Chapter 8

Discussion and Conclusion

Discussion

ATMs are pivotal players in the regulation of AT homeostasis and metabolism. ATMs exhibit heterogeneity influenced by the local microenvironment, metabolic state, and disease conditions. In a lean state, ATMs are primarily derived from bone marrow and are involved in maintaining tissue homeostasis and remodelling (Cho et al., 2007). During obesity, the proportion of ATMs increases in both mouse and human adipose tissue (Harman-Boehm et al., 2007; Weisberg et al., 2003). These are monocytes derived macrophages forms CLS around dead adipocytes (Cinti et al., 2005). These ATMs mediates chronic inflammation associated with obesity. Furthermore, "metabolic activation" conditions, similar to metabolic dysfunction, closely mimic the characteristics of ATMs *in vivo*. This represents a distinct subpopulation "**MMe"** that extends beyond the well-known M1/M2 paradigm (Kratz et al., 2014).

We established an *in vitro* model of MMe using THP-1 cell line. Firstly, we observed that MMe accumulates Lipid droplets within its cytoplasm. Several studies have reported the increased lipid content in ATMs (Lumeng, DeYoung, Bodzin, et al., 2007; Xu et al., 2013) and MMe (Kratz et al., 2014).

We observed that our THP-1 derived MMe similar to Peripheral blood mononuclear cell (PBMC) derived MMe (Kratz et al., 2014), express CD36, PLIN2 and ABCA1 on its surface and can easily be distinguished from M1 and M2. MMe also exhibited FABP4 expressions. It is in line with a single cell RNA sequencing of ATMs in obese individuals show an enrichment of genes related to lipid metabolism that included CD36, Lipa, FABP4, and Lpl (Jaitin et al., 2019). On the other hand, M1 expressed CD319, CD38 and CD274. These markers are induced in human macrophages on LPS or IFNγ stimulation and promotes inflammation and glycolytic activity (Amici et al., 2018; Lai et al., 2021; Simmons et al., 2022). M2 macrophages exclusively overexpress CD209, in comparison to M1 and MMe. IL4 treatment to monocytes induces expressions of CD209 through STAT6 while IFNα, IFNγ and TGFβ negatively regulate CD209 expressions (Relloso et al., 2002).

Also, MMe were able to produce proinflammatory cytokines (TNFα, IL1β, IL6 and IFNγ), although to a lesser extent than M1, while M2 did not show upregulation of these genes. Several single-cell RNA sequencing studies have yielded additional insights into ATMs features. Macrophages residing in CLS within human adipose tissue are described as Lipid-

Associated Macrophages (LAMs), which are characterized by lipid droplets in their cytoplasma and pro-inflammatory cytokines secretion (Hill et al., 2018). FFAs released from the dead adipocytes are known to promote inflammation by binding to TLR4 and TLR2 and further inducing NF-κB pathway (Nguyen et al., 2007). In M1 macrophages, inflammation is prominently due to LPS and IFNγ stimulation, mimicking infection. This is also revealed in the enrichment analysis of M1 protein in mass spectrometer data. Major upregulated pathways involve the IFNγ signalling and other inflammatory pathways. Some of the exclusive proteins expressed are TLR inducible genes like STAT1, CXCL1, IRF5 (chapter 7). On the contrary, MMe showed expressions of CCL19 and CCL20 (chapter 7). CCL19 levels are upregulated during obesity in mice and humans (Hayashi et al., 2021; Kochumon et al., 2019). Further CCL19 levels are linked with BMI, inflammation and IR from humans AT (Kochumon et al., 2019). Inflammation during diabetes induces the expressions of CCL20 and leads to β-cell death and dysfunction in rats and humans (Burke et al., 2015).

Nutritional excess and inflammation results in ER stress. ER stress markers induced in human AT during obesity were found to be associated with BMI (Sharma et al., 2008). ER stress also manipulates the metabolic program in macrophages. For example IRE1α promotes inflammation by supporting glycolytic flux through stabilising Hif-1α (Guimarães et al., 2023). Similarly, CHOP induction in AT is responsible for altering macrophage polarization towards M1, further promoting inflammation and IR (Suzuki et al., 2017). We observed regulation of ER stress markers in M1 and MMe indicating its association with inflammation. Palmitate promotes phospholipid accumulation which leads to ER expansion as a result of ER stress (Kim et al., 2015). Unlike M1, MMe showed more inclination towards IRE1 pathway suggested by higher expressions of XBP1s. Selective and transient activation of IRE1/XBPs signalling reduces the metabolic dysfunction caused due to obesity (Madhavan et al., 2022). Stabilising XBPs improved hepatic glucose homeostasis and insulin sensitivity in HFD induced mice (Liu et al., 2016). However, ablating myeloid IRE1α attenuates the inflammation by regulating the macrophage polarization towards M2 type (Shan et al., 2017). Thus, metabolic activation of IRE1 exhibits differential functions that are tissue specific and context dependent (Huang et al., 2019).

Lipid accumulation in ATMs induces lysosomal biogenesis and lysosomal lipid metabolism independent of inflammation. ATMs from obese mice showed upregulated expressions of fatty acid transporters like CD36 and Msr1 as well as lysosome genes including LAMP1 and

LAMP2, Lipa and Ctsk (Xu et al., 2013). Inhibition of lysosomal function increases lipid accumulation in ATMs (Xu et al., 2013). They further confirmed that lipid catabolism in ATMs is lysosomal dependent and independent of autophagy (Grijalva et al., 2016). Similarly, MMe exhibited expression of Lamp1 and Lamp2 along with lysosomal exocytosis. MMe accumulated p62 and LC3-II indicating impaired autophagy (Coats et al., 2017; Kratz et al., 2014). In our *in vitro* model of MMe, we observed an increase in the expressions of lysosomal bigenesis genes LAMP1 as well as autophagy genes ATG5 and ATG7. Further, p62 and LC3-II accumulation indicates impaired autophagy (Yoshii & Mizushima, 2017). This needs to be further confirmed by blocking autophagy. Dysregulation of autophagy in liver during obesity in mice enhanced the ER stress and promotes IR (Yang et al., 2010). Similarly, LC3 levels are found to be enhanced in SAT from obese humans in comparison to lean individuals and correlated with inflammation and IR (Jansen et al., 2012). Blocking autophagy increased inflammation in mice and humans (Jansen et al., 2012). Thus, autophagy might limit the inflammation and protect the AT from IR.

Other than lipid overload and inflammation, ROS also mediates the induction of autophagy. Nutritional load, excessive FAO (mitochondrial and peroxisomal), inflammation, accumulation of cellular damage leads to mitochondrial dysfunction and results in oxidative stress (Fernández-Sánchez et al., 2011). Although we have not performed any experiment to look into oxidative stress, but it is well linked to obesity and an important feature of ATMs. Reduced total antioxidant capacity (TAC) has been found to be associated with obesity. In a meta-analysis, it is reported that serum TAC parameters are correlated with several physical and metabolic parameters associated to obesity (Anaya-Morua et al., 2023). Moreover, from our protein data, we observed that antioxidant capacity is increased in MMe indicated by Nrf2 expressions. Considering all these observations, we can understand that MMe activates autophagy as a result of lipid accumulation to improve inflammation and control oxidative stress. However, prolonged lipid stress leads to dysfunctional autophagy. This needs to be studied further by looking at mitochondrial activity and tracking autophagy and its flux at different time points and how do these process affect or affected by lipid accumulation.

We observed that MMe significantly overexpressed CD36 and PLIN2 and accumulated lipid droplets. These genes are also associated with lipid metabolism. It is well established that

the phenotype of macrophages is associated with a specific metabolic contour. Further, MMe also exhibited upregulation of PPARγ and PGC1α indicating its dependence on FA metabolism. However, MMe also demonstrated a higher expression of PDK1 and PKM2. These findings align with previous reports that suggested obese humans demonstrated elevated expressions of PPARγ, an important regulator of lipid metabolism, in ATMs. Additionally, elevated transcripts of PKM2 and PDK1 suggested that MMe has not compromised with glycolysis. This indicates its unique metabolic profile unlike M1 and M2, where M1 had high tendency towards glycolysis and declined OXPHOS, while M2 was inclined towards FAO. The association of metabolism with the macrophage phenotype was further studied by inhibiting these pathways. Blocking glycolysis reduced the expressions of pro-inflammatory cytokines in all three subtypes suggesting that glycolysis is important for inflammation (Soto-Heredero et al., 2020). Similarly, inhibiting fatty acid oxidation reduced the inflammation in M1. Interestingly, this inhibition did not alter the TNFα in MMe (chapter 5). This clearly indicates the unique metabolism in MMe. Unexpectedly, PPARγ antagonist (GW9662) increased the lipid accumulation in macrophages (Lehrke & Lazar, 2005) (chapter 5). These metabolic rewiring occurs based on the stimulations from tissue microenvironment. Interestingly, MMe also demonstrated peroxisomal fatty acid metabolism with enhanced transcripts of Acox1. Pathway enrichment analysis also revealed the upregulation of proteins involved in β-oxidation of very long chain fatty acid including Acox1 that indicates peroxisomal activity in MMe. Moreover, this analysis also identified the proteins involved in polyunsaturated fatty acids (PUFA) metabolism expressed in MMe. These are known to have immunomodulatory effects and reduces inflammation. Diets rich in alpha-linolenic acids are shown to have beneficial effect on CVD patients (Cambiaggi et al., 2023).

Obesity impairs AT homeostasis, where adipocytes are key players in controlling local microenvironment and macrophages heterogeneity in their activities and functions compensate the consequences. During lean condition, where macrophages display M2 phenotype to maintain metabolic health of AT by engulfing dead adipocytes and preserving insulin sensitivity, while obesity induces phenotypic changes in macrophages which results in inflammation and IR. This alters the adipocyte development and bioenergetics, thus affecting its function (Keuper et al., 2019; Li et al., 2023). Our data suggests secretome of MMe is important in development of insulin resistance. However, inflammation affects the

adipogenesis. Further research is required to better understand the macrophage - adipocyte interaction and its importance in obesity and IR.