation in MMe is controlled by lipid uptake. It suggests that signals or stimulations are critical in determining the metabolic shift and thus modulating macrophage phenotype.