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Veronica Dahik

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Équipe « Métabolisme lipidique cellulaire et systémique dans les maladies cardiométaboliques »

dirigée par le Dr. Wilfried Le Goff

Lipid rewiring by adipose tissue macrophages during diet-induced inflammation and insulin resistance

Par Veronica Dahik

Thèse de doctorat en Biologie

Dirigée par le Dr. Wilfried Le Goff

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Devant un jury composé de :

Pr. Bruno Fève	Président
Dr. Laurent Yvan-Charvet	Rapporteur
Pr. David Masson	Rapporteur
Dr. Jennifer Jager	Examinatrice
Dr. Fabienne Foufelle	Examinatrice
Dr. Wilfried Le Goff	Directeur de thèse

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ABBREVIATIONS AND ACRONYMS

(N)-PVM: (non)-perivascular macrophages	LAM: Lipid-associated macrophages
AA: Arachidonic acid	LD: Lipid droplet
ABCA1/G1: ATP-binding cassette A1/G1	LPL: Lipoprotein lipase
ASC: Adipose stem cells	LXR: Liver X Receptor
AT: Adipose tissue	MMe: Metabolically activated macrophages
ATM: Adipose tissue macrophages	MUFA: Monounsaturated fatty acid
BAT: Brown adipose tissue	NAFLD: Non-alcoholic fatty liver disease
BMDM: Bone marrow-derived macrophages	NASH: Non-alcoholic steatohepatitis
BMI: Body Mass Index	OXPFO: oxidative phosphorylation
CCL2: C-C Motif Chemokine Ligand 2	PE: Phosphatidylethanolamine
CEM: Collagen-expressing macrophages	PC: Phosphatidylcholine
Chol: Cholesterol	PL: Phospholipid
CLS: Crown-like structures	PldG: Phosphatidylglycerol
CVD: Cardiovascular disease	PUFA: Polyunsaturated Fatty Acid
DHA: Docosahexaenoic acid	ROS: Reactive oxygen species
DIO: Diet-induced obesity	SFA: Saturated Fatty Acid
DNL: De novo lipogenesis	SL: Sphingolipid
EC: Endothelial cells	SM: Sphingomyelin
EE: Energy expenditure	SREBP: Sterol Regulatory Element-Binding Protein
EI: Energy intake	T2D: Type 2 Diabetes
EPA: Eicosapentaenoic acid	TG: Triglyceride
ER: Endoplasmic reticulum	TLR: Toll-like receptor
FAO: Fatty acid oxidation	TRL: Triglyceride-rich lipoproteins
FAS: Fatty acid synthase	VAM: vasculature associated-macrophages
FFA: Free Fatty Acid	VLDL: Very-low-density lipoprotein
HFD: High fat diet	WAT: White adipose tissue
IR: Insulin resistance	

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ABSTRACT

Adipose tissue macrophages (ATM) are central players in obesity-associated inflammation and its comorbidities. ATM metabolism is strongly linked to their functional phenotype and dysregulated or disproportionate ATM responses to environmental lipid fluxes during diet-induced obesity (DIO) influence the activation of specific inflammatory and metabolic programs that contribute to the onset of obesity-induced insulin resistance. The mechanisms governing this metabolic adaptation of ATM in DIO are however poorly understood. Our study demonstrates that the membrane Atp-binding cassette g1 (Abcg1) transporter controls ATM functional response to fatty acids (FA) carried by circulating triglyceride-rich lipoproteins (TRL), which are abundant in high energy diets. Mice genetically lacking Abcg1 in the myeloid lineage present an ameliorated inflammatory status in adipose tissue and a reduced insulin resistance. Abcg1-deficient ATM exhibit a less inflammatory phenotype accompanied by a low bioenergetic profile and a modification of FA metabolism pathways. A closer look into ATM lipidome reveals a shift in the handling of FA pools, including a redirection of saturated FA from membrane phospholipids to lipid droplets, and leading to a reduction in membrane rigidity and neutralization of pro-inflammatory FA. ATM from obese human subjects presented the same reciprocal relationship between Abcg1 expression and this inflammatory and metabolic status. Abolition of this protective, anti-inflammatory phenotype in Abcg1-deficient ATM was achieved through the inhibition of lipoprotein lipase (Lpl) activity, thus delineating the importance of the Abcg1/Lpl axis in controlling ATM metabolic inflammation. Overall, our work identifies the rewiring of fatty acid pools by Abcg1 as a new major pathway orchestrating ATM plasticity and insulin resistance in DIO.

Key words: obesity, adipose tissue macrophage, Abcg1, lipid metabolism.

INTRODUCTION

Part I: Obesity

1. Epidemiology and etiology of obesity

Obesity has emerged as a global public health concern in adults as well as in children, and is currently ranked the fifth most common leading cause of death worldwide. The World Health Organization (WHO) defines obesity as an “abnormal or excessive fat accumulation that presents a risk to health” (World Health Organization 2021). Indeed, obesity is a major risk factor for the development of numerous chronic metabolic diseases including type 2 diabetes, non-alcoholic fatty liver disease and cardiovascular disease (CVD) (see chapter 2).

The unit of Body Mass Index (BMI) is the most widely used formula to assess obesity and is calculated by dividing weight in kg to height in m². Any individual with a BMI ≥ 30 kg/m² is clinically defined as obese, with certain cutoffs used to further classify obese subjects with increased risk of morbidity and mortality (Table 1). Despite its utility in health screenings, BMI as a measure of adiposity and cardiometabolic outcomes has been often challenged as it does not take into account body composition (i.e. muscle, bone and fat mass), which can be highly variable to patient sex, cardiovascular fitness and race/ethnicity. Waist circumference or waist-to-hip-ratio are two body size measures that have been incorporated in the clinical assessment of obesity and focus on body fat distribution rather than weight. The rationale for the use of these measurements for risk prediction relies on the observation that abdominal/visceral fat distribution (also referred to as android or male-type obesity) is often associated with increased metabolic complications. Inversely, fat accumulation in hips and thighs/subcutaneously (referred to as gynoid obesity and typically associated to the female body) is correlated to better health outcomes (Després 2012, Gadde et al. 2018) (Table1).

Obesity is now regarded as global pandemic whose numbers have nearly tripled over the last 3 decades. Although its prevalence is lower in children than in adults (5% vs 12%, respectively), childhood obesity has seen a much steeper and alarming increase. Systematic data analysis of disease burden attributed to obesity have shown that high BMI was accountable for 4 million deaths worldwide and 120 million disability-adjusted life-years. Moreover, almost 70% of those deaths were due to CVD (GBD 2015 Obesity Collaborators et al. 2017). Fully understanding the pathogenesis of obesity is therefore key to curbing the rise in its prevalence. In the following section, we will explore the main causes and consequences of obesity and obesity-related comorbidities, as well as the therapeutic options available today for managing them.

BMI	Category	Morbidity risk	Waist circumference (M [§] >102cm, F [§] >88cm)	Treatment
≤18.5	Underweight	Low (but risk of other clinical problems)	No increased risk	Lifestyle intervention (LI)
18.5-24.9	Normal	Average	Increased risk	-
25-29.9	Overweight	Increased	High risk	LI + Pharmacological intervention (PI)*
30-34.9	Obese type I	Moderate	Very high risk	LI + PI + surgery* [§]
35-39.9	Obese type II: moderate	Severe	Very high risk	LI + PI + surgery*
≥40	Obese type III: severe	Very severe	Very high risk	LI + PI + Surgery

Table 1: Risk of associated disease and treatment options according to BMI and waist circumference.

*If presenting certain comorbidities such as type 2 diabetes, hypertension, sleep apnea.

[§]Only for certain laparoscopic procedures.

[§]M: Male, F: Female.

1.1. Environmental/Lifestyle causes

Obesity is a complex disease that issues from the combination of numerous individual factors. However, fundamentally, obesity stems from an imbalance between energy intake (EI) and energy expenditure (EE). When energy consumption is higher than that required for daily expenditure, calories are stored as fat principally in adipose tissue. Fat deposition in non-physiological sites such as liver, muscle, heart and perivascular regions (ectopic fat depots) can also be observed, but only when adipose tissue storage capacity is compromised. Measure of EI is considerably subjected to bias and quite difficult to assess in large scale population studies. However, changes in dietary patterns have been evidenced over the past few decades in both urbanized and rural areas as a consequence of economic growth and food availability. These include an increase in portion size and in the consumption of unhealthy fats, processed meat and refined sugars, (Malik et al. 2013), all of which are low in nutritional quality but high in calories (energy dense foods).

The metabolic consequences of these habits have been assessed by epidemiological studies showing that a consumption of a western-type diet (high sugar, fat and animal protein intake, low vegetable/fruit, whole grain/nuts and fish intake) and industrially ultra-processed foods is strongly positively correlated to weight gain (4.5kg increase), the development of insulin resistance (IR) (2-fold increase) and adverse health outcomes (Pereira et al. 2005, Romieu et al. 2017, Elizabeth et al. 2020). Indeed, simple sugars, saturated fat and sodium have been associated to a higher glycemic load

(Martinez-Perez et al. 2021), as opposed to fiber and polyunsaturated fatty acids which have been shown to be inversely associated to obesity-related comorbidities such as insulin resistance and hyperglycemia in the DASH (The Dietary Approaches to Stop Hypertension) diet (Yashpal et al. 2022). The physiological effects of these nutrients are various and have been extensively studied in numerous settings. In terms of metabolic health, saturated fatty acids have generated the most interest as they have been consensually associated to a much higher cardiometabolic risk (Li et al. 2022b). Indeed, while certainly an important source of stored energy, this particular class of fat is remarkably bioactive and therefore able to influence cellular pathways (i.e. membrane structure and signaling, transcriptional regulation, production of metabolites, etc) linked to inflammation, intracellular lipid storage and overall metabolic disease. These mechanisms will be detailed later on. Interestingly, replacement of these fats by polyunsaturated ones has been shown to have positive effects on plasma lipid levels and glycemic control, while their substitution with carbohydrates has had little effect (Wu et al. 2019). Within polyunsaturated fatty acids, it seems however that ω -3 rather than ω -6 polyunsaturated fats uphold more health benefits and may indeed participate at decreasing the risk of obesity (Simopoulos 2016). Exposure to certain nutrients and food groups as well as chronic overfeeding therefore guide disease burden by tipping the balance towards a positive energy profile and promoting weight gain.

As opposed to EI, EE is easier to assess as it comprises the energy used to digest and metabolize food (called the thermic effect of food, between 8-10% of total EE); the energy cost of maintaining basal body function (resting energy expenditure, 60% of total EE); and the energy expended in physical activity (Hill et al. 2012). Among these three factors, physical activity is the more variable component of EE and its decrease has been shown to be positively associated to adverse cardiovascular profiles, including a higher BMI, blood sugar and lipid levels and blood pressure (Chau et al. 2014). The contribution of this form of EE to the pandemic of obesity has mostly been possible by the sustained sedentary lifestyle that came with industrialization and the transition to office-based work, motorized transportation systems and the substantial increase in screen time (Malik et al. 2013). Controlling both energy intake and expenditure is therefore key in preventing obesity and obesity-associated comorbidities.

1.2. Genetic and epigenetic factors

The heritability and genetic influence on adiposity and obesity has been studied in genome-wide association studies (GWAS), which have allowed to identify hundreds of genes associated to the development of syndromic and non-syndromic types of obesity. Syndromic obesity develops from chromosomal defects that are associated to genetic syndromes (i.e. Prader-Willi, fragile X, Bardet-Biedl,

Albright) presenting congenital malformations and developmental delay, while non-syndromic obesity is caused by mutations in a single (monogenic) or several (polygenic) genes capable of causing and contributing to the obese phenotype (Bairqdar et al. 2023).

Monogenic obesities are quite rare and develop early in childhood. They are caused by mutations in the hypothalamic leptin-melanocortin pathway which has a major role in regulating the energy balance. This system is activated by the adipose tissue-derived hormone leptin (*LEP* gene), that binds its receptor (*LEPR*) in the hypothalamus and induces the production of pro-opiomelanocortin (*POMC*). This in turn leads to the synthesis of the prohormone convertase 1 (*PCSK1*) and the α -melanocyte-stimulating hormone (α -*MSH*), with α -MSH being able to induce satiety by binding to the melanocortin 4 receptor (*MCR4*) (Dubern et al. 2023). As such, subjects with homozygous or heterozygous deficiency of any of these genes have been observed to develop severe obesity early in life notably as a consequence of major hyperphagia (Valette et al. 2013, Farooqi 2022). Other genes (i.e. single-minded homolog1 (*SIM1*), brain-derived neurotrophic factor (*BDNF*)) involved in or regulating the pathway mentioned above have also been linked with severe obesity (Dubern et al. 2023).

Regarding polygenic obesities, more than 140 single nucleotide polymorphisms (SNPs) have been associated with obesity-related metabolic traits (Romieu et al. 2017). For instance, subjects carrying a risk allele (rs9930506A>G) for the *FTO* gene (Fat mass and obesity-associated protein) have been associated to a higher BMI and incidence of T2D (Frayling et al. 2007). Other *FTO* SNPs have also been found to be significantly associated with adiposity and plasma lipids (total and LDL-cholesterol and triglycerides)(Duicu et al. 2016). Variants in the genes coding for *PPARGC1A* et *PPARGCB*, activators of *PPARG* (Peroxisome proliferator-activated receptor gamma) who regulates metabolic homeostasis, have also been reported as associated to visceral adiposity and IR (Franks et al. 2014). Gene variants resulting in low levels of hormones adiponectin, ghrelin, pancreatic polypeptide (PP) and peptide YY (PYY) have also been linked to the obese phenotype, as they are implicated in glucose and lipid metabolism and the brain-gut axis regulating appetite (Bairqdar et al. 2023).

Others studies have put forward the part of epigenetics in early-life metabolic dysregulations. Indeed, significantly different DNA methylation patterns have been observed in obese vs lean subjects and associated to obesity features (Cordero et al. 2015, Fall et al. 2017). Methylation of CpG sites at the carnitine palmitoyl-transferase 1A (*CPT1A*) or the ATP-Binding Cassette G1 (*ABCG1*) transporter loci have for example been associated with BMI and the metabolic syndrome (Gagnon et al. 2014, Demerath et al. 2015). Altogether, these data support the notion that (epi)-genetics are important contributors to obesity that should not be overlooked when studying the pathogenesis of this disease.

1.3. Other factors influencing obesity

Gut microbiota dysbiosis has also been associated to metabolic homeostasis. Indeed, bacteria in our intestine interact with ingested food, pathogens and immune cells and therefore represent a cornerstone linking metabolic and inflammatory systems. Its dysregulation has been associated to not only obesity, but also diabetes, CVD and more largely metabolic syndrome (Romieu et al. 2017). Sequencing technologies have allowed us to determine that obese patients often present an enrichment or depletion of certain bacterial groups, which has been linked to the host capacity to digest and absorb certain carbohydrates and lipids, thus influencing the energy balance and obese phenotype (Aron-Wisnewsky and Clément 2016). This has been validated by studies of fecal transfer from obese mice or humans into germ-free mice, in which the recipient, lean mice developed obesity and metabolic alterations in the absence of increased food intake (Turnbaugh et al. 2008, Ridaura et al. 2013).

Finally, other factors directly or indirectly influencing obesity include, but are not limited to sleep duration (as it relates to sleep quality but also the timing of meals)(Mazri et al. 2022), stress, smoking, household income and alcohol consumption. Ultimately, obesity is a multifactorial disorder and further efforts are necessary to better understand the mechanisms related to its pathogenesis in order to counteract and limit its impact on public health.

2. Comorbidities and complications of obesity

Obesity can have a significant impact on health-related quality of life given the burden it relays to the development of other diseases (Figure 1). Indeed, obesity can affect nearly every organ system, from the cardiovascular system to the neuroendocrine and immune systems, and health care costs attributable to overweight- and obesity-related diseases can come up to 26% of total expenses (Lette et al. 2016). Here we will focus on some of the more common comorbidities of obesity and how the association of these diseases results in what is referred to as the metabolic syndrome.

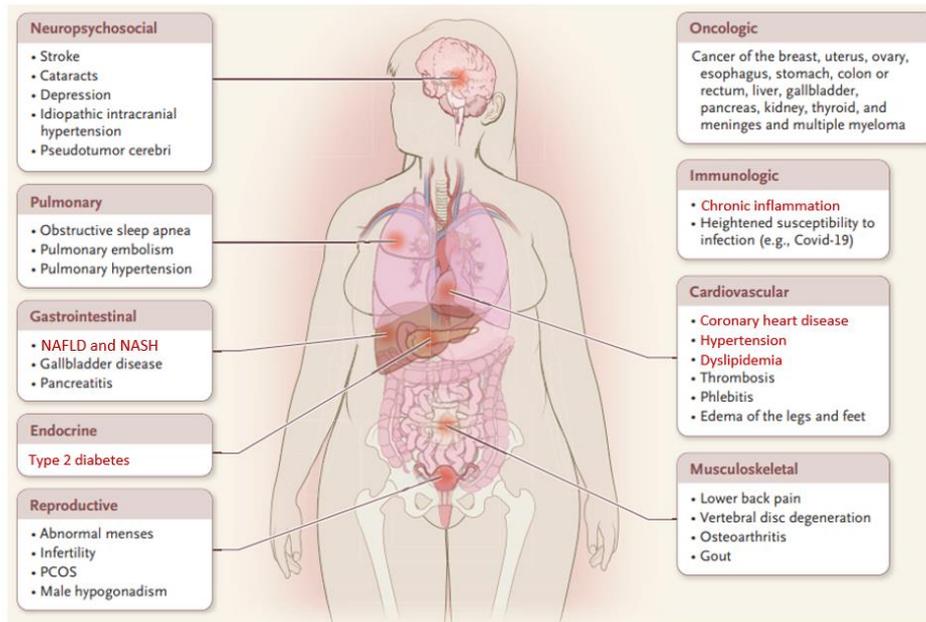


Figure 1: Obesity and its comorbidities. Obesity is a major risk factors for numerous physiological and psychological disorders. The comorbidities that we have developed in this manuscript are in red. if associated, these may constitute the metabolic syndrome. Adapted from Cypess, 2022.

2.1. Cardiovascular disease (CVD)

Obesity not only increases the risk but also worsens pre-existing conditions such as coronary heart disease/atherosclerosis, high systemic blood pressure, atrial fibrillation and heart failure. The Framingham Heart study was one of the first large, community-based reports confirming the significant role of obesity in cardiovascular disease, as it revealed that heart disease risk doubled with obesity and that 11% and 14% of cases of heart failure in men and women, respectively, were attributable to obesity alone (Kenchiah et al. 2002). This may stem from several different mechanism: unbalanced diets and chronic overfeeding can lead to dyslipidemia characterized by an increase in plasma triglyceride (TG) levels and plasma cholesterol (Chol) levels, notably those in low-density lipoproteins (LDL). This in turn results in ectopic lipid deposition in arteries, thus promoting macrophage foam cell formation, inflammation, and atherosclerotic disease (Csige et al. 2018). Epicardial and pericardial abnormal fat accumulation are also associated to a significant risk of atherosclerotic CVD and heart failure. Indeed, epicardial AT not only functions as mechanical support protecting arteries from cardiac contraction, but also serves as a significant energy source for adjacent myocardium. In accordance with this role, abnormal epicardial AT expansion during obesity has been associated with lipid infiltration in the atrial wall, leading to conduction abnormalities and atrial fibrillation. It has also been associated to inflammatory cytokine production, thereby potentiating atherosclerotic disease (Gaborit et al. 2017). Other direct or indirect mechanisms through which

obesity leads to CVD include hemodynamic changes, activation of the renin-angiotensin-aldosterone system that regulated blood pressure and vascular resistance, and endothelial dysfunction (Csige et al. 2018). Coherently, all of these CVD markers have shown to be positively correlated to BMI and intra-abdominal obesity (Varbo et al. 2015, Powell-Wiley et al. 2021).

2.2. NAFLD

Non-alcoholic fatty liver disease (NAFLD) is defined as the ectopic accumulation of fat in the liver independent of alcohol consumption and is one of the hallmark features associated with obesity (up to 90% prevalence in individuals with significant visceral obesity vs 3-15% in lean persons) (Cariou 2022). NAFLD is characterized by excessive intrahepatic TG content (steatosis), which can then lead to non-alcoholic steatohepatitis (NASH; inflammatory state) and even cirrhosis (advanced cell damage, fibrosis). Steatosis is the consequence of adipose tissue hypertrophy and dysfunction following excess energy intake: as the expansion capabilities of adipose tissue dwindle down, TG lipolysis from this tissue increases, which in turn results in enhanced hepatic fatty acid uptake. This combined with liver capacity to synthesize lipids (*de novo* lipogenesis), which also escalates during obesity, further contributes to the fatty phenotype and promotes lipotoxicity. While the liver is also capable of exporting lipids via TG secretion in very-low density lipoproteins (VLDL), the secretion and oxidation rate of lipids does not allow to compensate for the upregulation of these other mechanisms (Fabbrini et al. 2010).

2.3. Low-grade systemic inflammation

The inflammatory component of obesity has been evidenced in overweight and obese individuals who present altered serum levels of several inflammatory markers, including increased C-reactive protein (CRP) and cytokines interleukin (IL)-6, IL-8 and tumor necrosis factor alpha (TNF α) (Khanna et al. 2022). The initial trigger for inflammation is still not yet fully understood, but several potential mechanisms include an increased concentration of gut-derived LPS and other antigens as a consequence of enhanced intestinal permeability and microbiota dysbiosis; increased plasma concentration of non-esterified fatty acids (NEFA) from the diet that can directly activate inflammatory pathways such as those controlled by TLR2/4; and also stress signals resulting from abnormal adipose tissue expansion and consequent adipocyte death (Saltiel and Olefsky 2017, Ahmed et al. 2021).

As the principal energy storage organ, adipose tissue is capable of expansion by increasing both its cell size (hypertrophy) and cell number (hyperplasia); this faculty is however compromised during chronic nutrient overload leading to altered functionality and cellular stress. Markers of adipocyte stress include endoplasmic reticulum (ER) and mitochondrial dysfunction, unfolded protein response and reactive oxygen species (ROS) production (Ahmed et al. 2021). This in turn results in the release of

a cascade of cytokines (i.e. IL-6, TNF α) and chemokines (i.e. CCL2) that coordinately induce the recruitment of inflammatory macrophages issued from blood monocytes, but also the proliferation and activation of resident immune cells (mostly macrophages and neutrophils). Coherently, studies have shown that both adiposity and adipocyte size are positively correlated to adipose tissue macrophage (ATM) numbers in both visceral and subcutaneous AT in mice and humans (Weisberg et al. 2003). Adipocyte expansion also leads to hypoxia, extracellular matrix remodeling and fibrosis, all participating to local inflammation at the expense of tissue and cellular homeostasis (Karczewski et al. 2018) (Figure 2).

It is important to highlight that in the initial installment of obesity, it is indeed hypertrophic adipocytes that drive the inflammatory environment in AT; upon long-term caloric surplus however, this pivotal role in inflammation and inflammatory cytokine production is sustained by ATM (Hotamisligil et al. 1993, Lionetti et al. 2009). The precise role and mechanisms of ATM-dependent inflammation during obesity will be detailed later on (see chapter 3). Similar mechanisms of obesity-induced cellular stress and immune cell-mediated tissue inflammation can also be observed in liver, muscle, brain and pancreas (Saltiel and Olefsky 2017), the latter being particularly relevant for the pathogenesis of diabetes.

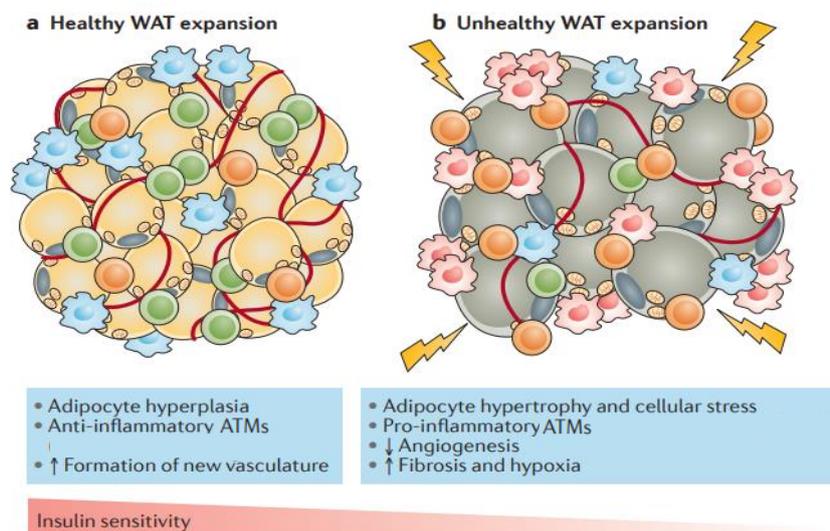


Figure 2: Adipose tissue physiological and pathological expansion. A) In physiological, lean conditions, AT expansion is characterized by adipocyte hyperplasia rather than hypertrophy and a rather low number of adipose tissue macrophages (ATM). Both adipocytes and ATM display high insulin sensitivity. In obese conditions, hypertrophic adipocytes induce the recruitment of inflammatory ATM, which heightens the inflammatory state and reduces insulin sensitivity. New vasculature formation is also impaired, leading to fibrosis and hypoxia. Adapted from Kusminski et al., 2016. ATM: light blue cells; pro-inflammatory ATM: red cells; hypertrophic adipocytes: grey cells; NK cells: orange cells; T-regulatory cells: green cells; Vasculature: red lines.

2.4. Insulin Resistance

Insulin signaling starts with its binding to the insulin receptor (INSR) at the plasma membrane of target cells, which activates the receptors intrinsic tyrosine kinase activity that phosphorylates the insulin receptor substrate (IRS), the PI3 kinase (PI3K) and the AKT protein. AKT then initiates the insulin response by activating or repressing downstream targets genes implicated in glucose and lipid homeostasis (Figure 3) (Czech 2017). As such, insulin is able to inhibit hepatic gluconeogenesis and hypothalamus-stimulated food intake through activation of the FOXO1 transcription factor, induce *de novo* lipogenesis (DNL) by upregulation of SREBP1c, promote GLUT-4 mediated glucose uptake by adipocytes and skeletal muscle and suppress adipocyte lipolysis by inhibiting adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (Li et al. 2022a). Given this plethora of biological functions, dysregulation of insulin signaling pathway has been found to be associated with the severity of obesity and insulin resistance (Guo 2014).

The causal relationship between obesity and the development of insulin resistance has been studied in numerous settings and has identified obesity-related chronic inflammation as one of the most important causes of peripheral resistance to insulin action. Activation of inflammatory pathways such as NLRP3, JNK or NF- κ B have indeed been shown to block insulin action by promoting phosphorylation and inactivation of IRS (Lionetti et al. 2009, Tanti and Jager 2009, Saltiel and Olefsky 2017), while their inhibition has proved able to maintain insulin signaling and sensitivity (Hirosumi et al. 2002, Vandanmagsar et al. 2011). It is important to note that given their role on inflammation, ATM have been identified as the main effectors in the development of local and systemic insulin resistance (Hotamisligil et al. 1993, Xu et al. 2003), with studies accordingly suggesting that obesity-triggered insulin resistance is in large part a consequence of chronic inflammation sustained by ATM.

It is important to note that cellular manifestations of T2D may materialize years before being able to fully clinically diagnose T2D, and that the disease is often preceded by an insidious but reversible phase of pre-diabetes (fasting glycemia between 1,10 – 1,25 g/L). Moreover, it has also been documented that hyper secretion of insulin may occur in obese subjects in the absence of IR, and that hyperinsulinemia can also be driver of IR (Shanik et al. 2008, van Vliet et al. 2020)(Figure 4).

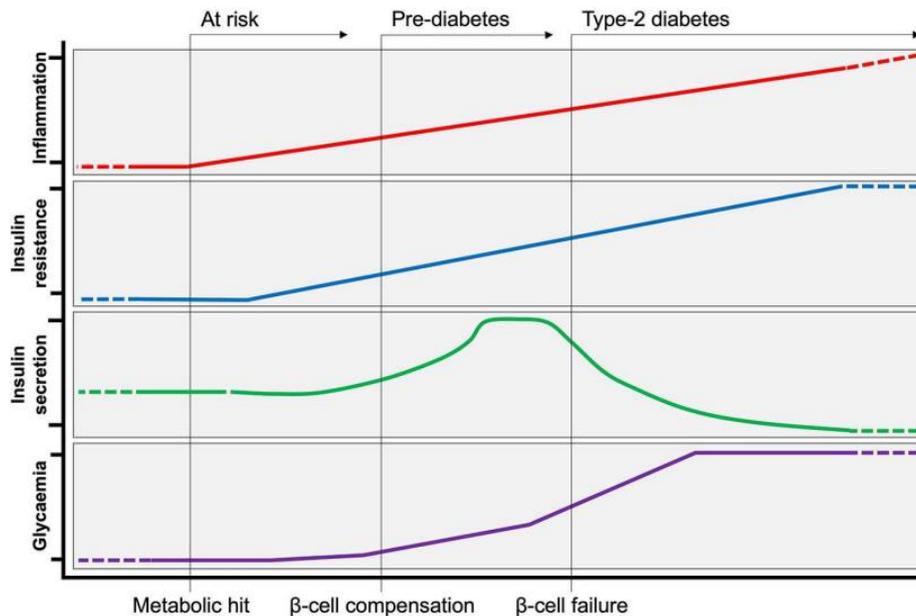


Figure 4: Evolution of T2D along with inflammatory status. Inflammation is one of the initial steps leading to peripheral insulin resistance following a metabolic challenge, which is then compensated by an increase in β -cell-dependent insulin secretion. However, as this mechanism is exhausted due to chronically elevated blood glucose levels, β -cell failure is imminent. T2D is installed, along with persistently high levels of inflammation. From Orliaguet et al., 2020.

To conclude, we see now that any of these conditions potentiate each other and are thus often found associated to one another (= the metabolic syndrome), making its prevention or management essential to improve overall health. We would also like to highlight the inflammatory trait observed in CVD, NAFLD and T2D and more globally in obesity, and how controlling inflammation could be an effective mechanism for maintaining metabolic homeostasis.

3. Treatments

3.1. Lifestyle interventions

Lifestyle modifications consisting of diet and physical activity are the core of obesity therapeutic management and its comorbidities, and require the building of a skill set of strategies and behavioral knowledge in order to properly implement and maintain these adjustments. Weight management may begin with the introduction of dietary changes aimed at a reduction of 170 to 250 kcal per day or 325

to 480 kcal per day for a 10% and 20% weight loss, respectively (Hill et al. 2012). It is important to note that the type of diet (i.e. low-carbohydrates or low-fat diets, vegetarian diets, etc.) is not a predictor of success; instead, studies have shown that dietary adherence is key to substantial weight loss (Ryan and Kahan 2018), as only 10-20% of individuals actually achieve $\geq 10\%$ weight loss over 1 to 2 years (Cariou 2022).

Increased physical activity is another pillar of comprehensive lifestyle therapies, especially since evidence suggests that exercise combined with diet is more effective at improving body composition (loss of fat mass but preservation of lean and muscle mass) than any of these interventions alone (Romieu et al. 2017). Guidelines recommend 150-300 minutes of aerobic moderate-intensity physical activity per week for weight loss, and similar endurance training times for greater reduction of visceral AT and improvement of metabolic parameters (reduced LDL-cholesterol and TG levels, increased muscle glucose uptake) even without weight loss (Ryan and Kahan 2018, Heffron et al. 2020, Powell-Wiley et al. 2021).

Introduction of any or a combination of these lifestyle adjustments has proven beneficial for preventing obesity-related complications. For instance, adherence to a Mediterranean diet (high vegetable/fruits, nuts, cereals, whole grains, olive oil, fish and low sugar, red meat and dairy products) and other similar diets (i.e. Nordic, DASH, vegetarian diets) has shown to decrease the risk for major adverse cardiovascular events in subjects already at risk, even in the absence of weight loss (Gadde et al. 2018, Trautwein and McKay 2020, Powell-Wiley et al. 2021). Similarly, a low-fat diet in T2D patients was effective at promoting weight loss and reduction of waist circumference, but also at improving HbA1C, TG levels and high-density lipoprotein (HDL) levels (Zhou et al. 2022). Exercise has been shown to significantly improve liver, pericardial and epicardial fat accumulation (Powell-Wiley et al. 2021), although significant and sustained weight-loss ($\geq 10\%$) has been required to fully reverse fibrosis and NASH (Cariou 2022). Finally, weight loss by dietary or exercise changes have also been associated with improved inflammatory status by reducing macrophage content in visceral and subcutaneous AT in mice and humans, then resulting in improved insulin sensitivity (Heffron et al. 2020).

It is important to note that weight recidivism is a frequent problem faced by people with obesity, notably due to a lack of compliance with the aforementioned dietary and exercise habits and perturbations in the levels of appetite-related hormones and resting energy expenditure (Busetto et al. 2021). Long-term management of obesity is therefore a challenge and can lead to more aggressive interventions involving pharmacological or surgical treatment.

3.2. Pharmacotherapy

Pharmacological intervention is preconized in obese subjects with BMI $>27 \text{ kg/m}^2$ presenting comorbidities and in those with BMI $> 30 \text{ kg/m}^2$ (Table 1), and is often offered when lifestyle modifications fail to reach the objective or to sustain lost weight. If more than 5% weight-loss is not achieved at 3 months starting therapy, new treatment options should be considered; however, if effect and tolerance are attained, it is recommended to continue the medication long term (Gadde et al. 2018, Heffron et al. 2020). There are currently four drugs available for the management of obesity (efficacy, dose and side effects reviewed elsewhere (Ryan and Kahan 2018)), although only two of them are commercialized in France. Orlistat is an agent that decreases intestinal fat absorption through the inhibition of pancreatic lipases responsible for the hydrolysis of dietary fat. As expected, this facilitates weight loss (~3%) and has also shown to improve metabolic and inflammatory parameters including HbA1C, LDL-cholesterol and insulin sensitivity. There is also the glucagon-like peptide-1 (GLP-1) agonist liraglutide, which was initially used for the treatment of T2D but has now been approved for obesity as daily subcutaneous injections (Deng et al. 2022). While it exerts similar beneficial effects on weight loss and metabolic/anti-inflammatory effects as orlistat, liraglutide mechanism of action relies on increasing insulin secretion in pancreatic β cells while also reducing glucagon (a hormone that increases blood glucose levels) production in pancreatic α cells (Heffron et al. 2020). Semaglutide is another GLP-1 agonists that has been demonstrated to exert a more significant weight loss (up to 15%) and cardiometabolic improvements (Wilding et al. 2021) than liraglutide, and has recently been approved for prescription in France in adults with a BMI $\geq 35 \text{ kg/m}^2$.

We would like to mention that there are a number of other classes of drugs with beneficial effects on peripheral fat accumulation, insulin sensitivity, glucose tolerance and inflammation that are prescribed for management of monogenic obesities and obesity-associated disorders such as NAFLD, T2D and CVD; these are however beyond the scope of this manuscript.

3.3. Bariatric surgery

Bariatric surgery is considered a treatment option in obese patients with a BMI \geq to 40 kg/m^2 or as low as 30 kg/m^2 depending on the presence of at least one obesity-related comorbidity and also on the type of surgery to be performed (Table 1) (Gadde et al. 2018). There are several common bariatric surgical procedures, all of them based on restrictive or malabsorptive mechanisms for weight loss and cardiometabolic risk improvement. Among them, Roux-en-Y gastric bypass presents the higher sustained weight loss (more than 30% and durable for at least 5 years) than others, plus the benefit of markedly contributing to T2D remission (Heffron et al. 2020). Long-term care and adapted nutritional supplementation are however necessary in most cases, and complications (i.e. nutritional deficiencies including vitamin D and calcium deficiency, ulcers, bowel obstruction, among others) may manifest

even years after surgery. Another adverse outcome associated to bariatric surgery is suboptimal weight loss (less than 40-60% of excess body weight over 2 years) and/or weight regain (30-70% weight increase depending on the type of procedure) (Noria et al. 2023). The factors influencing postoperative weight regain included dietary, psychiatric and genetic factors, but may extend to cellular (adipose) plasticity as maintenance of post-surgery benefits have been associated to adipose tissue function (Hoffstedt et al. 2017).

In summary, obesity is a disease of multifactorial etiology and associated with the development of serious comorbidities, all contributing to reduce individuals' quality of life. Although several therapeutic options are available today, prevention and management of obesity has proven far from easy, mostly due to the difficulty of attaining long-term weight loss and thus actually reducing cardiometabolic risk. These challenges not only highlight the importance of prevention, but also the need of further research on promising targets such as immune cell metabolism as a way of effectively preventing the onset and progression of obesity.

Part II: Adipose tissue

Adipose tissue is a loose, connective tissue formed of several types of cells including adipocytes (main fraction, specialized in storing metabolic energy in the form of cytoplasmic vesicles called lipid droplets) and a non-adipocyte fraction (the stromal vascular fraction, involved in AT homeostasis). Under physiological conditions, adipose tissue architecture and function are tightly regulated. Under obese conditions, however, many of its features such as localization, size and metabolic behavior are altered. In this section, we will give a brief overview of the different types and colors of AT depots, with a particular focus on white adipose tissue (WAT) and its role in health and disease.

1. Types of adipose tissue

1.1. White adipose tissue

i. Localization

As mentioned in the first part of this manuscript, human WAT is often separated into visceral (vWAT, 15% of total body fat under physiological conditions) and subcutaneous (scWAT, 80% of total body fat under physiological conditions) depots, these localizations being associated with higher and lower magnitude of metabolic disease risk, respectively (Gadde et al. 2018). scWAT tissue is found superficially in dermal, facial, or abdominal and gluteofemoral regions, while vWAT is located in deeper regions surrounding the organs (i.e. pericardial, perirenal, omental, gonadal) (Figure 5). It is important to note that these depots are mostly conserved between species, although some differences between mice and humans have been evidenced regarding the vWAT. For instance, the inguinal and gonadal WAT depots are particularly developed in mice, especially during diet-induced obesity. Omental (intra-abdominal) WAT is only found in humans, although most research agrees that this depot is largely comparable to inguinal or gonadal AT (Börgeson et al. 2022).

ii. Origin

White adipogenesis begins with the commitment of a pluripotent stem cell from the mesoderm ($Myf5^-$ or $Wt1^+$ mesenchymal stem cells) into the adipocyte lineage, then giving rise to an adipocyte precursor (preadipocyte) that differentiates and matures into an adipocyte with full functional properties (Ye et al. 2023). This latter phase is regulated by a number of molecules, among which PPAR γ has been identified as a major player. Indeed, overexpression of this receptor is sufficient to induce adipose differentiation (Tontonoz et al. 1994). Other pro-adipogenic factors include C/EBPs, Kruppel-like factors (KLFs), and BMP receptors which actually work in concert with PPAR γ to regulate adipogenesis (Ye et al. 2023). The modulation of PPAR γ expression or activity was therefore considered as a potential target for the treatment of obesity, which led to the development of synthetic PPAR γ agonists called thiazolidinediones (TZD). Although they did manage to improve glucose tolerance and

insulin sensitivity, they presented significant side effects (i.e. weight gain, fluid retention, edema, congestive heart failure, bone fracture, among others) that hampered its use (Luo and Liu 2016). Instead, partial PPAR γ modulators (SPPARM) have been developed to improve dyslipidemia and insulin sensitivity with minimal undesired side effects as compared to TZD (DePaoli et al. 2014, Yamashita et al. 2020).

WAT develops in the second trimester of pregnancy and is fully established by birth (Saely et al. 2012). As such, adipocyte numbers are quite stable during adulthood, with studies showing that adipocyte generation rate is not altered during obesity (Spalding et al. 2008). It is however important to note that thus far, studies investigating fat turnover have focused solely on cell division with little to no attention to postnatal adipocyte progenitor recruitment (Hepler and Gupta 2017), which could significantly alter the adipocyte pool and turnover, especially in obese states.

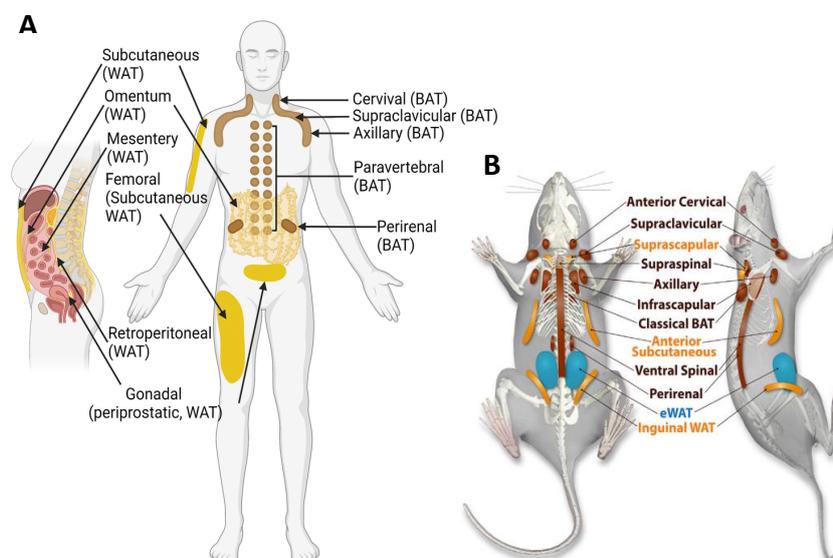


Figure 5: Human and mouse adipose tissue type and distribution. Principal human (A) and mouse (B) AT depots from frontal and sagittal planes. BAT depots are in brown, WAT depots are in yellow (human) and blue (mouse) and WAT pads with high browning capacity are in orange (mouse only). From Mukherjee et al., 2022 and Zhan et al., 2018.

iii. Morphology and function

White adipocytes (25-200 μ m of size) present a particular morphology characterized of a single, large lipid droplet that takes up about 90% of the cell volume while the nucleus and organelles are flattened and located at the periphery. Few elongated and thin mitochondria are also present (Saely et al. 2012) (Figure 6). WAT mass increases with age and ranges from 15 to 25% total body mass in adult men and 20-30% in adult women under lean conditions (Gallagher et al. 2000, Ibrahim 2010, Cypess 2022). In individuals with obesity, this tissue can account for up to 50% of total body mass

(Maniyadath et al. 2023). WAT primary function is to store calories in the form of TG during the post-prandial state, and release them as free fatty acids (FFA) if needed to fuel peripheral organs during fasting. The release of lipids from WAT is dependent on a number of parameters and molecules, among which we find insulin. The sensitivity of WAT depots towards insulin is therefore key for tissue proper function (see section 2). Besides this role in lipid storage, WAT also serves as a major endocrine organ that secretes numerous molecules (i.e. leptin and adiponectin, IL6, IL4, etc.) that regulates systemic energy homeostasis and assure a permanent crosstalk between adipocytes and other cell types (i.e. immune cells such as macrophages) within AT. Together, these roles have helped place this tissue at the forefront of metabolic disorders such as obesity. Finally, white adipocytes also provide a layer for mechanical protection against injury and thermal insulation.

1.2. Brown adipose tissue

i. Localization

Contrary to the initial notion that BAT is restricted to fetal development and neonates, studies using positron emission tomography (PET) and radioactively labeled fluorodeoxyglucose (FDG) have allowed to detect metabolically active BAT in adults (Zhang et al. 2018). This has allowed to up its volume from 0.1 to 0.5% of total body mass in humans (2 to 5% of body mass in mice) to up to 8.5% (Saely et al. 2012, Cypess 2022). BAT in infants is mainly found in the interscapular region, although this depot significantly diminishes with age. In adults, BAT presence is located to the axillary, cervical, supraclavicular and paravertebral and perirenal regions. In mice, brown adipocytes can be found in similar locations that include cervical and inter- and subscapular regions (Sanchez-Gurmaches et al. 2016) (Figure 5).

ii. Origin

BAT emerges mid gestation from mesodermal progenitor cells expressing Myf5⁺, but also the transcription factors Pax3 and Pax7. Engagement towards the adipocyte lineage is defined by the expression of PRDM16, which determines a transcriptional switch from myoblasts to brown adipocytes. Other genes such as BMP7 and Ebf have also been implicated in brown adipogenesis through the expression of PRDM16 and UCP1 (Sanchez-Gurmaches et al. 2016, Ye et al. 2023). Interestingly, another type that brown adipocyte that does not come from Myf5⁺ progenitors has also been evidenced. Indeed, studies have shown that certain BAT depots such as cervical and perirenal BAT arise from Myf5⁻ precursors (Luo and Liu 2016). Moreover, Myf5⁻ brown cells have also been identified in WAT depots, probably stemming from the activation of dormant stem cells (Saely et al. 2012) or from WAT browning.

iii. Morphology and function

As opposed to WAT, brown adipocytes are characterized by small but multiple lipid droplets and a particularly high density of large, spherical mitochondria and express important levels of uncoupling protein 1 (UCP1) (Figure 6) conferring these organelles a high oxidative capacity (Ye et al. 2023). Brown adipose tissue (BAT) main function is to dissipate energy through heat, a process called nonshivering thermogenesis which relies largely on β -oxidation of fatty acids. Besides this energy source, brown adipocytes also have a significantly high rate of glucose uptake, which can not only serve as for ATP production and FA synthesis, but has also implicated this tissue in the regulation of energy balance, obesity and insulin resistance. Increasing brown adipocyte mass through BAT transplantation was indeed revealed to upregulate insulin-stimulated glucose uptake in endogenous BAT, WAT, and heart, which was accompanied by improved glucose tolerance, insulin sensitivity and body weight despite a high-fat diet (Stanford et al. 2013). Finally, BAT can also act as an endocrine organ capable of secreting a number of molecules (i.e. FGF21) that can regulate glucose homeostasis and contribute to the thermogenic machinery. Despite this high metabolic profile however, this tissue is relatively resistant to obesity-induced inflammation (Luo and Liu 2016).

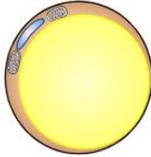
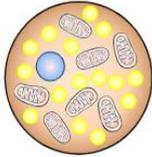
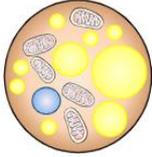
	White adipocyte	Brown adipocyte	Brite adipocyte
			
UCP1	UCP1 negative	UCP1 positive	UCP1 positive
Mitochondrial density	Low mitochondrial density	High mitochondrial density	Intermediate mitochondrial density
Lipid droplets	Single lipid droplet	Numerous multilocular lipid droplets	Mixture of small and large lipid droplets
Function	Lipid storage Secretion of adipokines (leptin/adiponectin)	Thermogenesis Secretion of adipokines	Thermogenesis Secretion of adipokines

Figure 6: Morphological and functional differences between white, brown and beige adipocytes. White adipocytes are characterized by a single lipid droplet taking up most of the cellular space and are implicated in lipid storage. Brown adipocytes present multiple small lipid droplets and a high mitochondrial density. Beige/brite adipocytes present an intermediate phenotype. Adapted from Evans et al., 2019.

1.3. Beige/brite adipose tissue

i. Localization

Given the transient state of beige adipocyte (white adipocytes that adopt a brown-like phenotype = browning), there is no particular depot of beige AT. It seems however that in mice, the location of

beige adipocytes was mostly observed in the inguinal region (Vitali et al. 2012) (Figure 5). This has been attributed to a differential capacity of distinct WAT depots to undergo browning, with inguinal WAT being able to better respond to browning stimuli than gonadal/epididymal WAT by upregulating genes implicated in thermogenesis (Jia et al. 2016). In regards to human beige AT, it has been principally observed in the supraclavicular and neck regions (Cheng et al. 2021).

ii. Origin

Beige adipocytes can either originate from the trans-differentiation of mature white adipocytes, or from the *de novo* differentiation of AT progenitors in WAT depots (Myf5⁺ cells). The differentiation process is induced by a number of stimuli, including cold exposure, exercise or hormonal regulation. Among the factors that stimulate browning we find norepinephrine, FGF21, BMP7, among others (Brestoff and Artis 2015, Maniyadath et al. 2023). Interestingly, WAT browning capacity has been found to be reduced in white adipocytes from obese individuals in comparison with lean subjects (Carey et al. 2014).

iii. Morphology and function

Beige adipocytes are characterized with an intermediate phenotype between white and brown adipocytes in terms of lipid droplet size and number of mitochondria (Ye et al. 2023)(Figure 6), thereby representing the bridge between lipid storage and thermogenesis and highlighting the relevance of WAT browning as a therapeutic target in obesity and T2D. This notion has been supported by the fact that selective ablation of beige but not brown adipocytes by targeting the PRDM16 gene negatively impact diet-induced obesity, insulin resistance and steatosis (Cohen et al. 2014).

For the remaining of this manuscript and given the prominent role of WAT in the development of obesity and diet-induced inflammation and IR, we have chosen to focus only on this particular AT type. Nevertheless, the contribution of other colors of AT to whole body homeostasis and cardiometabolic outcomes should not be overlooked. These are detailed elsewhere (van Marken Lichtenbelt et al. 2009, Virtanen et al. 2009, Kusminski et al. 2016, González et al. 2017, Morigny et al. 2021).

2. White adipose tissue in health and disease.

2.1. Metabolic role

Lipid turnover in WAT is governed by a delicate balance between lipid input (synthesis and storage in lipid droplets) and lipid output (removal by lipolysis or export). On average, the lipid content of an adipocyte is renewed six times on a 10-year lifespan, as assessed by ¹⁴C measurement, and that lipid turnover rate strongly increases with BMI, suggesting an increase in lipid input but a decrease in output

(Arner et al. 2011). These mechanisms are controlled by a number of nutritional and hormonal regulations which will be detailed below.

i. The lipid droplet (LD)

LD assembly begins with the accumulation of small amounts of esterified cholesterol and TG in the ER. The resulting oil lens is a lipid droplet intermediate that expands and is released from the ER membrane in a mechanism that is called budding. Phospholipids (PL) and other surface proteins (i.e. seipin, perilipins) then surround the nascent LD and facilitate its growth and maturation (Olzmann and Carvalho 2019). The main function of LD is to maintain a readily available stock of FA that can be mobilized when needed (lipolysis, see below). Another, equally important role is to protect the cell against FFA-induced lipotoxicity. Indeed, FA can exert deleterious activities (i.e. disrupt membrane order, participate in the synthesis of cytotoxic lipid species such as ceramides, induce activation of inflammatory pathways, etc) when found in their free form. Esterification and sequestration of FFA in the form of TG inside LD therefore represents a protective mechanism where these lipids are rendered neutral.

ii. Lipid storage and synthesis

Most of the lipids that are stored in adipocyte LD originate from TG-rich lipoproteins (TRL) in the circulation such as VLDL and chylomicrons. Lipoprotein lipase (LPL) allows the hydrolysis of these TRL and the subsequent release of FFA, that then enter the adipocyte either through passive diffusion or through interaction with membrane-specific receptors such as the translocase CD36, fatty acid binding proteins (FABPs), caveolin 1 (Thompson et al. 2010). Diffusion-mediated FA transport across the lipid bilayer occurs by rapid FA flip-flop into the internal cellular membrane (Simard et al. 2008) and is mostly associated to low plasma long-chain FA concentrations. On the contrary, FA transport through membrane transporters is slower but can be stimulated by dietary and molecular stimuli such as insulin (Chabowski et al. 2007). Of note, uptake of larger lipid particles (i.e. remnant VLDL still containing FA) is also possible but is rather mediated by other receptors such as VLDLR. Once FA are inside the cell, they can then be converted into Acyl-CoA before esterification into a glycerol-3-phosphate TG backbone by sequential action of several acyltransferases including AGPATs and DGATs (Morigny et al. 2021), which allows their final storage in LD (Figure 7).

TG can also originate from endogenous glucose by a process called *de novo* lipogenesis (DNL). In this case, glucose enters the cell through the GLUT4 transporter and is then metabolized into citrate via glycolysis and the tricarboxylic acid (TCA) cycle. Citrate is the first substrate required for DNL, as it allows the production of acetyl-CoA and malonyl-CoA which are in turn used for palmitate (PA) synthesis by fatty acid synthase (FAS). PA can then be esterified into a TG backbone for storage,

although certain modifications in terms on saturation degree and chain length can be performed beforehand (Morigny et al. 2021). While DNL is stimulated by both insulin and glucose either by upregulation of transporters implicated in FA and glucose uptake, or simply by increasing substrate availability, high-fat diets have been shown to inhibit this mechanism (Song et al. 2018). In accordance, DNL in WAT has been found to be significantly decreased in obese individuals compared to lean subjects (Eissing et al. 2013). This process is nonetheless essential for AT homeostasis, as adipocyte-specific inhibition of DNL was observed to cause insulin resistance and inflammation in chow and HFD-fed mice (Vijayakumar et al. 2017), and to be associated with metabolic risk markers in humans (Eissing et al. 2013).

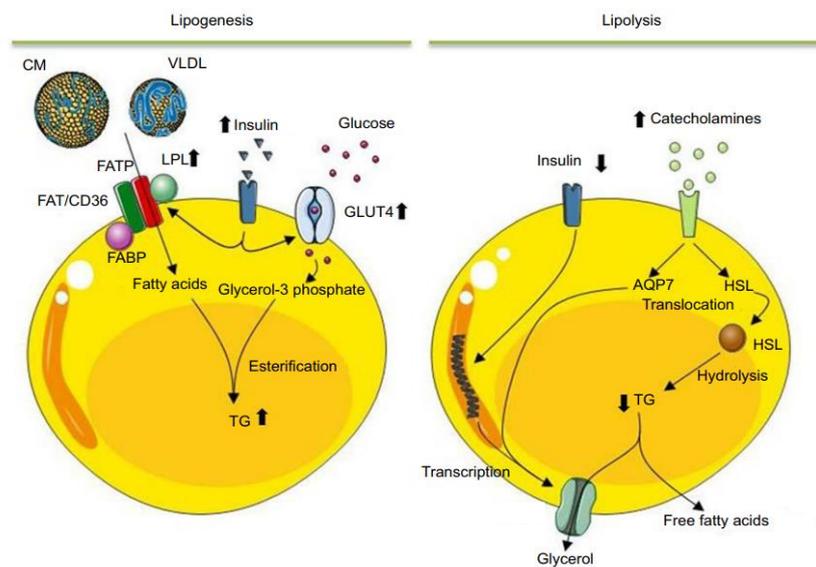


Figure 7: Lipogenesis and lipolysis pathways in mature adipocytes. In lipogenesis, triglyceride (TG)-rich lipoproteins such as chylomicrons (CM) and very-low-density lipoprotein (VLDL) are hydrolyzed by lipoprotein lipase (LPL) thus resulting in the liberation of FA acids (FA). These FA enter the adipocyte and are esterified for storage in the form of TG. These TG can then be hydrolyzed by the opposing mechanism called lipolysis, which is mediated by the sequential action of several intracellular lipases. The resultant FA can then be secreted into the circulation. Adapted from Badimon et al., 2015. FABP, fatty acid-binding protein; FAT/CD36, fatty acid translocase; FATP, fatty acid transport protein; GLUT4, glucose transporter type 4; HSL, hormone-sensitive lipase.

iii. Lipid release

Periods of high energy demand such as fasting, physical exercise or stress induce the mobilization of lipid stores in LD. TG breakdown is performed by the sequential action of three lipases: first, adipose triglyceride lipase (ATGL) catalyzes TG hydrolysis into diacylglycerol (DAG), which is then converted into monoacylglycerol by the hormone sensitive lipase (HSL). The final step involves monoacylglycerol lipase (MGL) for the release of the last FA from the glycerol backbone. The signals that mediate this process are insulin, catecholamines and natriuretic peptides, although certain inflammatory cytokines

such as TNF have also been found to participate in its regulation (Morigny et al. 2021) (Figure 7). Furthermore, the activity of lipases is highly dependent on their interaction with other proteins, as well as on their localization near the LD membrane. For instance, ATGL recruitment to the LD surface and subsequent activity depend on its interaction with CG1-58 and perilipin 5, the first factor enhancing ATGL lipolytic action and the latter inhibiting it (Wang et al. 2011). Other pathways of lipid mobilization alternative to lipolysis have been described and include lipophagy (LD are engulfed by lysosomes and degraded after the action of lysosomal acid lipase (LIPA)), and the release of LD-derived exosome-like vesicles (Flaherty et al. 2019, Zhang et al. 2022). Both these mechanisms are active in lean states, although their rate significantly goes up in obesity (Flaherty et al. 2019), suggesting they are highly dependent on adipocyte size.

2.2. Endocrine role

Originally thought of as a simple reservoir, AT is now known as a major endocrine organ that can synthesize and release a large number of molecules (i.e. hormones, cytokines, growth factors) collectively called adipokines. This allows adipocytes to communicate with other cells within the AT but also with extra-adipose tissues and thus influence whole body homeostasis.

i. Leptin

Leptin is a 16kDa protein coded by the *Ob* gene and secreted in proportion to fat mass (Greenberg and Obin 2006). In obesity, leptin secretion levels are significantly increased, studies have shown that chronically elevated concentrations of this adipokine induce resistance to its action in target cells by activation of specific inhibitors such as the suppressor of cytokine signaling 3 (SOCS-3) (Münzberg et al. 2004). Since its receptor is expressed in most cells, leptin has a plethora of targets and functional responses involving lipid and glucose metabolism, energy intake and expenditure, among others. As already seen, leptin signaling in the hypothalamus controls satiety and energy expenditure through the leptin-melanocortin pathway (Farooqi 2022), thereby facilitating body weight loss. Besides this major role, leptin is also able to inhibit hepatic gluconeogenesis (Rossetti et al. 1997) and increase insulin sensitivity in skeletal muscle and BAT while blunting it in WAT (Pereira et al. 2021). These beneficial effects on liver and muscle have been attributed to leptin capacity to stimulate fatty acid oxidation and glucose uptake in these tissues in a JAK/STAT3, MAPK and PI3K-dependent manner (Wein et al. 2007, Obradovic et al. 2021). This adipokine has also been found to reduce both insulin and glucagon secretion *in vitro*, but its contribution to β -cell mediated insulin secretion *in vivo* is still unclear. This has been mostly the result of inefficient targeting of β -cells only (Pereira et al. 2021). Coherent with its actions, leptin or leptin receptor deficiency has been characterized as deleterious, given that both human and mice (*ob/ob* background) lacking this gene are hyperphagic, develop early-

onset obesity and severe insulin resistance (Lindström 2007, Dubern and Clement 2012). These metabolic parameters are normalized following daily injection with recombinant leptin (Pelleymounter et al. 1995, Dubern and Clement 2012), although it also seems to enhance ATM infiltration. This has also been accompanied by enhanced cytokine production and bioenergetic capacity of ATM. Interestingly, if leptin receptor deficiency is performed in myeloid cells only instead of whole body, the impairment of leptin signaling has been revealed to be beneficial regarding obesity-induced IR and inflammation (Monteiro et al. 2022). Altogether, these observations capture the complex role of leptin in obese states, and highlight the importance of specific targets for the treatment of this disease.

ii. Adiponectin

Adiponectin is a 30kDa protein encoded by the AdipoQ gene and is the highest secreted adipokine by AT (Greenberg and Obin 2006). It harbors insulin-sensitizing properties, notably by improving glucose and lipid metabolism in muscle and liver. This is mediated by its binding to at least 3 membrane receptors: T-cadherin, which induces adiponectin recruitment at the cell surface, and AdipoR1 and AdipoR2, which promote glucose uptake, fatty acid oxidation and lipid clearance via AMPK, PPAR α and p38-MAPK-dependent pathways (Kusminski et al. 2016, Choi et al. 2020). Unlike leptin, adiponectin levels are inversely correlated to BMI, IR and hyperinsulinemia, and low levels of this adipokine are associated with increased risk of T2D and metabolic syndrome (Greenberg and Obin 2006). As such, mice constitutively expressing adiponectin have been shown to be protected against diet-induced hyperlipidemia and glucose intolerance (Kusminski et al. 2016). Regarding inflammation, adiponectin has been revealed to have both beneficial and detrimental roles according to effector tissue, with adiponectin levels being positively correlated with inflammatory status and disease progression in autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease, but negatively correlated to those same parameters in metabolic disease (Choi et al. 2020). For instance, in atherosclerotic plaques, studies have shown that adiponectin is capable of inhibiting macrophage recruitment and foam-cell formation by lowering IFN γ and CCL2 secretion (Sargolzaei et al. 2018). In AT, it is able to induce macrophage anti-inflammatory polarization via an AMPK- and PPAR γ / α -dependent pathways, resulting in the upregulation of IL-10 and mannose receptor CD206 expression (Stijn et al. 2015). Finally, it has also been associated to mild ER stress and anti-apoptotic properties (Kusminski et al. 2016, Oh et al. 2020).

iii. Others

Aside from the two adipokines reviewed above, WAT secretome potentially comprises around 600 proteins implicated in a vast array of biological processes. These include: inflammation and macrophage recruitment (i.e. IL-6, TNF α , IL-8, IL-1, CCL2, CXCL10), lipid and glucose metabolism (i.e.

ANGPTL4, resistin, chemerin), blood pressure (i.e. renin and angiotensin), cell adhesion (i.e. PAI-1), vascular growth (i.e. VEGF), among others (Fasshauer and Blüher 2015). These molecules can be secreted by all the different cell types that are present in WAT, although the contribution of the adipocyte fraction vs the stromal vascular fraction to their secretion changes dramatically under obese conditions. Indeed, it has been revealed that inflammatory cytokines and chemokines are primarily released by the non-fat fraction of WAT, particularly by adipose tissue macrophages in vWAT and that their production is considerably enhanced during DIO (Fain 2010, Siklova-Vitkova et al. 2012). This is consistent with the fact that ATM numbers significantly rise under obese conditions.

3. The stroma vascular fraction

SVF profiling has long been of interest in the understanding of adipose tissue biology, with flow cytometry being the go-to method for the characterization of the different SVF cell types according to the expression of distinct cell markers. The emergence of single cell transcriptomics then came to complement this method and allowed to identify up to 19 clusters of SVF cells in human AT that can be broadly regrouped into 3 major classes: stem/progenitors cells, vasculature-associated cells and immune cells (Figure 8). More importantly, several of these populations saw their frequency and proportion change during obesity, with endothelial and myeloid cell number positively correlating with patient BMI (Hildreth et al. 2021). Similar clusters and associations have been seen in lean and obese mice WAT (Sárvári et al. 2021).

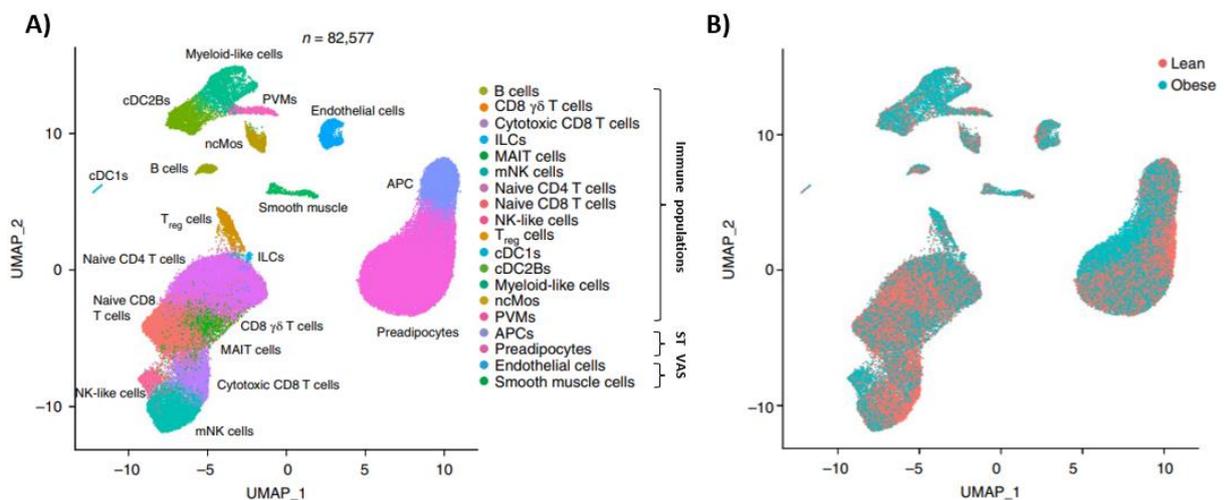


Figure 8: Cellular heterogeneity of the stromal vascular fraction of human AT revealed by single cell RNA-sequencing. A) UMAP plot depicting the different cell types found in human WAT. B) UMAP plot indicating the variation in the proportion of these populations during steady-state and obese conditions. Adapted from Hildreth et al., 2021. ILCs: innate lymphoid cells; MAIT: mucosal-associated invariant T cells; mNK: mature natural killer; cDC1s: conventional type 1 dendritic cells; cDC2Bs: conventional type 2B dendritic-like cells; ncMos: non-classical monocytes; PVMs: perivascular macrophages; APCs: adipocyte precursor cells; ST: Stem cells, VAS: Vasculature-associated cells.

3.1. Adipose stem cells (ASC)

ASC are multipotent mesenchymal stem cells that are able to differentiate towards multiple lineages including cardiomyocytes, fibroblast, chondroblasts, hepatocytes, myoblasts and adipocytes, among others. They reside near the vasculature and are characterized by the expression of numerous surface markers such as CD34, CD90, CD105, PDGFR α/β , but they are CD45- and CD31-negative (corresponding to leukocytes and endothelial cells, respectively. See below). However, some of these markers may change according to when and where the stem cell was originated (i.e. Myf5⁺ in retroperitoneal but not inguinal depots, SMA⁺ in adulthood but not in the developmental phase) (Shin et al. 2020), supporting the notion that ASC are a highly heterogenous population. Indeed, when studying their adipogenic potential, studies have shown that ASC ability to grow, differentiate and react to environmental cues is different between WAT depots. For instance, ASC from scWAT present a higher differentiation capacity into adipocytes than those from vWAT, which might potentially explain why vWAT adipocytes preferentially respond with hypertrophy instead of hyperplasia in obesity (Badimon and Cubedo 2017). vWAT ASC have also been found to secrete higher levels of pro-inflammatory cytokines IL6 and CCL2 (Silva et al. 2017). It is important to note that obesity further enhances the differences between these depots but also induces other transcriptional and secretory changes that contribute to impaired ASC function. The loss of the *stemness* phenotype (proliferation, differentiation and regenerative potential) is reduced as a consequence of obesity-induced chronic inflammation and T2D. This can also be accompanied by a reduction of insulin sensitivity, an increased production of ROS, and an altered energetic metabolism (Badimon and Cubedo 2017, Shin et al. 2020). The capacity of ASC to recover from the obesogenic stimuli is not accomplished for all of their functions even after weight loss (Silva et al. 2015).

3.2. Vasculature-associated cells

Capillaries consist of thin layer of endothelial cells (EC), encased by pericytes and supported by smooth muscle cells in a hierarchical and branched structure surrounded by connective tissue (Ramakrishnan and Boyd 2018, Ioannidou et al. 2022). AT is a highly vascularized tissue in which EC and adipocytes continuously communicate and impact each other renewal and function. EC represent around 8% of all cells in WAT and are classically identified by the expression of the surface marker CD31, although several subsets have been identified and are as follows: FABP4, CD36, LGALS1 for EC associated to fatty-acid handling microvasculature EC, the largest population; LYVE1 for lymphatic vasculature EC, mostly present in vWAT; and VCAM, PECAM, ACKR1, among others, for classic EC (Vijay et al. 2020). These cells participate in trans-endothelial nutrient uptake and transport, LPL-anchoring and subsequent interaction with TRL for hydrolysis, production of signaling molecules (i.e. VEGF for

angiogenesis) and even facilitate adipogenesis by serving as a niche of ASC. During obesity, all these functions are impaired notably due to rarefaction of capillaries that accompanies abnormal AT expansion. In this context, the angiogenic response of EC is inhibited by the inflammatory environment of obesity (Nijhawans et al. 2020). This induces a reduction of blood flow and the development of AT hypoxia, further contributing to inflammation. Indeed, WAT of overweight and obese subjects revealed to present 44% lower capillary density and 58% lower VEGF, which positively correlated with increased ATM infiltration (Pasarica et al. 2009). Obesity-induced EC-dysfunction has also been shown to trigger vascular permeability and pro-inflammatory cytokine expression by these cells, thus enhancing IR in WAT (Ioannidou et al. 2022).

3.3. Immune cells

Single cell and single nucleus RNA-seq studies have revealed that the immune population (identified by the CD45 marker) within SVF is quite heterogeneous and comprised of a multitude of cells comprising T lymphocytes, natural killer (NK) and non-NK cells (29% of immune cells); dendritic cells, ATM and monocytes (67%); and three minor populations of mast and B cells (4%), altogether representing 30 to 40% of total cells within the SVF in lean conditions. So far, only T cells have been identified as presenting a different proportion according to the type of depot, with CD4⁺ T cells being enriched in omental WAT and CD8⁺ T cells being abundant in scWAT (Massier et al. 2023). Regarding their response to diet, ATM have been found to be the most significantly upregulated population in both obese mice and humans (Hildreth et al. 2021, Sárvári et al. 2021), with studies showing that ATM may account for 40 up to 60% of all cells in obese WAT, including adipocytes, and that this enrichment is directly proportional to adipocyte size and BMI (Weisberg et al. 2003).

This substantial accumulation of ATM is of particular interest in the pathogenesis of obesity through several mechanisms. ATM participate to maintaining AT plasticity and remodeling by buffering FFA from hypertrophic and dying, necrotic-like adipocytes. In states of chronic FA surplus however, ATM lipid buffering capacity is overwhelmed, which induces cellular stress and the production of a vast repertoire of inflammatory cytokines (IL-1 β , IL-6, TNF α) (Figure 8). This in turn drives IR, low-grade inflammation and AT dysfunction. Specific depletion of these cells by intraperitoneal injection of clodronate liposomes or diphtheria-toxin mediated genetic ablation thus protects mice from high fat diet-induced weight gain, improves insulin sensitivity, glucose homeostasis and inflammatory status (Patsouris et al. 2008, Feng et al. 2011, Bu et al. 2013). Similarly, selective targeting of epididymal ATM genes implicated in lipid and inflammatory pathways (LPL and TNF α , respectively) confirmed their definitive role in obesity-associated metabolic disorders by altering ATM lipid and glucose metabolism and modifying whole-body glucose tolerance (Aouadi et al. 2013, 2014). ATM-mediated inflammation

and IR during DIO represent the core of my research, and further details on these cells (origin, markers, metabolism and dysfunction) will be developed on the next section of this manuscript.

As for the other immune cell types in SVF, studies have shown that CD4⁺ Th1 and CD8⁺ T cells actively contribute to the cytokine pool in obese AT as well as the activation of resident ATM (Vijay et al. 2020). Coherently, lymphocyte-deficient mice have been observed to be more susceptible to HFD-induced insulin resistance and obesity (Lee et al. 2011). B-cells are also able to trigger local inflammation by the production of cytokines and the activation and support of local Th17 cells. (Khanna et al. 2022). Mast cells, NK cells and dendritic cells have also been implicated in modulating ATM infiltration and T cell activation (Kawai et al. 2021). In dendritic cells, this has been linked to an altered antigen presenting capacity resulting from a drastically distinct transcriptomic profile following HFD, which was observed to favor fatty acid oxidation, ROS accumulation and inflammation (Chen et al. 2022). Finally, neutrophils have been found to initiate a rather early inflammatory cascade by the production of IL-1 β and the recruitment of ATM (Kawai et al. 2021) (Figure 9).

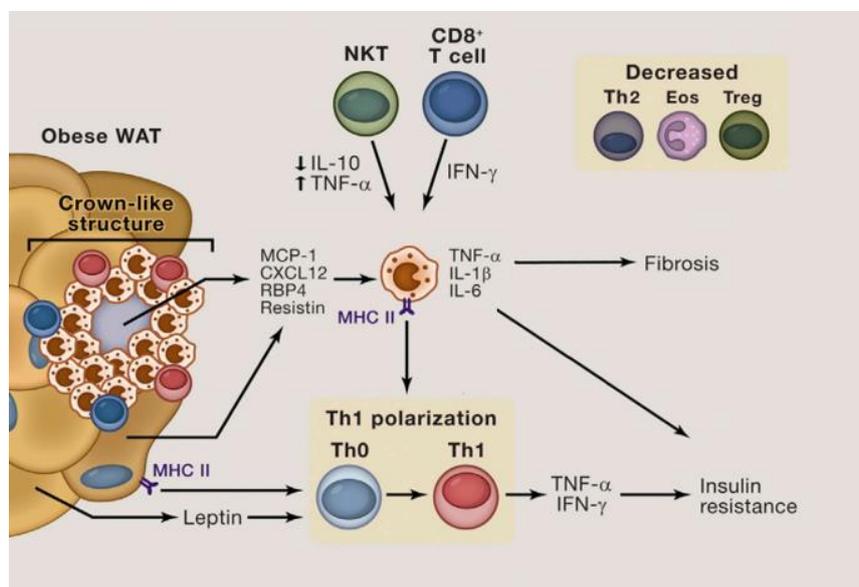


Figure 9: Obese adipose tissue is characterized by an increased infiltration of ATM and a pro-inflammatory cytokine immune response. Adipocyte hypertrophy during obesity is associated with a significant recruitment of ATM responsible for the installment of an inflammatory environment leading to insulin resistance. Other immune cells participate to inflammation by promoting activation/recruitment of ATM or secreting themselves inflammatory molecules. NKT: natural killer cells; Eos: Eosinophils; MCHII: major histocompatibility complex II. Adapted from Brestoff et al., 2015.

Overall, DIO compromises WAT function through several mechanisms, including adipocyte and adipose progenitors dysfunction, loss of vascularization and hypoxia, and high ATM recruitment (Figure 4). While these effects are manageable and reversible during short-term caloric excess, it is important to know that obesity has long-lasting consequences on AT biology and its responsiveness to metabolic

stimuli even after obesity remission, notably that of the immune compartment, which may ultimately worsen metabolic health (Cottam et al. 2018, Lee et al. 2018).

Part III: Adipose tissue macrophages in obesity-induced inflammation

1. ATM origin

1.1. Monocyte recruitment and myelopoiesis

AT from healthy subjects is populated by resident ATM originating from embryonic yolk-sac progenitors and are maintained via proliferation during adulthood, with little contribution of bone marrow (BM)-issued monocytes (Félix et al. 2021). However, obesity-induced inflammation has shown to drive the migration and recruitment of Ly6C/G⁺ monocytes that undergo differentiation in AT. Indeed, by assessing the expression of the Cd45.1 (donor) or Cd45.2 (recipient) allele in lethally irradiated, high-fat diet (HFD)-fed mice, Weisberg et al., demonstrated that around 80% of ATM from obese rodents were Cd45.1+, thus establishing obesity-induced ATMs were essentially BM-derived (Weisberg et al. 2003). Among the chemokines responsible for this recruitment, the chemokine CCL2 and its receptor CCR2 have been attributed a major role in this process, as mice deficient for either of these genes have reduced ATM content and altered metabolic parameters (Weisberg et al. 2006). Aside from the CCL2/CCR2 axis and other chemokine-signaling modules (i.e. CCL3, CCL5), it appears that obesity-derived ligands such as FFA and IL1-β promote myelopoiesis itself, thus directly contributing to the ATM pool (Nagareddy et al. 2014, Griffin et al. 2018).

The question that remains is whether ATM are phenotypically different when originating from embryonic precursors versus bone marrow monocytes. In this regard, circulating monocytes are known as a quite heterogenous cell population, with differences both in phenotype and function. Classical (CD14⁺CD16⁻ in humans; Ly6C^{hi}Ccr2⁺CX3CR1^{lo} in mice) and intermediate (CD14⁺CD16⁺) monocytes are more inflammatory, whereas non classical monocytes (CD14^{dim}CD16⁺⁺) are related to tissue repair (Pecht et al. 2016). Classical monocytes also present higher migratory and lipid storage capacities along with metabolic gene signatures for enhanced glycolysis and mitochondrial activity, as revealed by transcriptomic profiling (Schmidl et al. 2014). Most of these subclasses are reported to be increased in obesity (Schipper et al. 2012).

1.2. Proliferation and local regulation of ATM numbers

Proliferation of resident ATM is another mechanism responsible for increasing ATM content. As such, a time-dependent increase in ATM proliferation under DIO was observed *in vitro*, *in vivo*, and in human fat explants. Despite the divergency on the proportion of proliferating ATM (from 10-50% in mice vs 5-18% in humans), all studies agreed the highest rate was seen in ATM surrounding adipocytes in what is called a crown-like structure (CLS). Potential stimuli were found to be IL-4/IL-6 and CCL2, as neutralization/deficiency of either of these molecules led to impaired ATM proliferation and lower ATM presence in AT (Amano et al. 2014, Haase et al. 2014, Braune et al. 2017).

In addition to proliferation, evidence suggests that local stem cells progenitors may give rise to ATM, notably given that pre-adipocytes isolated from AT express ATM markers and are able to carry out macrophage-like functions such phagocytosis (Boutens and Stienstra 2016). Further studies are however warranted.

Most likely, both local proliferation of resident ATMs plus monocyte migration and proliferation contribute to the excessive ATM content in obese AT, although they do so at different stages. These two events were addressed in a rodent model of selective irradiation, where the epididymal AT of recipient Cd45.2 mice was shielded to protect resident ATMs from irradiation, prior to the reconstitution with BM from Cd45.1 donor mice. An 8-week HFD induced a significant increase in total CLS-associated ATM content as expected; however, only a minimal percentage (<2%) of these macrophages were BM-derived. Interestingly, when HFD feeding was pursued for another 4 weeks, the percentage had shifted to nearly 22%. EdU incorporation assays indicated that both resident and BM derived ATMs had a vast proliferating capacity which was under the control of the IL-4/STAT6 pathway. Yet, whereas resident ATMs were highly proliferative at the early onset of obesity, BM derived ATMs did so at a later stage (Davies et al. 2013, Zheng et al. 2016). Additional research is very much needed to better comprehend these mechanisms that may ultimately help target ATM-mediated inflammation.

2. ATM heterogeneity in obesity

2.1. The M1/M2 continuum concept of macrophage classification

Multiple macrophage activation and polarization states have been described in literature and vary along a continuum that resembles the Th1/Th2 inflammatory response (Locati et al. 2020) (Figure 10). In lean AT, resident ATM seem to adopt an “alternative activated”, M2 phenotype characterized by the expression of surface marker CD206, along with pan-macrophage markers CD11b, CD64, CD163, CD301 and F4/80 (the latter in mice) or CD14 (in humans). They present an anti-inflammatory phenotype characterized by the expression of molecules such as arginase-1, Ym1 and IL-10 (Figure 10) (Mosser and Edwards 2008, Yao et al. 2022, Nawaz et al. 2023). Their bioenergetic status is rather low and depends on oxidative phosphorylation (OXPHO) rather than glycolysis for energy production. These ATM perform tissue surveillance and remodeling functions by buffering necrotic-like adipocytes and debris in a process called efferocytosis. For this, M2 ATM organize in CLS around dying adipocytes and buffer lipids released from these cells. Although CLS are only occasionally found in lean AT, the lipid buffering and storing capacities of M2 ATM help maintain tissue homeostasis by impeding ectopic lipid accumulation and lipotoxicity in the first stages of energy surplus (Russo and Lumeng 2018).

By contrast, the M1, or “classically activated” macrophages have been suggested to represent the principal ATM population in obese states as a consequence of monocyte recruitment. They are characterized by the expression of CD11c (in mice and humans) or CD16 (in humans only), as well as pro-inflammatory cytokines such as TNF α , iNOS, IL-6, IL-1 β and CCL2 and a high bioenergetic status dependent on glycolysis (Russo and Lumeng 2018, Nawaz et al. 2023). Evidence suggests that M1 ATM accumulate massively around CLS in obese WAT, as the rate of hypertrophic and dying adipocytes goes up. This shifts in the ratio of M1 and M2 ATM is evidenced by an increased production of pro-inflammatory cytokines, which promote inflammation, further AT dysfunction and support IR (Lumeng et al. 2007).

While the M1/M2 dichotomy has been often employed in obese settings, it poorly portrays the various challenges and metabolic functions performed by ATM during obesity. Consistent with this postulate, both M1 and M2 phenotypes have been observed on a single, heterogenous population of ATM in obese mice (Li et al. 2010). This, coupled with the lack of detection of classical M1 markers in obese humans (Kratz et al. 2014) has suggested the presence of an ATM subset characteristic of obese states, which has been under much focus in these recent years.

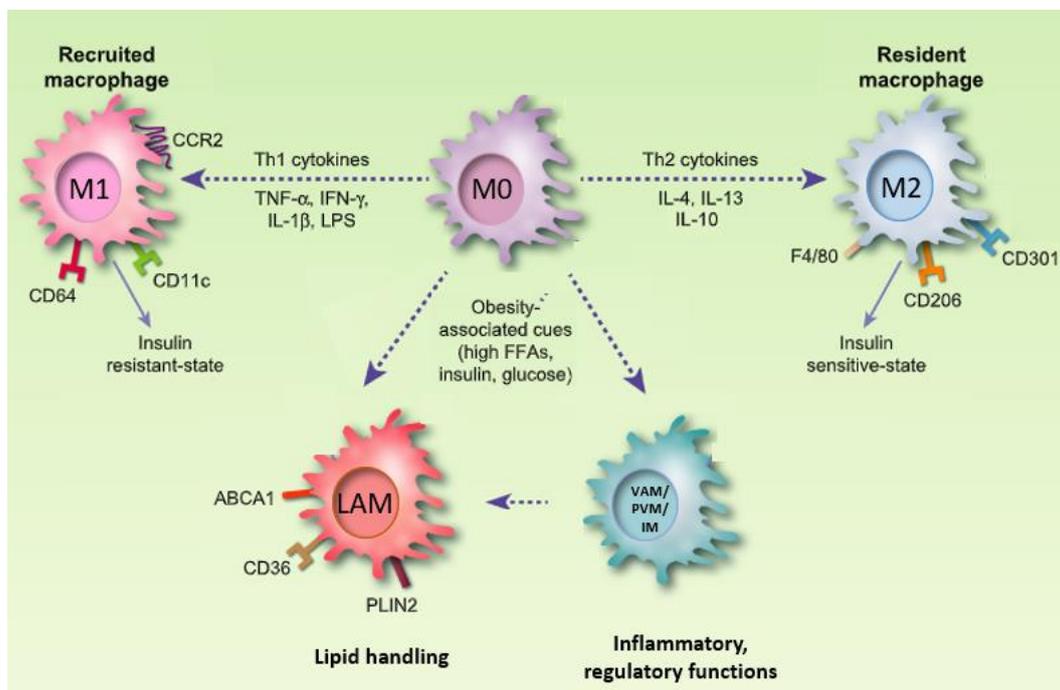


Figure 10: Adipose tissue macrophage polarization profiles. Depending on the stimuli, macrophages (M0) can differentiate into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages. The particular environment of obesity (rich in FFA, insulin and glucose) gives rise to lipid-associated macrophages (LAM) that are specialized in lipid handling. Other macrophages subsets have also been observed. Adapted from Russo and Lumeng, 2018. VAM: Vasculature-associated macrophages; PVM: perivascular macrophages; IM: inflammatory macrophages.

2.2. Metabolic classification of ATM

The advancement in single-cell technologies for in-depth analysis of adipose immune cell populations have made it possible to better understand the full heterogeneity and plasticity of mice and human ATM under obesogenic stress. This has led to the identification of several ATM subpopulations, where some are more metabolically active (i.e. lipid-associated macrophages) or more inflammatory (i.e. inflammatory macrophages) and regulatory (i.e. (non)-perivascular macrophages).

i. Lipid-associated macrophages

One of the first accounts of an ATM phenotype in obesity independent of classical activation was evidence by Xu and coll., where it was proposed that obesity induces a program of lysosomal lipid catabolism that influences adipocyte health and lipid clearance in both adipocytes and ATM beyond their inflammatory phenotype. Indeed, the authors observed that ATM from obese AT display increased lysosomal biogenesis which directly influences ATM intracellular lipid content by governing FA catabolism but has no effect on M1 polarization. Coherently, inhibition of this pathway was evidenced to promote FA accumulation in these cells (Xu et al. 2013). Further reports indicated that activation of macrophages with insulin, glucose and palmitate, all of which are readily available in DIO, generated a metabolically active macrophage phenotype (MMe) that is mechanistically distinct from M1 or M2 states. These ATM specifically express high levels of lipid-associated proteins such as ABCA1, CD36, PLIN2 along with lysosomal surface proteins and are capable of both beneficial (lysosomal catabolism of lipids) and detrimental (inflammatory cytokine production) roles in obesity that seem to be regulated by the NOX2/TLR/MyD88 axis (Kratz et al. 2014, Coats et al. 2017). As such, NOX2 inhibition in myeloid cells was found to impair dead adipocyte clearance by ATM through defective lysosomal lipid metabolism, thus resulting in ectopic fat accumulation and insulin resistance. Interestingly though, these adverse effects were only seen in mice who underwent a long-term (16 weeks) HFD. Instead, Nox2-deficient mice under a 8-week HFD displayed a protective phenotype, attributed to NOX2 capacity to activate inflammatory cytokine expression (Coats et al. 2017). Other similar, highly catabolic ATM are the MARCO⁺ macrophages, whose role in lipid metabolism and metabolic health during DIO have been observed to rely on the PI3K/AKT pathway by inducing the expression of the lipid buffering macrophage receptor with collagenous (MARCO) protein (Brunner et al. 2020).

From then on, high-resolution single-cell or single-nucleus RNAseq studies of murine and human obese eWAT allowed to identify a unique ATM subpopulation highly reactive to lipids and now consensually termed lipid-associated-macrophages (LAM) (Figure 10). LAM have been proven to be the predominantly expanded ATM subset in obese AT and preferentially recruited from BM monocytes

to CLS. Their persistence has also been evaluated during weight loss, showing that despite weight normalization this particular population remains higher than lean controls, and that weight regain prompts it to increase to numbers much more important than the obese group (Cottam et al. 2018). LAM signature is characterized by expression of CD9 and CD63 (lysosomal endocytosis), Lpl, Lipa, Fabp4/5, CD36, Trem2 (for lipid metabolism) and Atp6v0d2, among other ATPases (involved in OXPHOS), as well as a dual expression of canonical CD206 and CD11c. All these markers are present in both mice and humans (Hill et al. 2018, Jaitin et al. 2019, Hildreth et al. 2021, Sárvári et al. 2021). LAM have also been shown to present a hypermetabolic phenotype characterized by both increased OXPHOS and glycolysis and a pro-inflammatory capacity independent of Hif-1 α (Boutens et al. 2018, Serbulea et al. 2018b). Accordingly, these parameters are reduced in ATMs from lean patients (Serbulea et al. 2018b).

This LAM metabolic program is thought to be under the control of Trem2, as both whole body and hematopoietic Trem2 deficiency was found to enhance HFD-induced weight gain, adipocyte hypertrophy and death, hypercholesterolemia, glucose intolerance, inflammation and IR (Jaitin et al. 2019, Liu et al. 2019). This role has however been contested following studies suggesting that Trem2 beneficial actions regarding metabolic health are independent of its expression on ATM, instead showing that they are a result of altered sphingolipid metabolism (Sharif et al. 2021). More studies are therefore needed to properly identify the molecular drivers of the LAM phenotype. Of note, a smaller subset of LAM has been identified as expressing proliferative genes (termed proliferative LAM, P-LAM), indicating that LAM may expand by proliferation if needed (Sárvári et al. 2021).

ii. Other ATM subsets

Inflammatory macrophages (IM) represent another ATM population that has been described in WAT and that are markedly increased in obesity (Figure 10). They are characterized by the expression of numerous cytokines and chemokines such as CCL3L1, TNF and CXCL3, thus contributing to AT inflammation alongside LAM. Analysis of surface marker expression revealed that they are somewhat similar to M1 ATM, given that they are CD206⁻CD11c⁺ (Hildreth et al. 2021).

Various studies have also identified a population of resident ATM involved in vascular development and extracellular matrix maintenance and remodeling (Chakarov et al. 2019, Silva et al. 2019, Sárvári et al. 2021). These vasculature-associated macrophages (VAM), also called perivascular macrophages (PVM) have been found to be negatively correlated to obesity (approximately 40% decrease in numbers), although it is not clear if this is due to a differentiation of PVM into LAM and IM, as it has been found that obesity leads to a marked increase in the expression of genes involved in lipid handling in PVM which significantly overlaps with LAM markers (Sárvári et al. 2021). Nonetheless, PVM are

classically identified by the expression of Lyve1 but also M2 markers such as CD206, CD163 and Clec10A. Interestingly, they have also been observed to produce high levels of chemokines (CCL3, CCL3L1, CCL4 and CCL2), suggesting that they may directly contribute to adipose tissue inflammation and systemic insulin resistance. A similar population of regulatory non-perivascular macrophages (NPVM), as well as collagen-expressing macrophages (CEM), have also been observed in lean and obese eWAT, with NPVM equally able to transition to a LAM-like signature (Sárvári et al. 2021).

Finally, antioxidant (Mox) and iron-rich (MFe^{Hi}) macrophages have also been observed in AT but have been reported to be predominant in lean states (Orr et al. 2014, Serbulea et al. 2018b, 2018a). Overall, we see that ATM functions are quite heterogenous and depend on the metabolic context (Table 2), with obesity rather reprogramming ATM towards lipid uptake, storage and catabolism (=LAM phenotype).

ATM subset	Highly expressed genes	Stimuli	BMI correlation	Roles in obesity	Reference
M1	CD11c	Pathogens TLR ligands (LPS), SFA Hypoxia Cytokines (CCL2, TNF, IFN γ)	+	Pro-inflammatory cytokine production (IL-1 β , TNF α , IL-6, iNOS...)	(Lumeng et al. 2008, Orliaguet et al. 2020)
M2	CD206, CD163	Helminths Hormones Apoptotic adipocytes Cytokines (IL-4, IL-10)	-	Anti-inflammatory cytokine production (IL10) Fight against infection Tissue homeostasis	Idem
LAM	CD9, Lpl, Lipa, CD36, Trem2, ATPases	Dietary lipids Apoptotic adipocytes Cytokines (CCL2, IL-6...)	+	Lipid handling Phagocytosis Cytokine production (IL-10, IL-6, TNF α ...)	(Hill et al. 2018, Jaitin et al. 2019, Hildreth et al. 2021, Sárvári et al. 2021)
P-LAM	Pola1, Kif11, Kif15	Dietary lipids Apoptotic adipocytes	+	Idem	Idem
MME	Abca1, CD36, Plin2, ATPases	Glucose Insulin Palmitate	+	Idem	(Kratz et al. 2014, Coats et al. 2017)
MARCO	Marco, CD206, CD11c	Apoptotic adipocytes	+	Lipid handling	(Brunner et al. 2020)
IM	Ccl3l1, Tnf, Cxcl3, CD11c	Cytokines/chemokines (IL-6, TNF α , CCL2, CXCL10...)	+	Cytokine production (IL-1 β , TNF α , IL-6, iNOS...)	(Hildreth et al. 2021, Sárvári et al. 2021)
VAM/PVM	Lyve1, CD163, CD206, Clec10a	Oxidative stress and redox/hypoxia	-	Vasculature development and organization Lipid handling	Idem + (Silva et al. 2019)
NPVM	CD74, Fcrls, Eat5	Apoptotic adipocytes PPAR signaling	-	Lipid handling	(Hildreth et al. 2021, Sárvári et al. 2021)
CEM	Col5a2, Col3a1, Tgfbr3	Oxidative stress and redox/hypoxia	-	Extracellular matrix homeostasis	Idem
MOX	Txnrd1	Oxidized phospholipids	-	Production of antioxidant enzymes	(Serbulea et al. 2018b, 2018a)
MFE ^{HI}	Ferritin light and heavy chains, CD163, Ferroportin	Iron	-	Iron regulation	(Orr et al. 2014)

Table 2: ATM subpopulations in adipose tissue.

3. Mechanisms controlling ATM polarization.

ATM polarization states can be driven by non-metabolic/canonical stimuli (i.e. cytokines, chemokines, transcription factors) and metabolic effectors (i.e. intracellular fatty acid metabolism and bioenergetics). For instance, M1 ATM arise in response to IFN γ , LPS, TNF, IL-1 β or IL6, while M2 ATM do so in the presence of IL-4 and IL-10, LAM ATM respond to the particular environment of obese ATM (Mosser and Edwards 2008, Kratz et al. 2014). Adipocytes play an important part in guiding these activation states in both lean and obese conditions, as they actively contribute to the expression of inflammatory molecules such as IL4, TNF α or CCL2 which dictate ATM profile (Engin 2017). The molecular mechanisms initiated by this crosstalk results in the activation of several signaling pathways (Figure 11) and metabolic adaptations, which we will review below.

3.1. *Transcriptional control of ATM profile*

i. Surface/nuclear receptors and transcription factors

TLRs have often been placed at the center of inflammatory signaling and macrophage activation. They are structurally conserved molecules that directly or indirectly interact with numerous ligands such as LPS or FA. TLR2 and TLR4 have been particularly implicated in diet-induced inflammation, poor glycemic control and insulin resistance (Gupta et al. 2017) through the activation of four major transcriptional programs driven by interferon regulatory factors (IRFs), the activator protein (AP)-1, NF- κ B and JAK/STAT (Drareni et al. 2019). IRFs are a family of transcription factors controlling the pro-inflammatory type-1 interferon response in macrophages and have thus been implicated in DIO and metabolic inflammation. IRF5 for instance is associated to normal adipocyte expansion, and inhibition of his factors has been found to restrict adipocyte size, induce ATM accumulation and pro-inflammatory polarization and the development of insulin resistance (Dalmas et al. 2015, Orliaguet et al. 2022). AP-1 inflammatory capacity relies on its induction of the JNK and MAPK proteins that control apoptosis (Ameyar et al. 2003). JNK has been extensively studied in obesity and insulin resistance, with studies showing that inhibition of this kinase is able to reduce body weight gain and ameliorate metabolic homeostasis (Tuncman et al. 2006). In a similar manner, NF- κ B promotes insulin resistance by the production of inflammatory cytokines. Accordingly, its inhibition in myeloid cells has been found be beneficial for maintaining insulin responsiveness (Arkan et al. 2005). Lastly, the JAK/STAT pathway has been associated to both pro-inflammatory (STAT1/5) and anti-inflammatory (STAT3/6) ATM profiles through stimulation of inflammatory cytokine production and reducing lipid accumulation, respectively (Gong et al. 2017, Drareni et al. 2019).

As for nuclear receptors, PPARs (mostly PPAR γ , but also β and δ) expression has been linked to the M2 phenotype, as it is capable of promoting oxidative and lipid metabolism, plus inhibiting M1-

associated signaling (Mosser and Edwards 2008, Orliaguet et al. 2020). Inversely, the hypoxia inducible factor (Hif)-1 α has been found to induce the expression of the GLUT1 transporter and other glycolysis intermediates, as well as IL-1 β , thereby supporting a pro-inflammatory state (Sharma et al. 2020). Its deletion in myeloid cells has therefore been seen to protect against HFD inflammation and the recruitment of ATM to CLS (Takikawa et al. 2016). The role of LXR in macrophage polarization is also present but will be detailed further down.

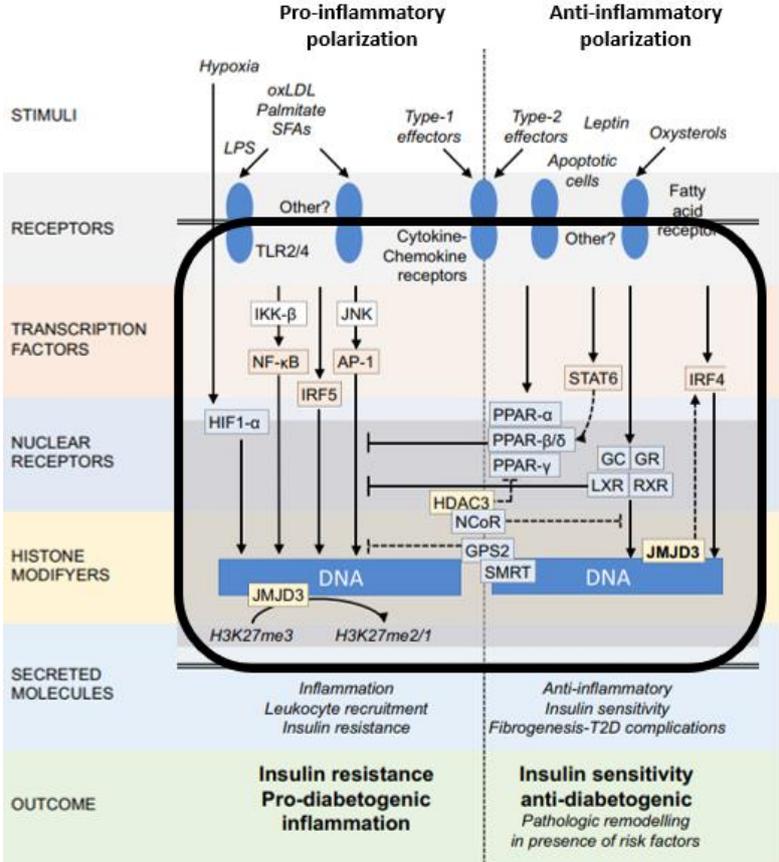


Figure 11: Major transcriptional pathways involved in macrophage polarization and the outcomes of these states at tissular (secreted molecules) and systemic level. Macrophage activation profiles are governed by a multitude of molecules that include surface and nuclear receptors, transcription factors and transcriptional repressors (histone modifiers). Adapted from Drareni et al., 2019.

ii. Epigenetic mechanisms

Epigenetic mechanisms controlling transcriptional activity or the accessibility of transcription factors to target genes has been evidenced in macrophages upon a number of stimuli (Figure 11). Activation of the transcription corepressor NCoR, for instance, is observed following TLR4 activation. Although NCoR is mostly responsible for repressing inflammatory pathways by forming a repressive complex with HDAC3, its inhibition in macrophages has also been seen to influence the activation of lipogenic genes that participate in the synthesis of anti-inflammatory ω -3 FA (Glass and Saijo 2010, Li

et al. 2013). The corepressor GPS2 has also been implicated in macrophage activation during obesity and T2D in mice and humans, and macrophage-specific knockout of this factor was seen to increase pro-inflammatory gene signaling that translated into impaired insulin sensitivity (Fan et al. 2016). Finally, repressive epigenetic marks promoting pro-inflammatory macrophage profile mediated by the action of KDM6B (also known as JMJD3) have also been observed (De Santa et al. 2009).

3.2. Metabolic control of ATM profile

i. Intracellular lipid metabolism

The metabolism of dietary/exogenous lipids (uptake, storage and or/clearance, as seen above) by ATM modifies the cell's intracellular metabolic status and the activity of specific pathways implicated in endogenous lipid synthesis. This regulation of ATM intracellular lipid metabolism is achieved essentially through two major families of transcription factors, the sterol regulatory element-binding protein (SREBP) and the liver X receptor (LXR), who translocate to the nucleus and induce or repress the transcription of genes involved in FA and cholesterol synthesis and efflux, thus altering ATM function.

FA synthesis in macrophages is a process activated by both LXR and SREBP1 when FA and glucose levels are high, and depends on the action of FAS (DNL, see chapter 2 for details). DNL has been found to be required for monocyte differentiation into macrophages (Ecker et al. 2010), and its inhibition in ATM through FAS deficiency has been revealed to have beneficial effects on glucose tolerance and IR by reducing ATM recruitment to obese AT and proinflammatory cytokine expression (Wei et al. 2016). Interestingly, when looking at the transcription factors alone, SREBP1 has been revealed to exert both pro-inflammatory and anti-inflammatory roles in a biphasic, time-dependent manner, with an early response associated to enhanced inflammatory cytokine production and impaired FA synthesis, and a late, resolutive phase contributing to anti-inflammatory lipid-mediator production that counteracts lipotoxicity (Shimano and Sato 2017, DeBose-Boyd and Ye 2018). LXR activation has also been observed to exert anti-inflammatory, insulin-sensitizing effects in mice by reducing cytokine and chemokine production, promoting lipid efflux and the production of anti-inflammatory lipid-mediators (Archer et al. 2013, Wang and Tontonoz 2018, Endo-Umeda et al. 2022). This has been attributed to LXR and SREPB capacity to modulate the expression of genes involved in polyunsaturated FA synthesis. Among their target genes we find 3 different enzyme families: ACLS3-4 for FA activation, the ELOVL family for elongation and FADS1-2 for desaturation. Deficiency on any of these enzymes following LXR or SREPB inhibition in macrophages has thus been found to lead and increased inflammatory response (Varin et al. 2015, Oishi et al. 2017, Jalil et al. 2019).

As opposed to DNL, cholesterol (Chol) synthesis is a mechanism activated when intracellular Chol levels are low, but it can also be stimulated by insulin. It involves the SREBP2 isoform that acts in concert with the cleavage-activating protein (SCAP) and the insulin-induced gene (INSIG) for transcriptional induction of genes implicated in Chol synthesis and uptake (i.e. LDLR), while inhibiting those implicated in lipid efflux (i.e. ABCA1/G1)(DeBose-Boyd and Ye 2018, Luo et al. 2020). The Chol biosynthetic pathway involves more than 15 enzymes that catalyze over 30 reactions. The starting point comprises 3 successive condensations steps of acetyl-CoA to generate HMG-CoA, which is then reduced into mevalonate by the rate-limiting enzyme HMG-CoA reductase. Numerous additional oxygenation and reduction steps either culminate in Chol synthesis or give rise to Chol oxygenated derivatives called oxysterols. These sterols can then bind LXR and activate its transcriptional activity, which in turn helps eliminate excess Chol (Wang and Tontonoz 2018). Interference with any of the steps involved in Chol metabolism has been associated with altered ATM function. For instance, myeloid deletion of HMG-CoA reductase has been shown to significantly reduce ATM accumulation, TNF α , IL-1 β and CCL2 expression, improved glucose tolerance and insulin sensitivity (Takei et al. 2020). Of note, statins, which are the pharmacological inhibitors of HMG-CoA reductase and are the forefront hypercholesterolemia/dyslipidemia treatment have shown to inhibit ATM pro-inflammatory polarization in both mice and humans by reducing Chol inflow into the cell, but also by attenuating the TLR4/MyD88 pathway therefore decreasing AT inflammation (Abe et al. 2008, Kauerova et al. 2021).

ii. Bioenergetics

As briefly mentioned before, different ATM subsets have different bioenergetic requirements that are supported by the glycolytic and/or mitochondrial pathways. Glycolysis is an anaerobic pathway that takes place in the cytosol and converts glucose into pyruvate through sequential action of several enzyme-catalyzed reactions, its end-products being ATP and NADPH. In aerobic conditions, pyruvate then enters the mitochondria, where it is decarboxylated into acetyl-CoA before entering the tricarboxylic cycle. This generates the production of reducing agents that donate electrons necessary to fuel the electron transport chain in the mitochondria and produce ATP in a process called oxidative phosphorylation (OXPHO). If pyruvate is scarce, cells can directly metabolize fatty acids through OXPHO (= fatty acid oxidation, FAO) to assure energy production. If however oxygen is limiting, cells can divert pyruvate into lactate production (El Kasmi and Stenmark 2015)(Figure 12).

Given their role as a source of energy, these pathways are able to dictate the magnitude of ATM effector function, with M1 and M2 macrophages relying on glycolysis and OXPHO, respectively, and LAM displaying a mixed phenotype. Conversely, inhibition of any of these metabolic routes alter ATM polarization state, with inhibition of glycolysis abolishing pro-inflammatory cytokine production, and

OXPHO reduction preventing M2 polarization (Van den Bossche et al. 2016, Boutens et al. 2018, Orliaguet et al. 2022). The mechanism governing the transition between a highly inflammatory to a highly oxidative phenotype has been attributed to the interferon regulatory factor 5 (IRF5), as mice deficient for this transcription factor have shown increased oxidation rates as a consequence of the derepression of the mitochondrial matrix protein GHITM and an increased mitochondrial surface area (Orliaguet et al. 2022). Similar implications of OXPHO controlling ATM phenotype have been suggested by a recent study in which OXPHO dysfunction selectively ablated pro-inflammatory lipid-handling in LAM, thus preventing insulin resistance and ectopic lipid accumulation (Wculek et al. 2023). It is important to mention that TG accumulation in LD seems to impair mitochondrial activity and promote apoptosis by inducing cytochrome dysfunction (Aflaki et al. 2011), thereby underlying the multitude of effects that obesity can have on the activation of these pathways. It also highlights the promise that ATM metabolic reprogramming represents as a strategy to achieve beneficial (anti-inflammatory while able to handle large amounts of lipids) polarization states despite exposure to dietary fatty acids during obesity.

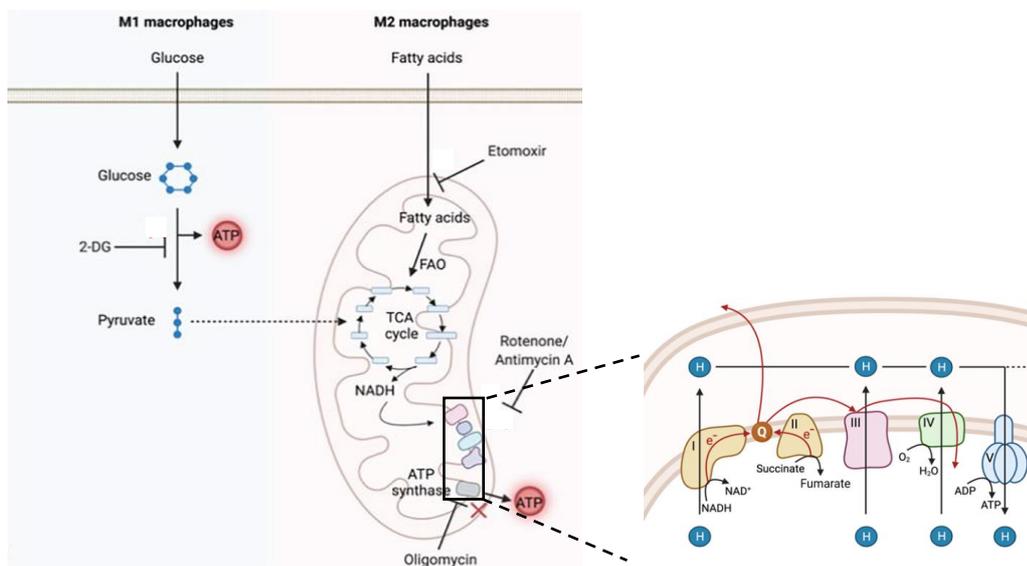


Figure 12: ATM energy metabolism and polarization status. Pro-inflammatory ATM rely on glucose for energy production (glycolysis), whereas anti-inflammatory ATM rely preferentially on the oxidation of fatty acids by the mitochondria (OXPHO). FAO: fatty acid oxidation. ETC, Electron transport chain; TCA cycle, tricarboxylic acid cycle; 2-DG: 2-deoxuglucose. Adapted from Thibaut et al., 2022 and Zhao et al., 2019.

4. Lipid handling by ATM

ATM are able to uptake and store lipids primarily by buffering cholesterol and FFA from circulation, or when these are released from dying, necrotic-like adipocytes. One of the first accounts of lipid-laden ATMs in human obesity was observed by Shapiro and coll., who managed to associate “AT foam cell”

presence to fasting glucose and insulin levels (Shapiro et al. 2013). This increase in lipid-laden ATMs was maximal at peak adipocyte size (Muir et al. 2018). Given that the origin (i.e. circulating vs adipocyte-issued) of these lipids is determinant to macrophage activation, the following section will concentrate on the mechanisms of lipid handling by obese ATMs and its implication in macrophage pro- or anti-inflammatory signaling (Figure 13).

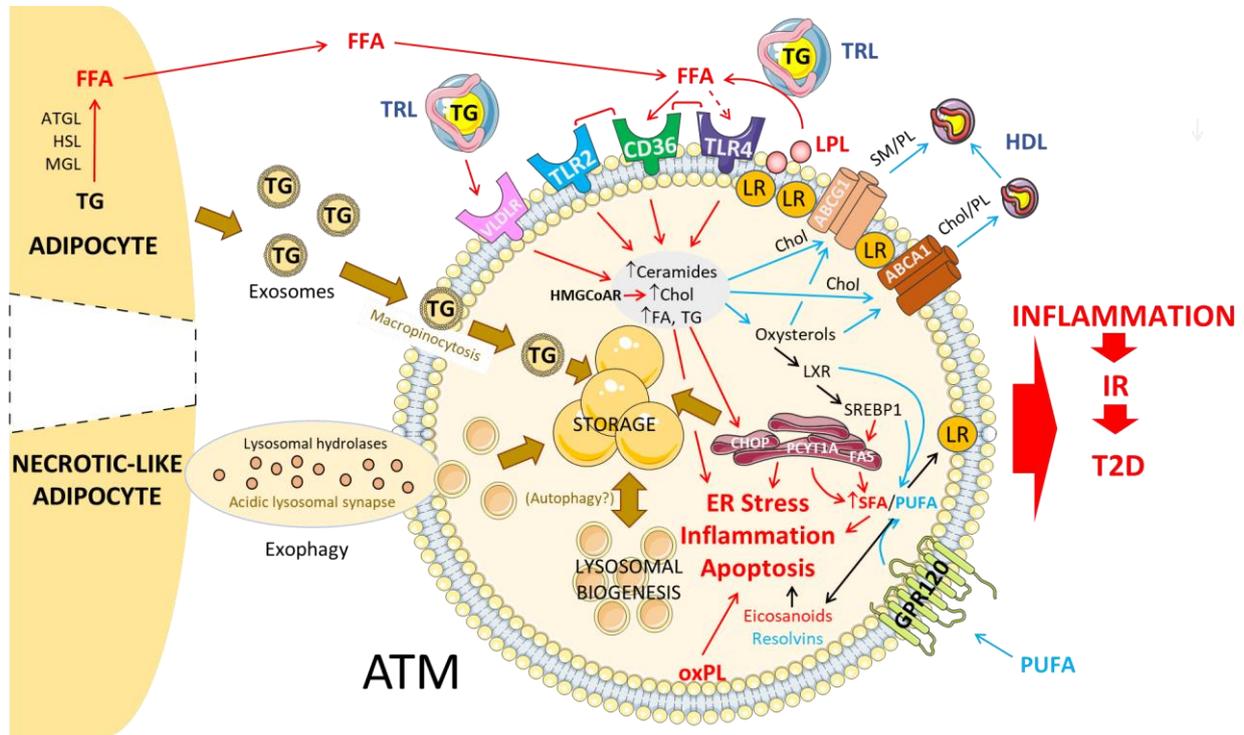


Figure 13: Lipid handling in adipose tissue macrophages in diet-induced obesity. In DIO, adipose tissue macrophages are metabolically activated by FFA released by lipolysis of TG from adipocytes or from TRL by lipoprotein lipase, through CD36, TLR or other specific receptors such as VLDLR. In CLS, necrotic-like adipocyte clearance in acidic lysosomal synapses through a mechanism of exophagy also contribute to lipid-laden ATM. Internalization of adipocyte-derived exosomes is possible. As a result, ATMs are characterized by an increased content of TG, free Chol, ceramides and FA, which can either be synthesized into other lipids, be stored in lipid droplets or catabolized through the lysosomal pathway. The accumulation of free cholesterol and sphingolipids including sphingomyelin into membrane lipid rafts promotes the recruitment of TLR4 at the cell surface and contributes to the activation of inflammatory signaling pathways. ABCA1 and ABCG1 transporters promote the efflux of Chol from ATM to high-density lipoproteins allowing the reduction of lipid raft formation, thus ensuring an optimal LPL activity which participates to lipid-laden ATM. Fatty acid synthesis, elongation and desaturation are under the control of LXR and SREBP1 thus exerting an important role in the relative cellular content of saturated and polyunsaturated FA. Increased cellular levels of free Chol and SFA trigger ER stress, inflammation and apoptosis, which can be alleviated by an enrichment in PUFA through mechanisms dependent or independent of the GPR120 receptor. Increased turnover of phosphatidylcholine as well as oxidation of phospholipids also participate to the inflammatory status of ATM. As a whole, rewiring of lipid metabolism in ATM promotes adipose tissue inflammation and contributes to the establishment of insulin resistance and type 2 diabetes. ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; ATGL, Adipose triglyceride lipase; Chol, cholesterol; FAS, fatty acid synthase; FFA, free fatty acids; HMGCoAR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HSL, hormone-sensitive lipase; IR, insulin resistance; LPL, lipoprotein lipase; LR, lipid rafts; LXR, liver X receptor; MGL, monoglyceride lipase; oxPL, oxidized PL; PCYT1A, phosphate cytidyltransferase 1; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelin; SREBP1, sterol regulatory

element-binding protein 1; T2D, type 2 diabetes; TLR, toll-like receptor; TG, triglyceride; TRL, triglyceride-rich lipoproteins; VLDLR, very low-density lipoprotein receptor.

4.1. Circulating lipids: mechanisms of uptake into ATM

i. LPL-dependent FA release

As mentioned before, FA are released from TRL by LPL-mediated hydrolysis before interaction with surface receptors such as CD36 and internalization within the cell. Macrophages are an important source of LPL, and LPL retention at the plasma membrane or inhibition by tetrahydrolipstatin has been shown to reduce TG accumulation in human macrophages (Olivier et al. 2012). Similarly, specific ATM LPL genetic deletion as performed by Aouadi et al. has been shown to result in reduced lipid storage in these ATM, but also increased FFA serum levels and glucose intolerance (Aouadi et al. 2014), thereby highlighting the importance of ATM lipid uptake for whole-body metabolic homeostasis. As for CD36, its deletion in mice has been associated to a significant reduction (60-70%) of FA uptake by adipose tissue (Coburn et al. 2000), macrophage anti-inflammatory phenotype and improved insulin resistance (Kennedy et al. 2011).

ii. The VLDL/VLDLR axis

TRL may also be directly endocytosed by other membrane receptors without prior action of LPL, although studies have shown that LPL presence may facilitate and even promote this interaction and consequential lipid uptake independently of its catalytic activity (Merkel et al. 1998). One of the principal receptors mediated in this endocytosis pathway is the VLDL receptor (VLDLR), which directly binds VLDL and other large remnant particles. VLDLR expression and proper function have been observed to represent an important mechanism implicated in DIO and diet-induced inflammation, as SNPs of this gene have been found to be associated with BMI, cardiovascular risk and the metabolic syndrome in humans (Crawford et al. 2008, Huang and Lee 2022). In accordance to this, VLDLR overexpression in HFD-fed has been shown to increase TG and C16 ceramide content within ATM, thus leading to ER stress, ATM pro-inflammatory polarization and insulin resistance. Improved AT inflammation and insulinemia were however achieved after adoptive transfer of VLDLR KO BM to Wt mice even under HFD as a consequence of decreased macrophage infiltration into AT (Shin et al. 2017).

4.2. Adipocyte-released lipids: mechanisms of uptake into ATM

i. Lipolysis-dependent lipid release

FFA may also originate from the lipolysis of hypertrophic adipocytes by the consecutive action of lipolytic enzymes ATGL, HSL and MGL, as already described. While less than in adipocytes, these enzymes have been shown to be also expressed in macrophages, particularly those specialized in lipid

handling (Chandak et al. 2010). As expected, ATGL macrophage deficiency or inhibition has been associated to an increased intracellular TG content and, concomitantly, to a pro-inflammatory response driven by the increased availability of inflammatory lipid precursors and the production of IL-6 even in the absence of exogenous lipids (Chandak et al. 2010, van Dierendonck et al. 2022). In a HFD context however, reports have shown that ATGL downregulation by the Hypoxia Inducible Lipid Droplet Associated (HILPDA) protein does not alter inflammatory parameter in ATM nor metabolic parameters despite significant reductions in ATM lipid levels (van Dierendonck et al. 2020). This uncoupling between intracellular lipid content and inflammation warrants further investigation. ATGL has also been implicated in macrophage chemotaxis, phagocytosis, apoptosis and its capacity to use FFA as an energy substrate in mitochondrial β -oxidation (Radovic et al. 2012). In terms of cardiovascular health, global knock-out of this enzyme has been found to lead to a more detrimental phenotype, in which large amounts of lipids accumulate in the heart and cause premature death (Haemmerle et al. 2006). To a similar extent, global or tissue specific deficiency of HSL also impair metabolic health by increasing CCL2 levels and ATM infiltration, hepatic steatosis, systemic IR and T2D (Albert et al. 2014).

ii. Exosomes

An alternative lipid source for macrophages has been described by numerous authors, where lipid-filled exosomes are released by adipocytes and contribute to the proinflammatory phenotype of ATM (Zhang et al. 2016, Xie et al. 2018, Flaherty et al. 2019). This mechanism is independent of lipase activation, given that inhibition of ATGL in adipocytes does not seem to alter the distribution of these exosomes, which were incidentally found to increase ATM TG content by 8-fold without the need of DGAT1/2-mediated FA esterification. Moreover, the exosomes were not only able to induce ATM activation and lipid storage, but also lead to ATM-like differentiation from BM progenitors by activating a gene program characteristic of macrophages that reside exclusively in AT (Flaherty et al. 2019). This very same role of exosomes in ATM differentiation has been found to participate in the development of ATM-mediated glucose intolerance and IR (Deng et al. 2009). Finally, a link between the lysosomal and exosome pathways has also been established, as exosomes were found to deliver TG to lysosomes for catabolism and FFA release (Flaherty et al. 2019).

iii. Exophagy

Exophagy is an active mechanism of lipid release specifically performed by dying, necrotic-like adipocytes around CLS. It entails the formation of a tightly sealed acidic lytic compartment where lysosomal enzymes (i.e. proteases, phosphatases, lipases) released by ATM allow the hydrolysis and subsequent internalization of large amounts of lipids, as well as dead adipocyte fragments, into these

macrophages. This form of lipid uptake has been shown to lead to lipid-laden macrophages, as evidenced by LipidTOX labelling, and has been associated to inflammatory signaling (Haka et al. 2016, Maxfield et al. 2020).

4.3. Fate of lipids in ATM

i. Storage in the form of LD

Despite slight differences in the disposition and constitution of LD – one large unilocular vesicle formed primarily of TG in white adipocytes vs various smaller vesicles composed of both TG and cholesteryl esters (CE) in ATMs –, LD biogenesis rate and lipid uptake are increased in obesity (visceral ATM > subcutaneous ATM). This pathway of lipid repartitioning is mediated by the induction of lipid-specific programs particularly in LAM and has been shown to contribute to the dysfunctional phenotype observed during energy surplus (Shapiro et al. 2013, Olzmann and Carvalho 2019). Indeed, exacerbated lipid accumulation in LD can lead to ROS production and ER stress by upregulating the expression of the unfolded protein response proteins, JNK and ERK signaling (Boden et al. 2008), therefore driving ATM proinflammatory phenotype. LD development is also linked to decreased fatty acid oxidation as macrophages commit to TG synthesis. An increase in the production of inflammatory cytokine (i.e. IL-6, IL-1 β) and lipid mediators (i.e. PGE2) as well as impaired phagocytic activity secondary to LD formation can also contribute to inflammatory macrophage activation (Castoldi et al. 2020). Many other mechanisms of lipotoxicity have been described, it is noteworthy to highlight that failure of packaging excess lipids into LD due to restricted storage capacity may also drive ATM inflammatory phenotype. Indeed, lipids stores in LD can also participate to the production of cytotoxic lipid species such as ceramides or saturated fatty acids that are capable of inhibiting the Akt pathway and directly contribute to the installment of insulin resistance (Tanti et al. 2013, Lipke et al. 2022). The effects of these lipid species on ATM profile will be detailed below.

ii. Lysosome-mediated lipid handling

The contribution of the lysosome system to inflammatory signaling and lipid handling has been the subject of numerous studies. Lipid catabolism, as it occurs in lysosomes, is mainly achieved by the action of LIPA, whose deficiency and overexpression have been shown to alter immune – efferocytosis, T cell and macrophage function – and metabolic – insulin signaling, VLDL metabolism, ATM lipid content, lipid mediator synthesis – homeostasis (Grijalva et al. 2016, Schlager et al. 2017, Li and Zhang 2019). In ATMs, the lysosomal program has been associated to the MMe and LAM phenotypes, as evidenced by the enrichment of lysosome-relates genes *Lipa*, *Lamp2*, among others, in both obese mice and humans (Xu et al. 2013, Hill et al. 2018, Jaitin et al. 2019). As such, inhibition of this pathway by chloroquine has been revealed to significantly enhance lipid accumulation in ATM LD and to reduce

AT lipolysis, altogether reducing circulating lipids levels consistent with an ameliorated metabolic phenotype on both mice and humans (Powrie et al. 1993, Xu et al. 2013). Defective lysosomal exocytosis following Nox2 inhibition has also been shown to compromise effective dead adipocyte clearance by ATM, thus contributing to metabolic disorders in DIO (Coats et al. 2017). Coherent with these observations, studies have shown that lysosomal lipid catabolism induces anti-inflammatory ATM polarization in bone marrow derived macrophages (BMDM) and peritoneal macrophages in response to FFA, as assessed by oxygen consumption rate and M2 markers CD206, Relm α and CD301 (Huang et al. 2014). Interestingly though, while short hairpin RNA treatment targeting LIPA did result in impaired M2 polarization (Huang et al. 2014), hindering lysosomal function has failed to be associated to the classic inflammatory M1 profile (Xu et al. 2013). This pathway of cell-intrinsic lysosomal lipid handling may therefore represent one of the stimuli leading to the LAM phenotype observed in DIO.

iii. Autophagy

A complementary mechanism relying on the lysosomal pathway is thought to be autophagy (also termed lipophagy). This form of lipid mobilization into lysosomes does not conform to a simple role in energy balance, but is also involved in major cellular responses. In AT, for instance, autophagy is necessary for adipogenesis and adipocyte differentiation (Singh et al. 2009). In liver and atherosclerotic plaque macrophages, autophagy is required for LD clearance and normal macrophage function (Ouimet et al. 2011, Zhang et al. 2022). In obese ATM, however, autophagy's role is less clear. In vitro experiments on BM-derived ATM and mice under HFD confirmed autophagy upregulation in DIO; it was nonetheless found dispensable for ATM activation and lipid uptake, as evidenced by both its genetic and pharmacological inhibition (Grijalva et al. 2016). Contradicting results were found using a model of RAW macrophages exposed to AT conditioned media plus the FA oleate (OA) as a lipid source. This study showed that autophagy was able to either decrease lipid uptake and LD formation in ATM when targeted at the early steps, or to support LD biogenesis when targeted at a later stage (Bechor et al. 2017). These discrepancies could be partly explained by the differences between macrophage models (BM-derived ATM vs RAW macrophages) and lipid substrates (whole AT vs FA), but also by the existence of a lipid storage site other than LDs that can nonetheless fuse with lysosomes, but does not follow the autophagic route, as hypothesized by Flaherty and colleagues (Flaherty et al. 2019).

5. ATM inflammatory signaling: a role for membrane dynamics

A distinct process by which FA and Chol alter ATM inflammatory response in the context of obesity is through their major role as membrane components. Membrane composition and optimal membrane

properties (i.e. rigidity vs fluidity) are crucial for a number of cellular processes such as glucose transport (Hresko et al. 2016), immune cell function and inflammatory signaling (Stulnig et al. 2001, Ruyschaert and Lonez 2015, Wei et al. 2016, Petkevicius et al. 2019), AT expansion (Pietiläinen et al. 2011), and even insulin signaling (Ginsberg et al. 1981, Park et al. 2013), all of which are compromised during DIO. Membrane dynamics are therefore of particular interest in the study of diabetes-promoting inflammation and IR.

5.1. Overview of membrane lipid composition

The membrane lipid bilayer is composed of three major lipid classes: glycerophospholipids (GPL), sphingolipids (SL) and sterols (mainly Chol) (Figure 14). GPL are synthesized by the incorporation of a FA in the sn-1 or sn-2 position of a glycerol backbone, which is further coupled to a specific head-group (i.e. ethanolamine, choline). Among GPL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant in all cellular membranes, comprising up to 75% of total PL. Other less represented GPL are phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylglycerol (PldG)(van der Veen et al. 2017, Harayama and Riezman 2018). PC and PE are asymmetrically distributed in the plasma membrane, with PC being enriched in the outer leaflet, whereas PE preferentially locates at the inner leaflet. PC/PE biosynthesis occurs mainly through the CDP-choline or CDP-ethanolamine pathway (the Kennedy pathway), although a minor amount of PE is also obtained through the conversion of PE into PC by consecutive methylations of PE head-group by PEMT or through the decarboxylation of PS by PS decarboxylase in the mitochondria (van der Veen et al. 2017) (Figure 15).

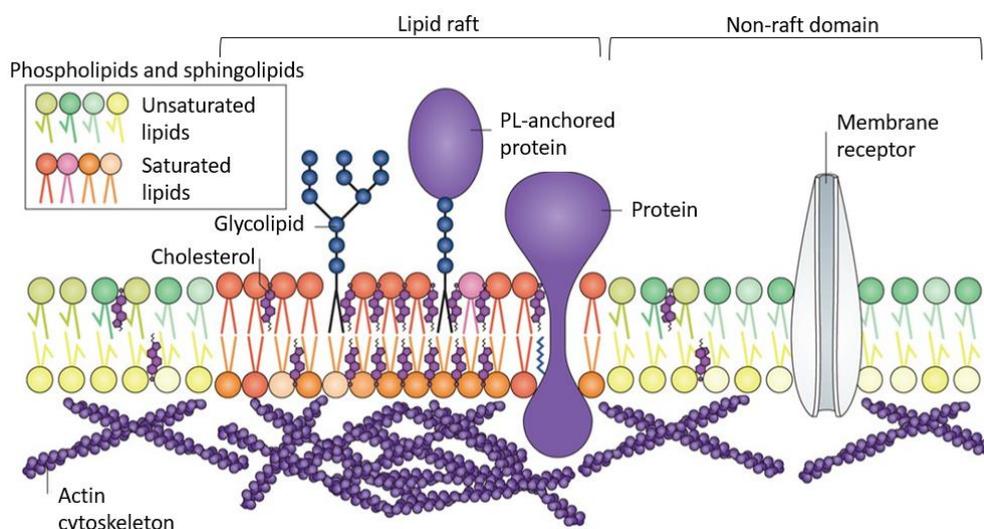


Figure 14: Membrane structure and composition. The membrane lipid bilayer is a highly heterogeneous structure containing phospholipids, sphingolipids and cholesterol. The proportions of these lipids in different regions (raft vs non raft domains) influences membrane properties but also protein anchorage and inflammatory signaling. Adapted from Sezgin et al., 2017.

Both the nature of the head-group and that of the FA can contribute to the overall properties of the membrane. As such, mono- or polyunsaturated FA (MUFA and PUFA, respectively) would increase membrane fluidity, while SFA would generate rigid domains. Membrane FA content remodeling can be performed by several mechanisms, the principal process implicating soluble or tissue-bound phospholipases (PLA) and lysophospholipid acyl transferases (LPLAT) that mediate the hydrolysis of the FA and its replacement into the resultant lysophospholipids, respectively, in a process called the Land's cycle (Hishikawa et al. 2014)(Figure 15).

Sphingolipids, on the other hand, are present at a lower abundance (<20%) and encompass ceramides, sphingomyelin (SM) and glycosphingolipids, with SM being the most abundant. The *de novo* synthesis pathway of ceramide begins with the condensation of a serine and a palmitoyl-CoA and involves multiple acylation and desaturation steps under the control of CerS and DES1 and DES2. Being both the end product and the precursor of more complex SL, ceramide is further metabolized in order to generate SM, glucosylceramide, sphingosine-1-phosphate, among others. It is interesting to note that SM and other complex SL can also be used the other way around to generate ceramides (Meikle et al. 2013, Harayama and Riezman 2018).

Lastly, Chol is the principal sterol synthesized by animal cells and is a highly abundant membrane component. In most cells, the Chol content of cellular membranes varies from <10-30%, increasing along the secretory pathway (ER < Golgi < Plasma membrane) (Subczynski et al. 2017). Due to its unique planar and rather rigid structure, Chol is able to decrease membrane fluidity by interacting with neighboring saturated SL and PL-anchored proteins. This particular interaction allows the creation of heterogenous, tightly packed and rigid microdomains (10-200 μ m) called lipid rafts that are implicated in the selective recruitment of other lipids and proteins necessary for intracellular signaling (Sezgin et al. 2017). Conversely, membrane regions with more unsaturated lipids and less Chol have been designated as the fluid, non-raft domains. Coexistence of both these domains assures regulation of distinct cellular processes as it controls mobility and translocation/anchorage of membrane receptors, intracellular proteins and cytoskeleton to the plasma membrane, thereby acting on their functionality and activation. Of note, excessive rigidification or fluidification of the plasma membrane hinders inflammatory signaling in macrophages (de la Haba et al. 2016). These changes in the context of obesity-induced ATM activation will be regarded in detail in the following sections.

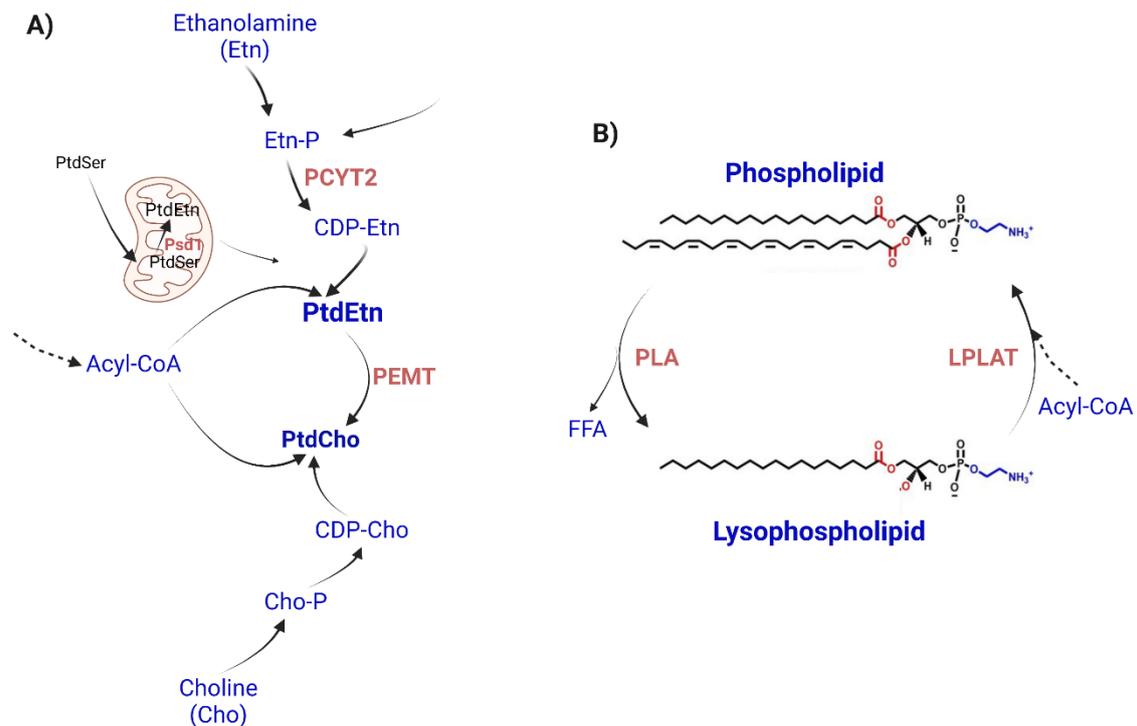


Figure 15: Mechanisms involved in phospholipid synthesis and remodeling. (A) The Kennedy pathway illustrating the steps involved in phosphatidylethanolamine (PtdEn) and phosphatidyl choline (PtdCho) synthesis, as well as the contribution of phosphatidylserine (PtdSer) to PtdEn production. (B) Schematic representation of the enzymes and substrates involved in the Land's cycle. PCYT2: Phosphatidylethanolamine cytidyltransferase; PEMT: Phosphatidylethanolamine N-Methyltransferase; PLA: Phospholipase; FFA: Free fatty acid; LPLAT: lysophospholipid acyl transferases.

5.2. Altered Chol content and membrane signaling

ATMs from obese mice are characterized by an accumulation of Chol, which was proposed to contribute to lipid-induced cytotoxicity during DIO (Prieur et al. 2011). Chol implication in inflammatory processes is mainly mediated by its role in lipid raft formation. For instance, these rafts have been found to favor the clustering and stabilization of the TLR machinery (Ruyschaert and Lonez 2015), with Chol loading of both plasma and endosomal membranes enhancing TLR4-dependent p38 activation in macrophages (Sun et al. 2009). This effect on inflammation was abrogated by Chol depletion in lipid rafts by ABCA1 and β -cyclodextrin, as it disrupted MyD88 recruitment to the membrane (Ito et al. 2015). Chol depletion was also able to reduce macrophage responsiveness to CCL2 (Umemoto et al. 2013). Similarly, changes in membrane Chol and lipid raft either by ABCG1 or FAS inhibition has shown to be associated with abnormal LPL retention at the plasma membrane and activity and disrupted Rho GTPase-dependent inflammatory signaling, respectively (Olivier et al. 2012, Wei et al. 2016). Rho GTPase decreased signaling was associated with reduced inflammation and IR, a phenotype which was rescued by exogenous sterols but not exogenous FA (Wei et al. 2016). Finally, accumulation of Chol in ER membrane was reported to induce cytotoxicity and apoptosis in

macrophages through activation of the cell death effector CHOP of the unfolded protein response (Feng et al. 2003).

5.3. Altered PL content and membrane signaling

i. Glycerophospholipids

An increasing number of studies have linked altered membrane GPL content to metabolic disorders. PC:PE ratio disturbance has been implicated in obesity-associated ER stress, inflammation, decreased insulin signaling and steatohepatitis (Li et al. 2006, Fu et al. 2011, van der Veen et al. 2017). Although most of these studies have focused on the effects of different GPL species on hepatocyte membranes, more recent data has found a similar relation between altered PC/PE levels and ATM activation. For instance, PC synthesis assessed by Pcyt1a expression and lysoPC content are increased in ATMs from genetically (*Lep^{ob/ob}*) obese, insulin-resistant mice. Myeloid-specific inhibition of endogenous PC synthesis improves *Lep^{ob/ob}* mice metabolic phenotype and lowers ATM inflammatory response to palmitate through reduced PC turnover at the plasma membrane of ATM, thus allowing incorporation of anti-inflammatory, membrane-fluidifying PUFAs (Petkevicius et al. 2019). Another study showed that LXR agonist-mediated insulin sensitizing and anti-inflammatory effects in obese mice were correlated to increased PC and PE species with high PUFA content (Archer et al. 2013). Mobilization of this PUFA pool from PE and PC therefore represents a protective mechanism in DIO. Coherent with this statement, hydrolysis of PUFA by ATM PLA2G2D has seen to attenuate adipose tissue inflammation by increasing WAT browning and the production of anti-inflammatory lipid mediators (Sato et al. 2020). Increased PldG has also been associated with anti-inflammatory ATM polarization, as daily PldG treatment of obese mice seemed to promote expression of M2 markers such as CD163 and CD301 and inhibit AT lipolysis, albeit through more indirect mechanisms (Kayser et al. 2019). It is interesting to note that overweight and obese subjects display increased serum levels of both PldG and PE, which are incidentally positively and negatively correlated to BMI and IL6 expression, respectively, suggesting a link between these PL and low grade, metabolic inflammation (Kayser et al. 2019).

ii. Sphingolipids

DIO has also been associated to dysfunctional SL metabolism. Among them, ceramides have been attributed highly inflammatory, insulin resistant properties that eventually lead to T2D. Ceramide accumulation is observed in plasma and visceral AT from obese women with metabolic syndrome and is accompanied by inflammatory marker expression (Choromańska et al. 2019). Pharmacological inhibition of short chain C16:0 ceramide synthesis has been correlated with lower weight gain in mice fed a HFD, as well as improved glucose tolerance and insulin sensitivity (Raichur et al. 2019). Ceramides

have been shown to induce pro-inflammatory ATM polarization and IR by activating the NLRP3 and TLR4 pathways (Shin et al. 2017, Kucuk et al. 2021). They have also been found to inhibit FAO, induce mitochondrial dysfunction and thus promote oxidative stress (Fucho et al. 2017). The role of SM in ATM-driven inflammation and IR has been less studied, but reports have shown that altered SM membrane turnover in mice is associated to a protective phenotype in diet-induced weight gain and IR by enhancing GLUT4 expression and insulin receptor activity (Song et al. 2019).

Globally, ATM metabolic activation in DIO is associated with a switch for more lipotoxic lipids. It is important to note that just as ATM membrane lipidome can influence inflammatory signaling, modulation of inflammatory pathways can also reshape lipid composition. This reciprocal relationship has been observed in lipid-loaded BMDM whose lipidome remodeling (mostly PC, but also cholesterol esters and TG) was observed after stimulation with a panel of TLR ligands (Hsieh et al. 2020). Lastly, we cannot rule out the contribution of other membrane lipids, such as phosphatidylinositol, phosphatidylserine, phosphatidic acid and plasmalogens to this signaling. Due to their low abundance in membranes, their effects might be masked by the other lipids. It is thus preferable to study membrane dynamics in relation to individual PL species and nature of the acyl chain.

5.4. FA nature and ATM-mediated inflammation

i. Saturated fatty acids

SFA are made up of a carbon chain containing only single bonds and are obtained through dietary intake of animal fats and plant oils or through DNL (small-chain SFA – 4 to 16 carbons – are synthesized by FAS, while those containing longer chains are obtained by elongation of already available SFA). These FA are known for their pro-inflammatory potential and are thereby positively associated with ATM inflammatory phenotype and proportion in obese AT (Poledne et al. 2019). SFA are implicated in NLRP3 activation; indeed, both palmitate (C16:0; the most abundant SFA) and stearate (C18:0) treatment of human macrophages is able to induce IL-1 β secretion by promoting rigidification of the plasma membrane and disrupting Na, K-ATPase activity and K⁺ efflux, a mechanism that has been long associated with inflammasome activation (Gianfrancesco et al. 2019). NLRP3 activation by SFA is also linked to the proinflammatory JNK pathway which has been found to exert a role in ATM-mediated IR and development of T2D during DIO (Talbot et al. 2014, Yung and Giacca 2020). Consistently, JNK-deficient mice were protected against glucose intolerance and IR, showed decreased ATM infiltration and M1-like polarization (Han et al. 2013, Hernandez et al. 2014).

Palmitate has also been found to induce NF- κ B activation in a TLR4 dependent manner, and macrophages from mice lacking TLR4 revealed blunted inflammatory cytokine production and increased insulin sensitivity (Shi et al. 2006). However, whether this SFA acts as a direct TLR4 ligand to

induce inflammation has been extensively challenged in recent years. The first piece of evidence showing PA is not a direct agonist of this receptor was brought in by Lancaster and coll., who demonstrated that PA treatment does not induce the conformational changes, dimerization nor endocytosis that characterize TLR4 active state. The absence of TLR4 in murine macrophages, although protective against PA inflammatory effects when used alone, is not sufficient to inhibit PA effects when cells had been previously primed with other TLR agonists. It is interesting to note that this priming of Tlr4-deficient macrophages was associated with a metabolic reprogramming that included upregulation of genes typical of M1-like metabolism and alteration of its lipidome, most notably ceramides, SM, PE plasmalogens and lysoPC/PE and cholesteryl esters (Lancaster et al. 2018). Similar observations have been made in studies for PA effect on ceramide production. Indeed, while PA alone was able to induce C16:0 ceramide synthesis and promote TNF α and IL-1 β secretion, this effect was independent of the presence of the TLR4 receptor (Schilling et al. 2013). Finally, palmitate is able to induce the expression of HIF-1 α in macrophages even under normoxic conditions (Sharma et al. 2020), providing another mechanism, independent of TLR4, for ATM activation during obesity. Other SFA such as lauric acid (C12:0) and stearic acid (C18:0) may also contribute to the overall inflammatory and insulin resistant profile seen in DIO (Wong et al. 2009, Seimon et al. 2010, Rogero and Calder 2018).

ii. Polyunsaturated fatty acids

As opposed to SFA, polyunsaturated FA (PUFA) present at least two or more double bonds in their acyl chain and are rather associated to an anti-inflammatory environment by inhibiting leukocyte chemotaxis, adhesion molecule expression and production of pro-inflammatory eicosanoids while upregulating that of specialized pro-resolving lipid mediators (SPM) (Calder 2017). When it comes to PUFA, the most abundant species are ω -3 and ω -6 PUFAs and are mostly obtained through diet (vegetable fats). The most prominent and commonly studied PUFA are α -linolenic acid (α -LA; C18:3n-3), arachidonic acid (AA; C20:4n-6), eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), the latter two sharing strong anti-inflammatory properties. Indeed AA, EPA and DHA are all increased in AT of T2D, obese women, with AA levels correlating to CD68 expression (Heemskerk et al. 2015). AA has been found to induce p38 and JNK signaling in human monocytes and also to generate a lipid-laden macrophage phenotype by promoting accumulation of TG, CE and AA itself in LD (Guijas et al. 2012). Increased intracellular AA content has also been associated to cell cycle arrest and apoptosis of macrophages (Pérez et al. 2006, Shen et al. 2018). Contradicting results have however arisen from the observation that AA is able to disrupt LPS- and SFA-mediated TLR4 activation in macrophages by directly binding to its co-receptor MD2 and decreasing the expression of its downstream targets NF- κ B and MAPK (Zhang et al. 2020). These differences may stem from the fact that AA functions are also a result of its ability to produce bioactive lipid mediators that can either

promote or instigate inflammation (Horrillo et al. 2010, Börgeson et al. 2012, Li et al. 2015a, Virtue et al. 2015, García-Alonso et al. 2016).

ω -3 PUFA such as DHA and EPA have been associated with increased membrane fluidity, repression of inflammation and insulin sensitivity in macrophages (Li et al. 2013, Mason et al. 2016). Indeed, anti-inflammatory M2 polarization and lipid catabolism is promoted in mice under a DHA/EPA rich diet, where shorter chain FA were used for β -oxidation while PUFAs were oriented to PL and pro-resolving lipid mediators production (Rombaldova et al. 2017). EPA alone also displays protective, insulin sensitizing effects, as supplementation of this PUFA prevented and even reversed AT inflammation and adipocyte hypertrophy, accounted for by adipocyte size, adipogenesis-related genes, ATM infiltration and FAO (LeMieux et al. 2015). DHA shares similar effects by promoting M2-like polarization in the AT of obese mice (Titos et al. 2011). These protective actions are most likely due to its ability to inhibit NF- κ B and NLRP3 inflammasome activation, as well as to target *de novo* ceramide synthesis and TLR4 recruitment into lipid rafts (Wong et al. 2009, Williams-Bey et al. 2014). Evidence also suggests that these FAs work in concert with the insulin sensitizing adipokine adiponectin, and that their neutralization augments IL-6, CCL2 and NOS2 expression, among others, as well as increases neutral lipid content within ATM (De Boer et al. 2015).

The discovery of the G-coupled protein receptor (GPCR) GPR120 and its interaction with DHA and EPA represent another mechanism accounting for the anti-inflammatory character of ω -3 PUFA (Im 2016). GPR120 is highly expressed in adipocytes and macrophages, and its deficiency abrogates the anti-diabetic effects that come with ω -3 PUFA supplementation. Indeed, DHA and EPA exposure on GPR120 null mice had no effect on monocyte/macrophage chemotaxis, M1-like and M2-like gene expression, as well as overall and tissue-specific insulin sensitivity (Oh et al. 2010, Im 2016). Importantly, the benefit of ω -3 PUFA for the treatment of metabolic disease has been observed in the ANCHOR trial, where supplementation of statin-treated type 2 diabetes women with a stable ω -3 FA (icosapent ethyl) reduced inflammatory parameters after a 12-week therapy (Ballantyne et al. 2012). Although less studied, MUFA also display anti-inflammatory properties that can oppose SFA action. A MUFA-enriched diet has been revealed to favor a more insulin sensitive phenotype in mice and humans, as well as reduced IL-1 β and NO secretion by macrophages, decreased the number of M1-like ATMs and limited adipocyte hypertrophy (Lima et al. 2014, Finucane et al. 2015, Souza et al. 2017).

Altogether, we see that ATM are at the crossroad of both lipid metabolism and lipid-mediated inflammation by adopting a specific program for handling these lipids as an attempt to control lipotoxicity, AT inflammation and the development of IR and T2D, but are ultimately overwhelmed. A better understanding of the mechanisms by which lipids shape ATM phenotype during DIO is thus

necessary to counteract this pathology, and will need the identification of new target genes capable of interacting with and modifying ATM lipidome. In that sense, my research also focused on a particular gene, *ABCG1*, which is strongly implicated in lipid metabolism, but whose contribution to ATM activation and ATM-mediated metabolic consequences during obesity have not yet been elucidated. The last chapter of this manuscript will therefore introduce and contextualize *ABCG1* role in cardiometabolic disease.

Part IV: ABCG1 in cardiometabolic disease

1. The ABC transporters in lipid-related disease

The ATP-binding cassette (ABC) transporters are a large superfamily of transport proteins encompassing 49 members that are subdivided in 7 families from A to G based on sequence homology. They actively transport several different molecules such lipids, ions, proteins, vitamins and other small or large inorganic and organic compounds across cell membranes by hydrolyzing ATP. Given this key role in overall cellular homeostasis, they are ubiquitous in nearly all organisms and are particularly expressed in brain, liver, pancreas, AT, intestine, among others (Ye et al. 2020). Dysfunction of any of these transporters is therefore associated to a wide variety of diseases and pathological conditions such as cardiometabolic disease and lipid disorder, Alzheimer's disease or even cancer multidrug resistance (Table 3).

Among the ABC transporters implicated in lipid or lipid-related compounds transport, mutations on ABCG5/G8 have been implicated in the development of Sitosterolemia, a hereditary disorder in which the absorption of plant sterols and dietary cholesterol is enhanced, conducting to a build-up of these lipids in plasma and tissue and an increased risk of premature CVD (Salen et al. 1985, Tzavella et al. 2017). ABCA2 and ABCA7 have been identified in Alzheimer's disease, with ABCA7 allowing for a particular link between cholesterol homeostasis and the immune system, particularly natural killer lymphocytes (Ye et al. 2020). Then, ABCA1 has been one of the most studied ABC transporters given its involvement in reverse cholesterol transport and the formation of HDL particles. As such, researchers have associated mutations in this gene with Tangier disease (Brooks-Wilson et al. 1999, Rust et al. 1999, Maranghi et al. 2019), a pathology characterized by a severe reduction in the amount of HDL in blood as well as ectopic cholesterol deposits particularly in tonsils, liver, lymph nodes and spleen. ABCA1 expression has also been correlated with impaired insulin secretion, increased TG and Chol levels, inflammation and foam cell formation (Brunham et al. 2007, Kruit et al. 2011, Chen et al. 2016, Shi et al. 2021).

Regarding ABCG1, no genetic disorders have thus far been associated to mutations of this gene. However, given that ABCG1 actively participates in Chol efflux, especially in macrophages in the context of atherosclerosis, its activity has been linked to a protective CVD phenotype. The whole spectrum of action of macrophage ABCG1 and its exact contribution to atherosclerotic disease is however still a subject of debate and need to be elucidated (Hardy et al. 2017). Here we aim to provide a comprehensive view of the role of this transporter in lipid homeostasis, and the gaps in the literature that prompted us to expand our current knowledge regarding ABCG1 implication in ATM-mediated inflammation during DIO.

ABC Transporter	Tissue distribution	Substrate	Pathological link (monogenic disease* or contribution [§])	Reference
ABCA1	Ubiquitous	Phospholipids, Chol	Tangier disease*, Alzheimer's disease [§] , Atherosclerosis [§] , T2D [§]	(Brooks-Wilson et al. 1999, Brunham et al. 2007, Shi et al. 2021, Holstege et al. 2022)
ABCA2	Brain, kidney, lung, heart, HSC	Chol, sterols, sphingolipids	Alzheimer's disease [§] , Cancer drug resistance [§]	(Davis and Tew 2018)
ABCA7	Brain, immune cells	Chol, phospholipids	Alzheimer's disease [§]	(Aikawa et al. 2019, De Roeck et al. 2019)
ABCA12	Lung, skin	Lipids	Harlequin ichthyosis*	(Rajpopat et al. 2011)
ABCB1 (MDR1)	Colon, epithelia, blood-brain barrier	Glycosylceramides	Inflammatory bowel disease [§] , ulcerative colitis [§]	(Brinar et al. 2013, Tarling et al. 2013)
ABCB4 (MDR4)	Hepatocytes, colon	Phosphatidylcholine	Progressive familial intrahepatic cholestasis 3*	(Weber et al. 2019)
ABCD1 (ALDP)	Enterocytes	Very long chain fatty acids	Adrenoleukodystrophy*	(Turk et al. 2020)
ABCG1	Lung, brain, spleen, heart, liver, macrophages	Chol, sphingomyelin	CVD [§] , obesity [§] , T2D [§]	(Hardy et al. 2017)
ABCG4	Brain	Chol, desmosterol	Alzheimer's disease [§]	(Dodacki et al. 2017)
ABCG5	Enterocytes, hepatocytes	Chol, plant sterols	Sitosterolemia*	(Salen et al. 1985, Tzavella et al. 2017)
ABCG8	Enterocytes, hepatocytes	Chol, plant sterols	Sitosterolemia*	(Salen et al. 1985, Tzavella et al. 2017)

Table 3: Non-exhaustive list of ABC lipid transporters implicated in human disease. Chol: Cholesterol; HSC: hematopoietic stem cells; CVD: cardiovascular disease; T2D: Type 2 diabetes.

2. The ABCG1 transporter

2.1. Structure and expression profile

The human ABCG1 transporter was first identified as a homolog of the *white* gene in *Drosophila* who is implicated in the transport of eye pigment precursors (Savary et al. 1996). The ABCG1 protein contains a C-terminal transmembrane domain with six α -helices and a N-terminal cytosolic nucleotide-binding domain consisting a Walker A, Walker B and Signature/C-loop motifs (ABC domain) that are required for ATP binding and hydrolysis (Figure 16). Mutations on amino acids present in either of these motifs have been shown to impair not only lipid transport, but also the trafficking of this transport to the plasma membrane (Ye et al. 2020). ABCG1 is a half transporter, meaning it needs to homodimerize or heterodimerize to be active, with evidence suggesting that it usually functions as a homodimer (Vaughan and Oram 2005). There are two functional transcript variants of ABCG1 in humans which differ only by the absence of a 12 amino acid internal segment (ABCG1(678), full-length variant; ABCG1(666), spliced variant). The shorter transcript has been observed to be the most prominently active form of ABCG1 in humans (Engel et al. 2006). Mice have been found to express only the shorter variant (Burns et al. 2013).

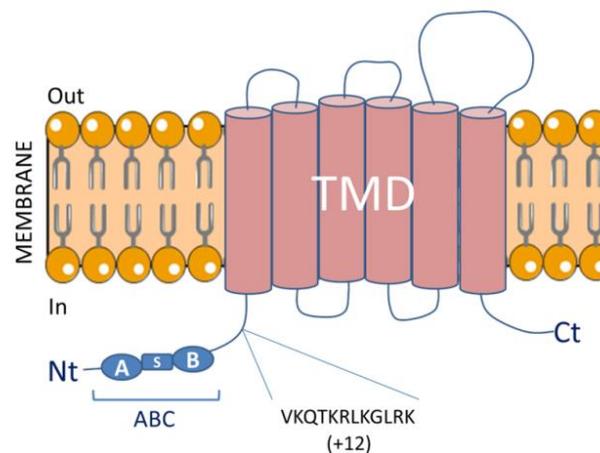


Figure 16: Structure of the membrane ABCG1 transporter. A: Walker A motif; B: Walker B motif; S: C-loop; TMD: transmembrane domain; Nt: N-terminus; Ct: C-terminus. From Hardy et al., 2017

The expression of this transporter is seen mainly in lung, brain, spleen, liver and macrophages and is under the control of the LXR pathway. ABCG1 is therefore stimulated by Chol and oxysterol (Chol oxidative derivatives) loading, but it can also be downregulated by PUFA, certain miRNA (miR33 and miR-23a-5p) and by the corepressor GPS2 (Ku et al. 2012, Ye et al. 2020). Indeed, depletion of GPS2 has been found to correlate to ABCG1 expression levels in mature adipocytes and in the AT of obese humans (Barilla et al. 2020). Although some studies suggested that ABCG1 was exclusively found in the ER, trans-Golgi network and the endosomal recycling compartments, others showed that ABCG1 is also

localized at the plasma membrane, where it is stabilized by actin filaments and exerts its action (Wang et al. 2006, Pandzic et al. 2017, Harris et al. 2018).

2.2. Function and mechanism of action

ABCG1 is specialized in the efflux of Chol and oxysterols, sphingomyelin and PL (mainly PC), thereby preventing the cytotoxic effects of lipid accumulation within the cell (Kennedy et al. 2005, Kobayashi et al. 2006, Sano et al. 2007). Besides lipids, it has also been reported as an exporter of liposoluble molecules such as vitamin E (Olivier et al. 2014). In terms of lipid transport, ABCG1 works in concert with ABCA1 to generate HDL: first, ABCA1 exports Chol to lipid-poor apolipoprotein A-I (apoA-I; nascent HDL), which is then further lipidated by ABCG1-mediated Chol efflux (HDL₂ and HDL₃) (Vaughan and Oram 2005, Gelissen et al. 2006). The mechanistic aspects of this transport have not yet been fully elucidated; however, several recent studies focusing on the cryo-electro structure of ABCG1 have allowed to identify molecular motifs involved in its interaction with Chol and propose a working model (Figure 17). In the absence of substrate, ABCG1 is presumed to be in an inward open state, the binding cavity open to the cytosol/plasma membrane (Skarda et al. 2021). The recruitment and alignment of Chol to this cavity have been found to be assisted by two sphingomyelin molecules, who create hydrophobic interactions with Chol to stabilize it (Xu et al. 2022). Consistent with this observation, studies have shown that ABCG1 efflux activity can be stimulated in a sphingomyelin-dependent manner (Sano et al. 2007). The hydrophobic site is located more at the center of the transmembrane domain and has been found to contain several amino acid residues (i.e. phenylalanine570/571, glycine164/242, lysine124, among others) that participate in closing the cavity to the cytosolic leaflet of the plasma membrane (Sun et al. 2021). Binding of ATP can then induce another conformational change that allows the adoption of an outward open conformation rich in phenylalanine residues, which are necessary for substrate release. Chol is then accessible to HDL particles. The inward-open conformation is expected to reappear after ADP diffusion (Xu et al. 2022). Mutation of any of residues implicated in Chol sensing and interaction with the transporter have thus been observed to inhibit ATP activity (Hegyi et al. 2022).

The consequences of ABCG1-dependent lipid efflux are numerous. For instance, ABCG1 allows the redistribution of intracellular sterols away from the ER and into endosomes, which could have implications in lipid-mediated ER stress but also contribute to an altered plasma membrane lipid composition. Moreover, Tarling and coll. showed that ABCG1 expression levels are positively correlated to SREBP1 activity and the activation of its target genes, again arguing for its role in lipid homeostasis (Tarling and Edwards 2011). Interestingly, it has also been seen to be associated with the expression of genes involved in cell adhesion, immune cell transendothelial migration and apoptosis

(Shen et al. 2019). Lastly, ABCG1 expression has also been required for membrane Chol-rich microdomain formation (Freeman et al. 2014). Overall, we have seen that membrane lipid composition is able to influence the activity of ABCG1 and, at the same time, ABCG1 function is able to change lipid distribution within the membrane and thus its structure by reorganizing/promoting efflux of lipids.

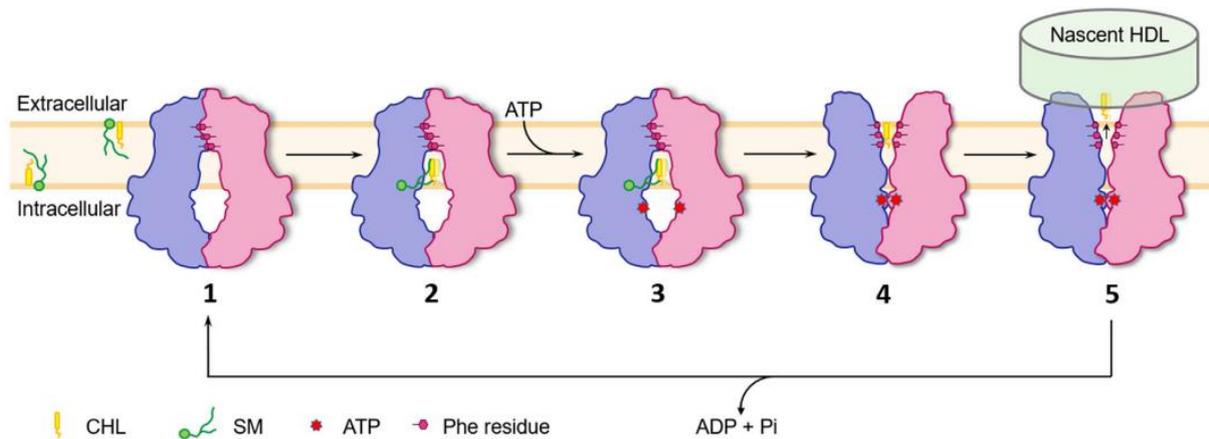


Figure 17: Proposed mechanism of action of the ABCG1 transporter in lipid efflux. The ABCG1 transporter (1) can adopt an inward facing conformation following cholesterol (CHL) and sphingomyelin (SM) interaction (2). Chol then moves through ABCG1 (3) and upon ATP binding it adopts an outward facing conformation whose exit cavity is rich in phenylalanine residues (Phe) (4). Chol exists the transporter (5) and ABCG1 goes back to its resting state. From Xu et al., 2022.

3. Roles of ABCG1 in lipid homeostasis and cardiometabolic disease

The role of ABCG1 as a lipid transporter led to believe it could be implicated in maintaining tissue lipid homeostasis in metabolic contexts. This potential contribution has mostly been studied in mice models featuring a genetic loss-of-function or gain-of-function of this transporter and fed a high-lipid diet. The first characterization of ABCG1 knockout mice revealed that the absence of this gene did not impact viability nor growth, and it was not associated with significant changes in body weight, blood lipids or any other metabolic parameters when fed a normal diet. Interestingly, this changed in a context of high-fat and high-cholesterol feeding, where it was observed that ABCG1 deficiency resulted in prominent Chol ester, TG and PL storage in hepatocytes and in macrophages from several different tissues, particularly lungs and liver. Overexpression of the transporter annulled these effects (Kennedy et al. 2005). This report contributed to place ABCG1 at the forefront of cellular lipid metabolism and guided researchers to explore its contribution to several pathophysiological conditions at a genetic and an epigenetic level.

3.1. *ABCG1* in cardiovascular disease

i. Impact of circulating lipid levels

Despite its contribution to whole-body lipid homeostasis, as seen above, mice lacking *ABCG1* do not present altered plasma lipid levels (Kennedy et al. 2005). In agreement with this experimental model, genome-wide association studies (GWAS) on *ABCG1* single nucleotide polymorphisms (SNPs) have failed to detect significant differences between *ABCG1* variants and serum TG, total Chol, LDL-cholesterol (LDL-C) or Lipoprotein(a) levels (Furuyama et al. 2009, Olivier et al. 2012). However, larger cohort studies did manage to associated two rare *ABCG1* SNPs located in the promoter region to modest reductions (<7%) in HDL-C levels only (Schou et al. 2012). Similar observations were reported with other variants, but depended on postprandial state, PUFA lipid intake, age or BMI (Abellán et al. 2010, 2011, Zago et al. 2022). Interestingly though, other *ABCG1* SNPs have also been associated to plasma LPL activity independently of it mass and of circulating TG levels (Olivier et al. 2012). As opposed to genetic marks, epigenetic studies focusing on cytosine guanine dinucleotide (CpG) methylation sites have nonetheless correlated *ABCG1* CpG not only to lower HDL-C, but also higher TG levels, SM, PC and lysoPC (Petersen et al. 2014, Jiang et al. 2021, Mazaheri-Tehrani et al. 2022), suggesting the importance of environmental stimuli on *ABCG1* function and lipid traits.

ii. Impact on monocytes/macrophages

While *ABCG1* effects on plasma lipid levels seem to be few, its contribution to CVD development and progression has been more documented. In humans, *ABCG1* expression in CD14⁺ monocytes has been inversely correlated to premature coronary heart disease independently of plasma lipid levels (Sivapalaratnam et al. 2012). Several *ABCG1* SNPs and hypermethylated loci have also been identified as associated with the risk of atherosclerotic disease, myocardial infarction and ischemic stroke (Xu et al. 2011, Liu et al. 2014, Peng et al. 2014, Li et al. 2015b, Hedman et al. 2017). Functional studies carried out on one of these *ABCG1* SNPs (rs57137919G>A) revealed that the resulting downregulation of its expression led to impaired Chol and oxysterol efflux and increased apoptosis in lipid-loaded human macrophages (Liu et al. 2014). Since this same defect in Chol efflux to HDL was seen in *ABCG1*-deficient mice macrophages (Kennedy et al. 2005), it was hypothesized that *ABCG1* expression could protect from atherosclerotic plaque formation by promoting Chol export from arterial macrophages to HDL. Indeed, lipid-laden macrophages/foam cells presence in the artery wall is one of the first events leading to atherosclerosis.

To test this hypothesis, several independent groups investigated the impact of BM transplantation of donor *Abcg1*^{-/-} mice into atherosclerotic-prone, recipient *Ldlr*^{-/-} and *Apoe*^{-/-} mice, as well as *Abca1/Abcg1* double knockout mice fed a high-Chol diet, but reported contradicting results. Consistent

with human data, two studies reported increased atherosclerotic plaque formation in the absence of ABCG1, this due to heavy intracellular lipid accumulation within macrophages (Out et al. 2006, 2008, Lammers et al. 2009). It is important to note that the double *Abca1/Abcg1* knockout model resulted in a much more important increase in intracellular lipids (Out et al. 2008), suggesting that these two transporters work in a synergistic, complementary way. Others showed however that *Abcg1* deficiency was instead protective, as it increased foam cell susceptibility to oxidized-LDL-mediated apoptosis, which was sufficient to clear macrophages from atherosclerotic plaques and reduce lesion size (Baldán et al. 2006, Yvan-Charvet et al. 2007, Tarling et al. 2010). This was supported by another report showing that ABCG1 deficiency was found to increase macrophage susceptibility to oxLDL by impairing the efflux of proapoptotic 7-ketocholesterol (Terasaka et al. 2007). To sort out these discrepancies, Meurs et al., reported that *Abcg1* role on atherosclerotic lesion development depends not only on the stage of the disease, but also on the length of the diet (Meurs et al. 2012). It appears then that macrophage *Abcg1* presence is initially beneficial as it promotes Chol efflux and prevents foam cell formation; when the disease advances, *Abcg1* activity becomes deleterious by protecting macrophage from oxysterol-induced apoptosis.

Beyond lipid transport, *Abcg1* deficiency in arterial macrophages has also been associated to enhanced inflammatory gene expression and signaling. For instance, myeloid deficiency of *Abcg1* on a *Ldlr*^{-/-} background has been found to induce IL-1 β and IL-18 production in monocytes and macrophages from mice fed a high-Chol diet. This have been observed to be under the control of the NLRP3 inflammasome and dependent on macrophage Chol accumulation (Westerterp et al. 2018, Yalcinkaya et al. 2023). Increased TLR4 recruitment at the plasma membrane and inflammatory signaling has also been seen in *Abcg1* deficient macrophages stimulated with LPS (Yvan-Charvet et al. 2008).

iii. Impact on other immune cells

Increased neutrophil infiltration and netosis has also been evidenced in atherosclerotic plaques of mice following global, neutrophil- or macrophage-specific *Abcg1* deletion (Yvan-Charvet et al. 2008, Yalcinkaya et al. 2023) and under a high-fat or high-cholesterol diet. This has been connected to ABCG1 capacity to inhibit proliferation of the hematopoietic cell compartment and progenitor cells, thus preventing neutrophilia but also monocytosis (Yvan-Charvet et al. 2010). Increased number of thymocytes and peripheral T lymphocytes have also been observed as a consequence of excessive Chol accumulation and T-cell receptor signaling in mice deficient for ABCG1 and fed a chow diet (Armstrong et al. 2010). Double *Abcg1* and *Abca1* deficient mice exhibit splenomegaly and extramedullary hematopoiesis by mobilizing hematopoietic cells from the BM in a signaling cascade under the control

of IL-23 and IL-17 (Wang and Westertep 2020). All these mechanisms may contribute to further immune cell infiltration and plaque destabilization.

iv. Impact on endothelial cells

In endothelial cells, the absence of this transporter has shown to increase lesion area by dramatically enhancing monocyte infiltration into the atherosclerotic plaque, consequently increasing macrophage number and macrophage lipid accumulation, as well nitric oxide production in *Abca1^{fl/fl}Abcg1^{fl/fl}Ldlr^{-/-}* mice (crossbred with Tie2Cre mouse strain for endothelial cell targeting) fed a western-type diet. This was found to be mediated increased inflammatory signaling in both human and mouse *Abca1/g1*-deficient endothelial cells (Westertep et al. 2016). ABCG1 role is therefore essential for maintaining homeostasis in all the cellular intermediaries of atherosclerotic disease.

3.2. ABCG1 in obesity

i. Clinical findings

In humans, ABCG1 expression has also been positively associated to adipocyte differentiation and fat mass and BMI in morbidly obese individuals or individual at risk of obesity and related metabolic conditions (Frisdal et al. 2015, Wahl et al. 2017). Its expression seems to be higher in scWAT vs vWAT but is found to decrease with age and be accompanied of increased propensity to developing the metabolic syndrome (Panteleeva et al. 2021). Weight loss does not seem to alter its expression in adipocytes nor in ATM (Capel et al. 2009, Campbell et al. 2013); on the contrary, several studies have shown that ABCG1 expression in AT is significantly upregulated during weight loss either by lifestyle changes or bariatric surgery (Aron-Wisnewsky et al. 2011, Johansson et al. 2012). Interestingly, other recent data has shown that *ABCG1* SNPs could influence the outcome of weight loss in obese women, as patients carrying the ABCG1 rs692383 G allele were less responsive to dietary intervention (Teixeira et al. 2020).

Epigenome analyses also showed a positive association between *ABCG1* CpG methylation levels in blood monocytes and other immune cells and BMI/waist circumference (Demerath et al. 2015, Ding et al. 2015). Other hypermethylated *ABCG1* loci have also been positively correlated to hypertriglyceridemia and, interestingly, to epicardial AT and the development of coronary artery disease (Miroshnikova et al. 2021), consistent with the notion that obesity enhances cardiometabolic risk. Importantly, methylation of ABCG1 locus has been identified as able to predict the development of obesity in a cohort of 394 subjects from the Framingham Offspring study (Lee et al. 2021), thereby sustaining ABCG1 potential involvement in the development of obesity. Of note, this study also found certain dietary factors such as fat-rich and carbohydrates-rich food as strong predictors of obese status. While we already know that dietary habits share a strong causal relationship with this disease, it is

important to mention that a recent study found a positive association between carbohydrate intake and ABCG1 methylation levels (Lai et al. 2023). These observations highlight the notion that environmental factors can influence cardiometabolic risk *via* epigenetic changes of ABCG1 locus.

ii. Findings in mice

The first account of ABCG1 mechanistic involvement in the pathogenesis of obesity was observed by Buchman and coll., who reported that targeted deletion of ABCG1 protected mice against HFD-induced obesity, glucose intolerance and fatty liver by decreasing adipocyte size and food intake, and increasing energy expenditure (Buchmann et al. 2007). The mechanism behind this phenotype was later elucidated by our laboratory (Figure 18), where it was shown that adipocyte ABCG1 decreases lipid raft formation by promoting SM from the plasma membrane. Low amounts of membrane SM ensure LPL release and activity, which can then hydrolyze TRL and contribute to the release of FFA. Remnant lipoproteins and FFA can then enter the cell and be stored in LD. Intracellular uptake of FFA and Chol leads to activation of PPAR γ , which upregulates a cascade of genes implicated in adipocyte maturation and lipid uptake/storage such as C/EBPs, FABPs or perilipins. This promotes adipogenesis and fat mass growth concomitantly with the development of obesity (Frisdal et al. 2015). Transcriptionally, ABCG1-dependent induction of adipocyte growth and hypertrophy is regulated by GPS2, whose inhibition has been found to trigger the commitment of stem cell progenitors towards the adipogenic lineage (Barilla et al. 2020). The histone deacetylase 9 (HDAC9) has also been implicated in ABCG1-mediated adipocyte differentiation, as decreased expression of this gene following fasting treatment was found to repress ABCG1 activity in adipocytes and decrease insulin and glucose metabolism (Khamis et al. 2020)

While these observations differ from those made by Kennedy et al., who not only did not observe a difference in weight gain between *Abcg1*^{-/-} mice and controls but also showed increased lipid content in lungs and liver (Kennedy et al. 2005), they brought into light the dual role of ABCG1 in lipid efflux and lipid accumulation depending on the lipid context. Indeed, the main difference between these studies was the diet employed, which was either enriched in fat (45%) (Frisdal et al. 2015), in carbohydrates and fat (52% and 15%, respectively) (Buchmann et al. 2007), or in both FA and Chol (21% and 1.25%, respectively) (Kennedy et al. 2005). These diets have been shown to elicit distinct metabolic and transcriptomic signatures, as well as different consequences on weight gain and lipid accumulation. For instance, LXR deficient mice have been shown to be resistant to DIO but only when challenged with a diet containing Chol. This phenotype has been observed to be mediated by Chol capacity to stimulate FA synthesis via LXR and SREBP1, a pathway that is not activated in FA-rich diets (Kalaany et al. 2005, Buchmann et al. 2007). Overall, these studies helped to determine that ABCG1 works as a metabolic

switch depending on the lipid context (Figure 19), thus placing this transporter as a promising therapeutic target in metabolic states characterized by high FA content such as obesity.

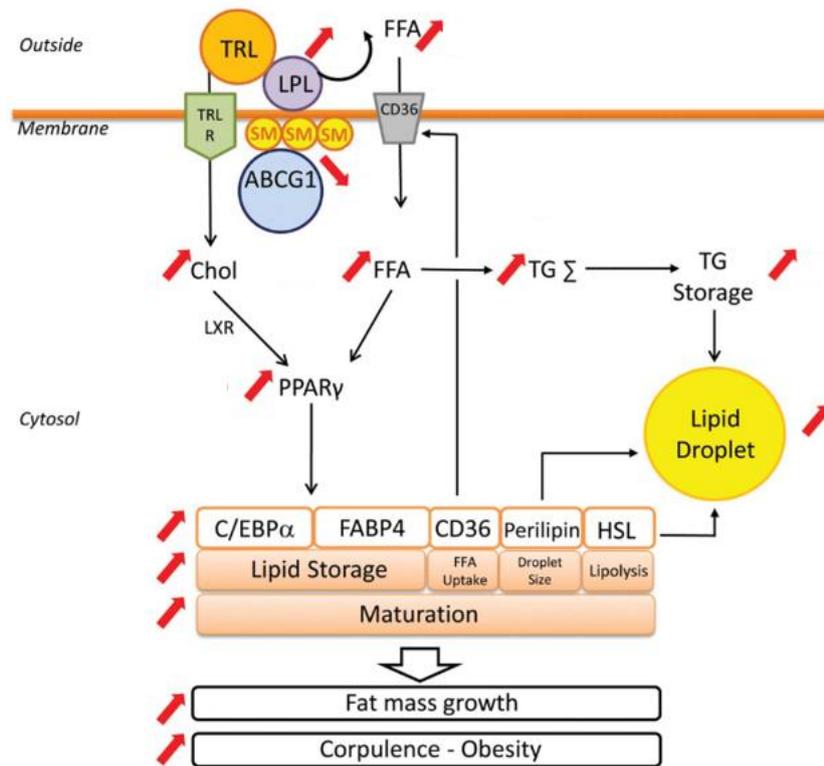


Figure 18: Role of adipocyte ABCG1 in regulating fat mass growth and the installment of obesity. ABCG1 promotes adipogenesis in a PPAR γ -dependent manner and ensures optimal LPL activity and subsequent TG storage in adipocytes by inducing SM efflux from lipid rafts. Adapted from Frisdal and Le Goff, 2015.

Further characterization of this transporter during obesity consistently showed that its expression in mice is robustly upregulated in epididymal AT during weight gain, but also during calorie restriction (Edgel et al. 2012). This study highlighted that the increase in *Abcg1* levels within AT of obese or calorie restricted *db/db* mice was mostly attributable to an induction of its expression in ATM rather than in adipocytes. These ATM were also evidenced to be lipid-laden, but were proposed to egress AT into the peritoneal cavity in an attempt to lower lipid levels within this tissue (Edgel et al. 2012). These results are however inconsistent with findings showing that ATM numbers are sustained in previously obese mice despite weight loss (Blaszczak et al. 2020). Moreover, others have shown in *ob/ob* mice that ABCG1 levels are instead increased in obese AT, although no estimates were made on the expression of this transporter in ATM alone (Duong et al. 2018). These last observations are supported by another study showing that ABCG1 is particularly enriched in anti-inflammatory, CD206⁺ ATM (Wei et al. 2015). Other landmark studies agree macrophage ABCG1 expression and function is dependent on ATM

phenotype. For instance, its expression and Chol efflux capacity are enhanced in metabolically active, obese ATM (which also display the CD206 marker) vs lean or M1-polarized ATM (Kratz et al. 2014, O'Reilly et al. 2019). It is important to note that all reports showed that ATM ABCG1-deficient mice (myeloid or genetic inhibition) fed either a chow or a HFD presented increased intracellular cholesterol levels and were not associated to any changes in body weight, composition or glycemic status (Edgel et al. 2012, Wei et al. 2015, Duong et al. 2018).

Overall, the role of ABCG1 in obesity and on ATM polarization profile, notably in LAM which are the most prominent ATM population during DIO, is still not fully defined. The differences in diets and genetic models for targeting this transporter make it difficult to fully characterize its implication in the development of this disease, and have not properly focused on the intrinsic inflammatory profile of ATM. Further research is thus needed to elucidate the mechanism through which ABCG1 drives ATM inflammatory status and should rely on more specific models of obesity (i.e. Diet-induced rather than genetically obese mice; High-fat rather than High-Chol diets) and of ATM targeting (i.e. myeloid/macrophage deficiency rather than whole body).

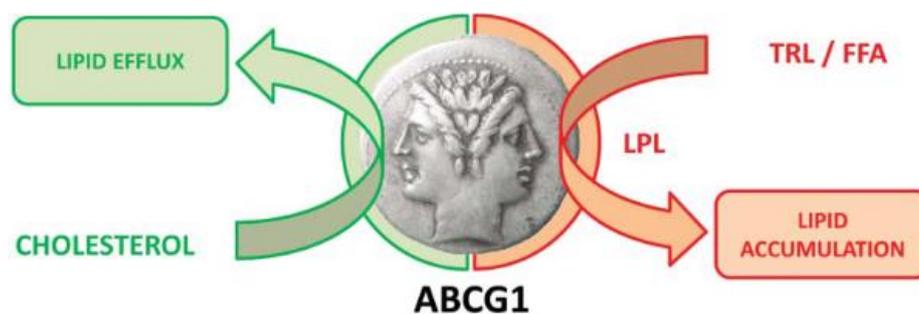


Figure 19: ABCG1 is a Janus-faced metabolic switch whose function depends on the lipid context. Whereas ABCG1 mainly promotes lipid efflux in a cholesterol-rich context, it induces lipid accumulation by controlling LPL activity in a FA-rich context. TRL: Triglyceride-rich lipoproteins; FFA: Free fatty acids; LPL: lipoprotein lipase.

3.3. ABCG1 in T2D

i. Clinical findings

Macrophages issued from subject with T2D presented reduced ABCG1 expression which translated into a 30% reduction in Chol efflux and a concomitant 60% increase in lipid accumulation (Mauldin et al. 2008). The reduction in ABCG1 mRNA levels has been found to be mediated in part by glucose, as observed in normolipidemic subjects who underwent an oral glucose challenge (Spartano et al. 2014), but also by polyunsaturated linoleic acid in *in vitro* differentiated human macrophages (Mauerer et al. 2009). Regarding ABCG1 SNPs, we and others have failed to detect any association of ABCG1 with

HOMA-IR and T2D (Schou et al. 2012, Frisdal et al. 2015). Numerous studies have nonetheless established a link between ABCG1 epigenetic modulation and insulin resistance/diabetes, again highlighting the importance of environmental cues. For instance, the Genetics of Lipid Lowering Drugs and Diet Network Study comprising of 837 non-diabetic participants revealed that CpG methylation in CD4⁺ T cells was significantly associated with fasting insulin levels and HOMA-IR (Hidalgo et al. 2014). The London life sciences prospective population (LOLIPOP), the Botnia prospective study and the San Antonio family heart study (SAFHS) then confirmed that ABCG1 methylation is a determinant of future T2D incidence (7.8% of the heritability of T2D explained), independently of BMI (Chambers et al. 2015, Kulkarni et al. 2015, Dayeh et al. 2016). Indeed, methylation of ABCG1 CpG sites in whole blood but also in adipose tissue were found to be associated with elevated fasting glucose levels, HbA1c and fasting insulin levels (Dayeh et al. 2016, Walaszczyk et al. 2018). Finally, ABCG1 methylation has been observed to be negatively modulated by statin treatment, with consequences on insulin and HOMA-IR outcomes among other T2D traits (Liu et al. 2020, Ochoa-Rosales et al. 2020). All these observations confirm that the interaction of ABCG1 with glucose and insulin metabolism can be regulated by epigenetic processes.

ii. Findings in mice

The role of ABCG1 in insulin secretion and glucose homeostasis is perhaps the most critical. It was first evidenced by Sturek and coll., who showed that *Abcg1*^{-/-} mice under a chow diet were unable to mount an appropriate insulin response after a glucose challenge due to defective insulin secretion from pancreatic β -cells (Sturek et al. 2010). They concluded that this inhibitory effect was the result of impaired insulin granule formation, storage and exocytosis, as ABCG1 deficiency led to an altered granule membrane Chol content (Sturek et al. 2010, Hussain et al. 2018). This was supported by the observation that *ABCG1* mRNA levels were markedly decreased in islets from hyperglycemic *db/db* mice compared to normoglycemic controls, and that glucose and the antidiabetic thiazolidinedione were able to significantly downregulate and upregulate ABCG1 expression, respectively (Sturek et al. 2010). Others then confirmed the role of this transporter in β -cell dysfunction and the development of chronic hyperglycemia as mice lacking ABCG1 presented increased fasting glucose levels, islet sterol levels, inflammation and macrophage infiltration. These defects were potentiated by the double inhibition of ABCA1 and ABCG1 (global ABCG1 deficiency coupled to β -cell ABCA1 deficiency on a chow diet) (Kruit et al. 2012), suggesting a complementary action of these transporters under this context.

Aside from pancreatic β -cells, diabetic mice have also been shown to present reduced levels of ABCG1 in macrophages from different tissues. Indeed, peritoneal macrophages isolated from *db/db* mice have displayed impaired efflux capacity leading to intracellular lipid accumulation (Mauldin et al.

2006), and the alteration of ABCG1-mediated Chol efflux in T2D has been shown to promote atherosclerosis development (Daffu et al. 2015). Foam cell formation in a diabetic setting has also been associated to adiponectin signaling, this adipokine being able to significantly increase ABCG1 expression without changing ABCA1, CD36 or SRBI levels, thus attenuating macrophage lipid storage (Wang et al. 2013). Furthermore, ABCG1 has also been implicated in diabetes-associated vascular diseases by promoting endothelial progenitor cell function (migration and differentiation) and reendothelialization after vascular injury (Shi et al. 2018).

As a whole, ABCG1 role in the development of DIO, IR and T2D is unequivocally present and its mechanisms of action are multiple. Indeed, ABCG1 maintains intracellular lipid homeostasis by redistributing/modifying membrane lipids, but also seems to control glucose and lipid metabolism by governing the availability of key molecules such as insulin and LPL. This multitude of effects and their consequences are however highly dependent on the tissue and cell type that are studied, but also on the length and type of diet. In obesity, further research is warranted using a more integrated and detailed approach, notably regarding ABCG1 contribution to ATM-mediated inflammation. Of note, FA nature and mechanism of uptake are also parameters that should be considered for future studies due to the role of ABCG1 in LPL activity, which hydrolyses FA from TRL (Olivier et al. 2012), but also given that SFA and PUFA seem to differentially modulate ABCG1 expression in macrophages (Ku et al. 2012).

OBJECTIVE

As I have thoroughly described, obesity is a condition characterized by the pathological expansion of adipose tissue leading to a disproportionate infiltration and accumulation of inflammatory macrophages. ATM are a subset of immune cells whose phenotype and function are highly dependent on their surrounding environment. In physiological conditions, they contribute to AT homeostasis and adaptive remodeling through the activation of phagocytic and anti-inflammatory pathways. In obese states, excessive influx of FFA as a consequence of increased circulating FA levels and adipocyte lipolysis and death steers ATM towards a metabolically active profile (LAM) that specializes in lipid handling, but is also enriched for factors/signaling molecules associated with inflammatory signaling (Coats et al. 2017, Hill et al. 2018). ATM-driven inflammation is known to be a key feature at enabling the instalment of insulin resistance both locally and systemically, thus allowing the development of T2D and other obesity- associated comorbidities. While the mechanistic links between inflammatory pathways and insulin signaling/resistance have been largely explored, the way lipids shape ATM metabolic activation during DIO is still not properly elucidated. This is of particular interest as the capacity of ATM to handle excess lipids has been associated to a protective ATM phenotype by recent scRNAseq studies (Jaitin et al. 2019, Hildreth et al. 2021).

The nature of lipids (i.e. SFA vs PUFA) metabolized by ATM, as well as their mechanisms of uptake (i.e. CD36-mediated uptake of FFA vs VLDLR-mediated uptake of TRL), have also been associated to distinct states of ATM polarization. Indeed, while SFA trigger inflammation and promote insulin resistance, PUFA, and more especially omega-3 PUFAs, hamper ATM inflammation (Oh et al. 2010). FFA activate ATM inflammatory cytokine production through TLR4,2/NF- κ B, JNK and NLRP3, whereas TRL such as VLDL seem to do so *via* the production of ceramides (Wen et al. 2011, Shin et al. 2017). Both mechanisms are also correlated to insulin resistance and may depend on LPL activity.

In this context, we set out to investigate the potential mechanisms involved in ATM plasticity and responsiveness to lipids during DIO. Given our lab's expertise, we focused on the ABCG1 transporter, whose role as a metabolic switch favoring lipid efflux or lipid accumulation depending on the lipid context has been proposed to be one of the main events regulating intracellular lipid homeostasis (Frisdal and Le Goff 2015). Indeed, numerous studies, including several from our laboratory, have put forward the importance of this transporter in foam cell formation in the context of atherosclerosis, as well as in the development of obesity and T2D traits in particular by controlling adipocyte differentiation and growth (Hardy et al. 2017).

The study of ABCG1 role on ATM activation and function has led to confounding and inconsistent findings, as seen before. It was however demonstrated that ABCG1 is implicated in FFA release from TRL and reuptake by macrophages by controlling the bioavailability of LPL through plasma membrane lipid remodeling (Olivier et al. 2012). This important role of ABCG1 on fatty acid metabolism suggests that ABCG1 might govern membrane lipid distribution and inflammation in ATM during obesity and insulin resistance.

To test this hypothesis and assess the causality of ABCG1 in fatty acid handling and membrane lipid remodeling in ATM, our project was organized based on 3 aims:

- 1) To characterize the function of macrophage ABCG1 in metabolic disorders associated to DIO

A mouse model specifically invalidated for *Abcg1* in the myeloid lineage (*Abcg1^{fl/fl}* x *LysM-Cre: Abcg1^{MACKO}*) was generated. Wt and *Abcg1^{MACKO}* mice were fed a high-fat diet for 8 to 25 weeks. The consequences of impaired macrophage *Abcg1* expression on the development of insulin resistance during DIO was explored both systemically (OGTT and ITT) and at the cellular level (insulin signaling). A complete phenotyping of AT (adipocyte size, inflammation) and liver (hepatosteatosis, inflammation) was also performed. ATM phenotype was assessed by flow cytometry (Fortessa, BD).

- 2) To elucidate the role of ABCG1 in ATM membrane lipid remodeling and inflammation

ATM were sorted from perigonadal WAT of Wt and *Abcg1^{MACKO}* mice fed an 8-week HFD. The impact of *Abcg1* deficiency on ATM activation was studied by the analysis of membrane lipid remodeling (phospholipids and sphingolipids, TG; LC-ESI/MS/MS) and inflammation (RNAseq). Results were validated in an *in vitro* model: bone-marrow derived macrophages were generated from Wt and *Abcg1^{MACKO}* mice and cultured in an obesogenic environment (insulin, glucose and VLDL). Metabolic phenotyping of ATM (Seahorse, glucose uptake) was additionally performed. RNAseq data was also compared and confirmed with public data (Sárvári et al. 2021).

- 3) To identify ABCG1-dependent macrophage populations in human adipose tissue

RNA-sequencing of ATM from obese patients (n=14, low vs high ABCG1) subjected to bariatric surgery (Geoffroy Saint-Hilaire Clinic) was carried out. Results were validated with public data generated from scRNA-sequencing studies (Hildreth et al. 2021).

The conclusions of this study strongly supported a non-canonical, deleterious function of ABCG1 in the rewiring of fatty acid metabolism in ATM during DIO. Such role involved a shift in the handling of different fatty acid pools between lipid droplets and the plasma membrane, with consequences on inflammatory signaling and metabolic circuits. These results are presented below.

RESULTS

Article

ABCG1 transporter orchestrates adipose tissue macrophage plasticity and insulin resistance in obesity by rewiring saturated fatty acid pools.

V.D. Dahik, P. KC, C. Materne, C. Reydellet, M. Lhomme, M. Ponnaiah, F. Deknuydt, E. Frisdal, L.M. Hardy, H. Durand, I. Guillas, P. Lesnik, I. Gudelj, G. Lauc, M. Guérin, A. Kontush, A. Soprani, M. Diederheim, O. Bluteau, N. Venticlef, W. Le Goff

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ABCG1 transporter orchestrates adipose tissue macrophage plasticity and insulin resistance in obesity by rewiring saturated fatty acid pools.

V.D. Dahik¹, P. KC¹, C. Materne¹, C. Reydellet¹, M. Lhomme², M. Ponnaiah², F. Deknuydt², E. Frisdal¹, L.M. Hardy¹, H. Durand¹, I. Guillas¹, P. Lesnik¹, I. Gudelj³, G. Lauc³, M. Guérin¹, A. Kontush¹, A. Soprani⁴, M. Diedisheim^{5,6}, O. Bluteau^{1,7}, N. Venteclef⁶, W. Le Goff¹.

1- Sorbonne Université, INSERM, Foundation for Innovation in Cardiometabolism and Nutrition (ICAN), UMR_S1166, F-75013 Paris, France.

2- Foundation for Innovation in Cardiometabolism and Nutrition (IHU ICAN), ICAN I/O data science (MP), ICAN omics (ML), ICAN BioCell Flow Cytometry (FD), 75013 Paris, France

3- Genos Glycoscience Research Laboratory, HR-10 000 Zagreb, Croatia.

4- Department of Digestive Surgery, Générale de Santé (GDS), Geoffroy Saint Hilaire Clinic, 75005, Paris, France.

5- Clinique Saint Gatien Alliance (NCT+), 37540 Saint-Cyr-sur-Loire, France.

6- INSERM UMR-S1151, CNRS UMR-S8253, Université Paris Cité, Institut Necker Enfants Malades, F-75015, Paris, France.

7- AP-HP Pitié-Salpêtrière-Charles Foix, Department of Endocrine and Oncological Biochemistry, F-75651, Paris, France.

Correspondence:

Wilfried Le Goff, Ph.D.

INSERM UMR_S1166

Faculté de médecine Sorbonne Université

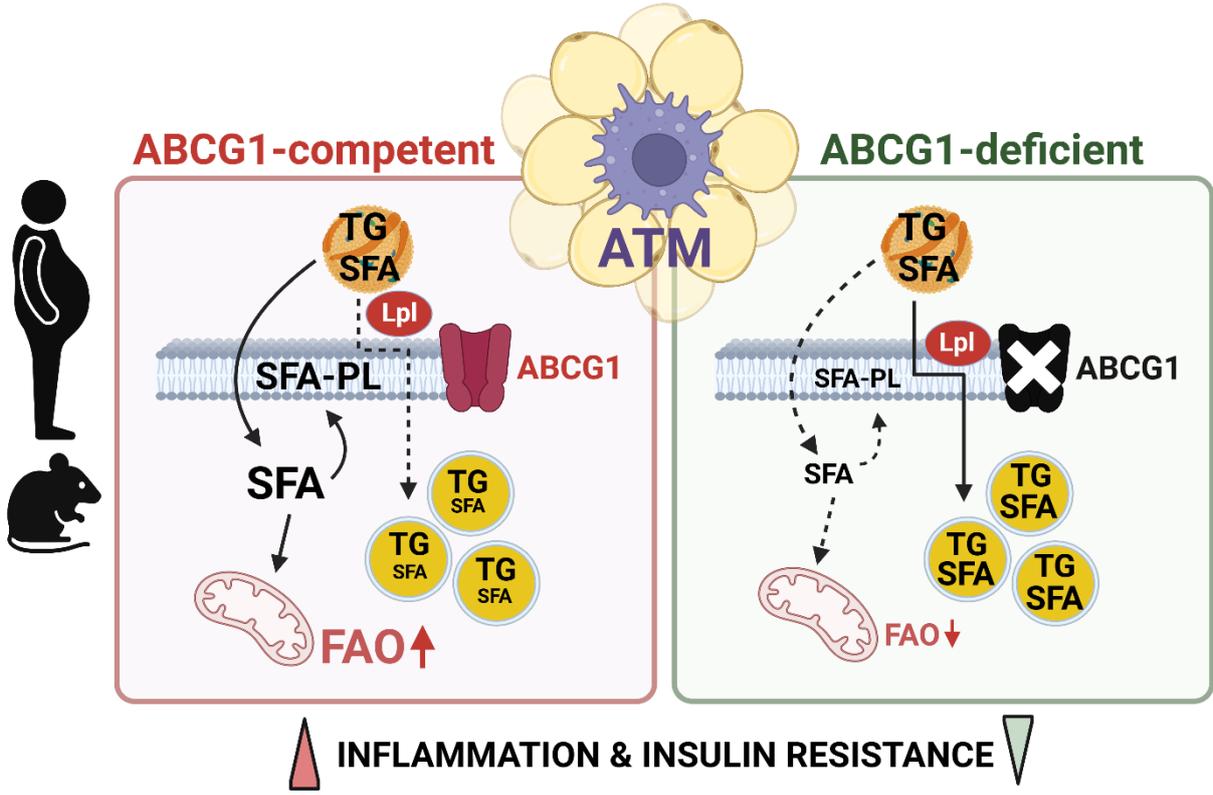
91, boulevard de l'Hôpital

75013 Paris

France

email: wilfried.le_goff@sorbonne-universite.fr

Graphical abstract



Abstract

In diet-induced obesity (DIO) adipose tissue macrophages (ATM) are exposed to significant lipid fluxes, which can influence the activation of specific inflammatory and metabolic programs and contribute to the development of obesity-associated insulin resistance and other metabolic disorders. However, mechanisms governing this metabolic adaptation of ATM in DIO are poorly understood. In the present study, we demonstrate that the membrane Atp-binding cassette g1 (Abcg1) transporter controls ATM functional response to fatty acids (FA) carried by triglyceride-rich lipoproteins (TRL), which are abundant in high energy diets. Mice genetically lacking Abcg1 in the myeloid lineage present an ameliorated inflammatory status in adipose tissue and a reduced insulin resistance. Abcg1-deficient ATM exhibit a less inflammatory phenotype accompanied by a low bioenergetic profile and a modification of FA metabolism pathways. A closer look into ATM lipidome reveals a shift in the handling of FA pools, including a redirection of saturated FA from membrane phospholipids to lipid droplets, and leading to a reduction in membrane rigidity and neutralization of pro-inflammatory FA. ATM from obese human subjects presented the same reciprocal relationship between Abcg1 expression and this inflammatory and metabolic status. Abolition of this protective, anti-inflammatory phenotype in Abcg1-deficient ATM was achieved through the inhibition of lipoprotein lipase (Lpl) activity, thus delineating the importance of the Abcg1/Lpl axis in controlling ATM metabolic inflammation. Overall, our study identifies the rewiring of fatty acid pools by Abcg1 as a new major pathway orchestrating ATM plasticity and insulin resistance in DIO.

Introduction

Obesity is a growing, clinically complex disease associated with a number of metabolic disorders including insulin resistance (IR), type 2 diabetes, dyslipidemia and non-alcoholic fatty liver disease, which are major risk factors for cardiovascular disease. In adipose tissue (AT), excess of dietary nutrients triggers a low-grade chronic inflammation which in turn drives the accumulation of adipose tissue macrophages (ATM) and the installation of IR. As such, targeting inflammatory pathways in macrophages have been shown to ameliorate obesity-induced metabolic disorders including IR^{1,2}.

The transcriptional and phenotypic changes that ATM adopt in response to high caloric intake have been widely studied³, although most studies have focused on macrophage inflammatory signature without taking into account tissue microenvironment. Beyond an inflammatory role, ATM contribution to maintaining adipose tissue homeostasis relies also on their responsiveness to the lipid-rich environment that accompanies diet-induced obesity (DIO). ATM are able to internalize circulating and adipocyte-released lipids through different mechanisms⁴ and are therefore able to influence adipocyte health. Moreover, several recent reports have highlighted the heterogeneity of ATM response during the different stages of obesity⁵, with beneficial phenotypes being linked to ATM capacity to transition to a more metabolic program⁶. Consequently, loss of the lipid uptake and storage functions through inhibition of lysosomal biogenesis and the lipid sensor *Trem2* has been shown to amplify AT metabolic dysfunction^{7,8}. These findings have allowed for the characterization of a macrophage subset highly responsive to lipids (lipid-associated macrophages, LAM) and capable of expanding during obesity until becoming the most abundant macrophage population in adipose tissue from both obese mice and humans^{8,9}.

Among the mechanisms taking part in LAM functional changes, cell energy metabolism has been identified as a major determinant of ATM capacity to successfully adapt to a lipid-rich context. Traditional macrophage markers have characterized pro-inflammatory M1 macrophages as a highly energetic population relying mainly on glycolysis, while a more quiescent, oxidative phenotype was attributed to anti-inflammatory M2 macrophages¹⁰. In the context of obesity however, LAM are characterized by a particular metabolically active phenotype governed by both glycolysis and oxidative phosphorylation (OXPHO)¹¹. Dysregulation of any such programs has been shown to alter ATM inflammatory profile^{12,13}, but also their capacity to utilize lipids as a form of energy via fatty acid oxidation¹⁴.

Another major determinant of ATM inflammatory response is the nature and the distribution of lipids (i.e. saturated vs unsaturated, free vs membrane lipids vs lipid droplets). In obesity, a major source of proinflammatory lipids such as saturated fatty acids (SFA) are provided by triglyceride-rich

lipoproteins (TRL), for instance very low-density lipoproteins (VLDL), and by adipocytes mainly through lipolysis. Previous studies have shown that SFA can activate inflammation through the TLR4/CD36 axis^{15,16} and that these deleterious lipid species are increased intracellularly in ATM from *ob/ob* mice¹⁷. Knock-out of very low-density lipoprotein receptor (VLDLR), which would allow the internalization of SFA-rich VLDL, has been reported to alleviate inflammatory cytokine expression in ATM¹⁸. Similarly, FA partitioning into other intracellular lipid pools such as plasma membrane, can also exert deleterious or beneficial effects on inflammation according to their saturation degree. This is often mediated by changes in membrane order and composition, with SFA-enriched membranes being associated with increased inflammatory signaling and IR¹⁹. Precise mechanisms controlling the partitioning of proinflammatory SFA into ATM upon DIO, as well as the impact of this latter on AT inflammation and IR are however not fully described.

Membrane ATP-Binding Cassette G1 (ABCG1) transporter was reported to exert an active export of membrane lipids to the extracellular compartment, with consequences on the bioavailability of several proteins²⁰. Genetic and epigenetic modulation of ABCG1 in humans is associated with obesity and insulin resistance, respectively^{21,22}. We previously demonstrated that the membrane transporter ATP-Binding Cassette G1 (*Abcg1*) controls the activity of lipoprotein lipase (Lpl) in macrophages by trapping it at the cell surface²³. Lpl is one of the main lipases that hydrolyses and releases SFA from VLDL thus allowing their uptake through VLDLR. Such a role of *Abcg1* in DIO, where circulating concentrations of TRL are elevated, could impact the distribution of SFA into ATM and participate to the development of metabolic disorders associated with obesity. In the present study, we brought to light a new mechanism through which the intracellular rewiring of proinflammatory saturated FA metabolism operated by *Abcg1* controls the metabolic activation of ATM and contributes to the development of AT inflammation and IR in DIO.

Materials and Methods.

Mice

Abcg1^{fl/fl} mice on a C57/BL6J background (Jackson Laboratories) were crossed with *LysM*^{Cre+/-} mice (Jackson Laboratories) to generate myeloid-specific *Abcg1* knockout mice (*Abcg1*^{MACKO}). Genotyping was performed according to the protocol from Deltagen. *Abcg1*^{MACKO} mice and littermate controls (Wt) were housed in a controlled environment (22°C, 12h light-dark cycle) with *ad libitum* access to a standard chow diet and water. At the age of 7-9 weeks, mice were fed a high-fat diet (60% fat, Research Diet#D12492) for 25 weeks. Body composition was analyzed by Nuclear Magnetic Resonance (Bruker Mini-spec+). Mice were sacrificed by cervical dislocation and tissues were collected, snap frozen and stored at -80°C or fixed in 10% formalin for further analysis. All procedures were approved and accredited (No. 02458.02) by the French Ministry of Agriculture and in accordance with the guidelines of the Charles Darwin Ethics Committee on animal experimentation.

Plasma parameters

For oral glucose tolerance test (OGTT) and insulin tolerance test (ITT), mice were fasted for 5h and administered 2g.kg⁻¹ of glucose by oral gavage and 1 UI.kg⁻¹ of insulin (Eli Lilly) by intraperitoneal injection, respectively. Glycaemia was measured at indicated time points using the Accu-Check Guide system (Roche). Fasting plasma insulin levels were quantified using the Mouse Ultrasensitive Insulin ELISA (Alpco). HOMA-IR values were calculated according to the formula HOMA-IR = [glucose (nmol/L) * insulin (μU/mL)/22.5], using fasting values. Plasma adipokines were measured with the MILLIPLEX Mouse Metabolic Hormone Expanded Panel assay (Millipore) and the MAGPIX device (Luminex) system according to the manufacturer's instructions. Plasma triglyceride (TG), total (TC) and free cholesterol (FC) and high-density lipoprotein-cholesterol (HDL-C) concentrations were quantified with an autoanalyzer (Konelab 20) using reagent kits (ThermoFisher).

Histological analysis

After fixation, tissues were dehydrated, cleared and embedded in paraffin. 5μm sections were stained with hematoxylin and eosin according to standard procedures. Mean adipocyte size was quantified using ImageJ software (Adiposoft plugin) and is defined as the average adipocyte area (μm³) from a randomly chosen field. At least 1000 cells were measured for each genotype. For immunohistochemistry staining, citrate-based antigen retrieval was performed in a 750W microwave for 15min followed by a 10-min incubation with H₂O₂ 3% for quenching of endogenous peroxidase activity. Slides were subsequently blocked and incubated with anti-F4/80 primary antibody (MCA497, Biorad, 1:300 dilution) followed by appropriate secondary antibody, as well as the avidin-biotin

complex as per instructed in the Vectastain ABC-HRP Kit (Vector Laboratories). Color development was performed with DAB (3,3-diaminobenzidine) substrate (DAKO Cytomation) and slides were counterstained with hematoxylin. Negative controls were carried out without incubation of primary antibodies. Quantification of immunohistochemical staining was performed using ImageJ software (Fiji). Images were acquired using a Zeiss microscope.

Stromal vascular fraction isolation

Gonadal (mouse) and omental (human) adipose tissue was excised, minced and digested in DMEM containing 2.5mg/ml collagenase D (Roche) and 1mg/ml collagenase type II (Sigma Aldrich), respectively, for 20min (human) or 30min (mouse) at 37°C. The lysate was dissociated through a 70µm pore cell strainer and centrifuged at 1,800 rpm for 5min. The stromal vascular fraction (SVF, cell pellet) was washed, resuspended in ACK lysis buffer for 5min at RT and centrifuged. Recovered cells were kept on ice for staining.

Flow cytometry and cell sorting

Cells were stained with a viability dye (Fixable Viability Dye, eBiosciences) for 15min, blocked with an FcR blocking reagent (Miltenyi Biotech) for 20min and stained for surface markers (Supp. Table 1). For intracellular lipid staining, Bodipy 493/503 (D3922, ThermoFischer) was added. For proliferation studies, AF700 coupled KI67 (561277, BD Biosciences) was incubated for 30min after permeabilisation with Foxp3 / Transcription Factor Staining Buffer Set according to provider's protocol (00-5523-00, ThermoFischer). Acquisition was performed on a LSR II Fortessa Analyzer (BD Biosciences). Cell sorting was performed on a Moflo Astrios EQ (Beckman Coulter) (human panel) or a FACSAria II (BD Biosciences) (murine panel). Data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Human samples

Omental adipose tissue biopsies were obtained during gastric surgery of 14 obese female subjects (mean BMI = 41 kg.m⁻²) admitted to the Geoffroy Saint Hilaire Hospital (Paris, France). Clinical and biological parameters of subjects are presented in Table 1. The Ethics Committee of CPP Ile-de-France approved the clinical investigations for all individuals and written informed consent was obtained from all individuals. Studies were conducted in accordance with the Helsinki Declaration.

Western blot

Mice were fasted for 5h and injected with 8 U.l.kg⁻¹ of Insulin (Eli Lilly). After 5 minutes, mice were sacrificed and tissues were collected. Samples were extracted in RIPA lysis buffer (1mM EDTA, 1mM EGTA, 50mM NaF, 50mM Tris, 10mM glycerophosphate, 20mM pyrophosphate, Triton 1%, pH 7.4)

containing protease and phosphatase inhibitors (Roche). Proteins were separated on a 4-12% Bis-Tris gel (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were subsequently blocked with casein for 1h at RT, then incubated with primary antibodies against pAKT-Ser473 (Cell Signaling, 9271) and AKT (Cell Signaling, 9272) overnight. After incubation with fluorescent secondary antibodies, proteins were visualized with the Odyssey infrared imaging system (LI-COR Bioscience) and quantified with ImageJ (Fiji).

Bone marrow-derived macrophage culture

Bone marrow cells from *Abcg1*^{MACKO} and Wt mice were isolated and differentiated in DMEM supplemented with 20% FBS, 30% L929-conditioned media, 2 mmol.L⁻¹ glutamine and 100 U.ml⁻¹ Penicillin/Streptomycin at 37°C in 5% CO₂ for 7 days as previously described²⁴. Adherent bone marrow-derived macrophages (BMDM) were then incubated with VLDL (50 µg protein.ml⁻¹) isolated from normolipidemic plasma by ultracentrifugation²⁰, glucose (30 mM), insulin (10 nM) and/or Lpl inhibitor, Tetrahydrolipstatin (10 µM; Sigma) for 24h. For extracellular flux analysis, BMDM were cultured in low binding plates, detached with PBS and re-plated at a density of 6.5 x 10⁴ cells/well in an 8-well Seahorse microplate (Agilent).

Extracellular flux analysis

The extracellular acidification rate (ECAR) and real-time oxygen consumption rate (OCR) of *Abcg1*^{MACKO} and Wt BMDM were measured on a Seahorse XF analyzer (Agilent). The Real-Time ATP Rate Assay (Agilent, 103592-100) was performed according to manufacturer's instructions. Briefly, cells were incubated in Seahorse XF DMEM medium supplemented with 1 mM pyruvate, 2 mM glutamine and 10 mM glucose for 1h at 37°C in a non-CO₂ incubator. Ports were loaded with oligomycin (1.5 µM) or rotenone/antimycin A (0.5 µM) and OCR and ECAR were measured in response to each injection. These measurements were used for calculation of glycolytic and mitochondrial ATP production rates. Results were normalized to total protein content using a standard BCA assay (Pierce).

For fatty acid oxidation analysis, cells were incubated in a substrate-limited growth media containing Seahorse XF DMEM medium supplemented with glucose (0.5 mM), glutamine (1 mM), 1% FBS and L-carnitine (0.5 mM) for 24h. On the day of the assay, former media was replaced with DMEM supplemented with glucose (2 mM) and L-carnitine (0.5 mM) and cells were incubated for 1h at 37°C in a non-CO₂ incubator. BMDM were then pre-treated with palmitate-BSA (200µM) and OCR was measured following the injection of 40 µM etomoxir, 1.5 µM oligomycin, 1 µM FCCP and 0.5 µM rotenone/antimycin A. Three consecutive measurements were performed for basal conditions and after each injection. Results were normalized to total protein levels quantified using the BCA assay (Pierce).

LPL activity measurement and glucose uptake

LPL activity was determined with a 50- μ L aliquot of culture medium using an LPL activity assay kit (Roar, New York, NY), according to the manufacturer's instructions. For glucose uptake cells were starved overnight, then incubated with glucose analog 2-deoxyglucose (2-DG) and lysed according to manufacturer's instructions (Glucose Uptake Fluorometric Assay Kit, MAK084, Sigma–Aldrich).

Quantification of liver fat content

Quantification of liver lipid content was performed by a modified Folch method. Briefly, approximately 50mg of tissue were mechanically homogenized in chloroform-methanol (3:1) solution, agitated for 2h and centrifuged at 2200rpm for 20min. The supernatant was collected and added to 50 μ L of chloroform-methanol (3:1) solution containing 1% Triton X-100. Solvents were evaporated at 60°C and the remaining fatty phase was used for quantification of TG content. Results were normalized to cell protein levels quantified using the BCA assay (Pierce).

Membrane rigidity

Cells were incubated with 20 μ M Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene; Invitrogen) at 37°C for 30min. Samples were then centrifuged and distributed in a 96-well plate at a density of 2×10^5 per well. Spectral data was acquired with the Gemini EM Microplate Reader and the SoftMax Pro software (Molecular Devices) at 440nm (for the ordered phase) and 490nm (for the disordered phase). The GP value was calculated with the following formula: $GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$.

Lipidomics

For lipidomic analysis, lipids from BMDM and ATMs were extracted using a modified Bligh and Dyer protocol. Briefly samples supplemented with a mixture of internal standards were mixed with 1600 μ L of acidified methanol:0.1N HCl (1:1 v/v) and 800 μ L chloroform as described elsewhere^{26,27}. The lower organic phase was dried, lipids were reconstituted into 40 μ L of LC/MS compatible solvent and lipids quantified by LC-ESI/MS/MS using a prominence UFLC and a QTrap 4000 mass spectrometer. For quantification of phospholipid and sphingolipids, sample (4 μ L) was injected to a kinetex HILIC 2.6 μ m 2.1x150mm column. Mobile phases consisted of water and acetonitrile containing 30mM ammonium acetate and 0.2% acetic acid. Lipid species were detected using scheduled multiple reaction monitoring (sMRM) in the positive-ion mode reflecting the headgroup fragmentation of each lipid class. For quantification of neutral lipids (CE, FC, DAG and TAG), sample (4 μ L) was injected to an Ascentis C18 2.7 μ m 2.1x150mm column. Mobile phases consisted of A (acetonitrile/water(60:40)) and B (isopropanol/acetonitrile (90:10)) in the presence of ammonium formate and formic acid. Lipid species were detected using sMRM in the positive-ion mode reflecting the neutral loss of (RCOO + NH₃) for

DAG and TAG, the product ion scan of m/z 369 (cholesterol – H₂O) for CE and FC. Quantification of phospholipids, sphingolipids and neutral lipids was performed in positive-ion mode. Sample was injected to a Kinetex HILIC 2.6 μ m 2.1x150mm column (Phenomenex, CA, USA). Mobile phases consisted of water and acetonitrile containing ammonium acetate and acetic acid. Lipid species were detected using scheduled multiple reaction monitoring (sMRM) and quantified using thirty-seven calibration curves specific for the 16 individual lipid subclasses and up to 12 fatty acid moieties. More abundant lipid species were quantified from a 10-fold diluted sample. An in-house developed R script was used to correct for isotopic contribution to MRM signals from HILIC separation as adapted from Ejsing CS et al²⁸. All internal standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). LC/MS grade or UPLC grade solvents were used without further purification and obtained from Sigma-Aldrich (St Louis, MO, USA). Structural elucidation of phospholipids, sphingolipids and triacylglycerides was a combination of experimental determination (fatty acid fragmentation) and theoretical extrapolation based on literature (Supp. Table 3). Statistical analysis and heatmap clusterisation was performed with MetaboAnalyst software 5.0²⁹.

Glycomics

Isolation of membrane proteins. The cells were spun down by centrifugation at 400 x g for 5 min at 4°C, and supernatant was discarded. 1ml of Triton lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton X-114) was added to microtube containing cell pellet. Samples were sonicated in 4 cycles for 20 sec (sonication amplitude was 50 %, sonicator probe was manually handled) on ice to homogenize the cells. Homogenized cells were incubated at 4 °C overnight. Afterwards, samples were centrifuged at 10,000 x g for 30 min at 4 °C. 200 μ L of sucrose cushion (6% (w/v), 10 mM Tris-HCl, 150 mM NaCl, 0.06 % Triton X-114) was added to a new 2 mL microtube and overlaid with the supernatant. Samples were incubated at 37°C for 20 min to achieve micellization and centrifuged at 400 x g for 3 min at 37°C. Lower phase was purified further by addition of 500 μ L of cold (4°C) PBS, followed by incubation for 20 min at 37°C and centrifugation at 400 x g for 3 min at 37°C. Upper phase was discarded and the purification protocol was repeated. Proteins were precipitated with methanol/chloroform/ultrapure water extraction in 4:2:3 ratio. Afterwards, samples were centrifuged at 9,000 x g for 1 min at 4°C. Upper phase was discarded, and cold methanol was added to the lower phase. Samples were centrifuged at 9,000 x g for 2 min at 4°C and supernatant discarded. Protein pellets were dried in speedvac overnight.

Deglycosylation, ProA labelling and UHPLC analysis: glycan release, labelling and clean up. N-glycans were released, fluorescently labeled, and cleaned up as described previously³⁰, with modification by using procainamide hydrochloride (ProA) as a fluorescent labeling agent for N-glycans. Briefly, proteins

were denatured with sodium dodecyl sulfate (SDS) (Invitrogen, USA) and by incubation at 65°C. After enzymatic release with PNGase F (Promega, USA), N-glycans were fluorescently ProA labeled in a 2-step procedure. First, 25 µL of procainamide mixture containing 4.32 mg of procainamide hydrochloride (Sigma Aldrich) in glacial acetic acid (Honeywell, Charlotte, NC, USA)/dimethyl sulfoxide (Sigma-Aldrich) (30:70) was added per sample, followed by incubation at 65°C for 1 h. Then, 25 µL of reducing agent solution consisting of 4.48 mg of 2-picoline borane (J&K Scientific, Beijing, China) in glacial acetic acid/dimethyl sulfoxide (30:70) was added per sample and incubated at 65°C for an additional 1.5 h. Samples were then cooled for 30 min at RT. Excess reagents were removed from the samples using solid phase extraction (SPE). For the SPE step, an AcroPrepAdv 1 mL 0.2 µm wvPTFE plate (Pall) was used as solid phase. The labeled glycans were eluted with ultrapure water and stored at -20°C until use.

HILIC-UHPLC-FLR analysis. Fluorescently labeled N-glycans were separated by HILIC on a Waters Acquity UPLC instrument (Waters, USA), which consisted of a quaternary solvent manager, a sample manager, and an FLR fluorescence detector set with excitation and emission wavelengths of 310 and 370 nm, respectively. Labeled N-glycans were separated on a Waters BEH-glycan chromatography column, 150 × 2.1 mm i.d., 1.7 µm BEH particles, with 100 mM ammonium formate, pH 4.4 as solvent A and acetonitrile as solvent B. The separation method used a linear gradient of 30-47% solvent A at a flow rate of 0.56 ml/min in a 25-minute analytical run. Samples were maintained at 10°C prior to injection, and the separation temperature was 60°C. Data processing was performed using an automated processing method with a traditional integration algorithm. Then, each chromatogram was manually corrected to maintain the same integration intervals for all samples. The chromatograms were all divided into 41 chromatographic glycan peaks (GP). The amount of glycans in each peak was expressed as % of the total integrated area. Statistical analysis and heatmap clusterisation was performed with MetaboAnalyst software 5.0²⁹

Quantitative RT-PCR

Total RNA was extracted from cell culture or tissues using the NucleoSpin RNA II kit (Macherey-Nagel) or TRIzol reagents (Euromedex) according to the manufacturer's instructions. Reverse transcription and real-time quantitative PCR using a LightCycler LC480 (Roche) with sequence specific primers (Supp. Table 2). mRNA expression levels were normalized to 3-4 housekeeping genes. Data were expressed as a fold change in mRNA levels relative to control values.

RNA-Sequencing of mouse and human ATM

MRNA library preparation was performed following manufacturer's recommendations (Kapa mRNA Hyperprep from Roche or SMARTer® Stranded Total RNA Sample Prep Kit - Low Input Mammalian from

Takara). Final samples pooled library prep were sequenced on Novaseq 6000 ILLUMINA with SP-200 cartridge (2x800Millions of 100 bases reads and 2x1600Millions of 100 bases reads), corresponding to 2x33Millions and 2x66Millions of reads per sample after demultiplexing. Quality control of the raw sequencing data was performed with FasQC v0.11.9. Adapter sequences and poor-quality reads were filtered using Fastp. Dragen (Illumina) was then used to align reads to the mouse and human genome and generate raw counts. Differential analysis were performed using DESeq2.

Public data

Single-cell RNA-Sequencing data. For murine ATM, single-cell RNA-Seq data were downloaded from https://github.com/JesperGrud/snRNAseq_eWAT (eWAT_R3_Immune.Rds) from Sarvari et al⁹. For human ATM, single-cell RNA-Seq data were downloaded from Gene Expression Omnibus (accession code GSE156110) from Hildreth et al.³¹. Clustering was performed according to the authors' instructions, and ABCG1 expression was analyzed in all macrophage subsets according to their lean/obese status and ABCG1 expression (no ABCG1 mRNA = ABCG1-negative cells). Gene scores were calculated with the Seurat AddModuleScore function. The lists of genes for each pathway were retrieved from <https://www.gsea-msigdb.org>.

Statistical analyses

Data are shown as mean \pm SEM. Experiments were performed in triplicate and values correspond to the mean from at least three independent experiments. Comparisons of 2 groups were performed by a 2-tailed Student's t test and comparisons of 3 or more groups were performed by ANOVA with Newman–Keuls post-test. All statistical analyses were performed using Prism software from GraphPad (San Diego, CA, USA)

Results.

***Abcg1* deletion in ATM reduces adipose tissue inflammation and insulin resistance.** To assess the expression of *Abcg1* in macrophages during diet-induced obesity, we first sorted ATM and liver macrophages from normal and 16-weeks HFD-fed mice. *Abcg1* mRNA levels were robustly increased in ATM upon HFD (3-fold, $p=0.001$) (Fig. 1A), while no changes were seen in liver macrophages (Fig. S1A). To study the contribution of ATM *Abcg1* in diet-induced obesity (DIO), we then placed *Abcg1* myeloid-deficient mice (*Abcg1*^{MACKO}) and their wild-type littermates (Wt) on a HFD for 8 to 25 weeks. *Abcg1*^{MACKO} mice displayed a significant decrease of *Abcg1* mRNA expression in gonadal white adipose tissue (gWAT) (Fig. 1B, -43%, $p=0.001$), with no compensatory increase of *Abca1* mRNA levels (Fig. S1B). We observed that weight gain, body composition and plasma concentrations of leptin and resistin were similar between genotypes (Fig. 1C-D, S1C). While fasting glycaemia and glucose tolerance were unchanged, fasting insulinemia and insulin sensitivity were improved in *Abcg1*^{MACKO} mice in comparison to Wt mice (Fig. 1E-G). As a result, insulin resistance index HOMA-IR was reduced upon myeloid *Abcg1* deficiency (Fig. 1H). In agreement with these findings, insulin-stimulated Akt phosphorylation (Ser473) was increased in gWAT of *Abcg1*^{MACKO} relative to Wt mice (Fig. 1I). No modulatory effects of myeloid *Abcg1* deficiency were seen on plasma lipid levels (Fig. S1D-F).

Although the mass of gonadal fat was not altered (Fig. 1J), *Abcg1*^{MACKO} mice displayed a significantly greater adipocyte size than Wt mice (Fig. 1K). This was however not accompanied by changes in the expression of key genes involved in adipogenesis (*Pparg*), lipogenesis (*Acc1*, *Fas*), lipid droplet formation (*Plin1/2*) or lipolysis (*Atgl*, *Hsl*) (Fig. S1G). Further analysis of gWAT sections revealed the number of crown-like structures (CLS) was reduced in *Abcg1*^{MACKO} mice (Fig. 1L), suggesting an attenuated adipose tissue inflammation upon *Abcg1* deficiency. In accordance to this, quantification of inflammatory genes in total gWAT from *Abcg1* myeloid deficient mice revealed a significant decrease of mRNA levels of *Trl4*, chemokines *Ccl2* and *Ccl5*, cytokines *Il1b*, *Tnfa* and *Il10* and macrophage makers *F4/80*, *Cd11c*, *Cd206* and *Cd9* (Fig. 1M), the latter being a known marker of lipid-associated macrophages (LAM)³².

Although *Abcg1* expression was also significantly reduced in both total liver and liver macrophages (Fig. S1H-I), no effect was observed in inflammation when mRNA levels of inflammatory cytokines, chemokines and macrophage markers were quantified (Fig. S1J). Nevertheless, HFD-induced liver steatosis was significantly improved in terms of liver histology and total triglyceride content in *Abcg1*^{MACKO} mice (Fig. S1K), an effect which did not result from an alteration of the expression of key genes involved in lipid metabolism (Fig. S1L). Overall, these results suggest that the reduction of insulin

resistance upon myeloid *Abcg1* deficiency might be driven through changes on ATM-dependent inflammation.

Macrophage *Abcg1* deficiency improves ATM inflammatory and metabolic phenotypes. ATM numbers and profile, we first analyzed ATM populations from gWAT of HFD-fed WT and *Abcg1*^{MACKO} mice by flow cytometry (gating strategy in fig. S2A). We observed a significant decrease in ATM numbers (identified as viable CD45⁺CD11b⁺CMHII⁺F4/80⁺CD64⁺) following *Abcg1* deletion both at 8 (Fig. 2A) and 25 (Fig. S2B) weeks of HFD. Since HFD-induced obesity alters dramatically the abundance of both monocyte-derived ATM and proliferative ATM, we also analyzed the proportion of Ly6C/G⁺ cells in gWAT from Wt and *Abcg1*^{MACKO} mice. We observed a significant reduction in the amount of Ly6C/G⁺ cells (Fig. 2B), while the proportion of proliferative cells remained similar between genotypes (Fig. S2C). These results suggest that the lower number of ATM in *Abcg1*^{MACKO} mice was most likely due to reduced monocyte-recruitment. Interestingly, while the expression of pro-inflammatory surface marker CD11c was unchanged, *Abcg1* deficiency was accompanied by a significant increase in the expression of CD206, a marker classically associated with an anti-inflammatory ATM phenotype (Fig. 2C-D).

Recent reports show that LAM are the most enriched subpopulation of ATM during obesity, and that non-perivascular (NPVM) and peri-vascular macrophages (PVM) go through a metabolic, LAM-like reprogramming⁹. To investigate which of these macrophage subpopulations preferentially expresses *Abcg1*, we analyzed published scRNA-seq of epididymal ATM from wild-type mice fed a low-fat (LFD) or high-fat diet (HFD). *Abcg1* was mostly expressed in LAM and proliferating-LAM with HFD (Fig. 2E). To test for potential transcriptional regulations in our model of macrophage *Abcg1* deficiency, we then sorted gonadal ATM from Wt and *Abcg1*^{MACKO} mice on an 8-week HFD for bulk RNA-sequencing (Fig. 2F-G). At this high resolution, several transcriptomic signatures were detected and the top regulated pathways included inflammatory response and signaling, oxidative phosphorylation and fatty acid metabolism (uptake, transport, catabolism) which were markedly downregulated in *Abcg1* deficient macrophages (Fig. 2H). This is consistent with previous reports underlying the importance of energetic and metabolic adaptation of ATM to fulfill their lipid-handling and inflammatory activities^{10,12}. Moreover, when filtering for LAM genes, we observed a marked reduction of this signature in ATM from *Abcg1*^{MACKO} mice, including genes such as *Trem2*, *Cd36*, *Fabp5*, *Lpl*, *Lipa* and coding for several ATPases (Fig. 2I). Altogether, these findings indicate that *Abcg1* deficient ATM exhibited an impaired inflammatory and metabolic phenotype otherwise enhanced during DIO.

ABCG1 expression is associated with pro-inflammatory and metabolic pathways in human ATM from obese individuals. To translate our findings into a human pathophysiological context, we analyzed public data from single cell RNA-sequencing of different ATM populations isolated from omental WAT from lean and obese individuals³¹. Interestingly, inflammatory response, oxidative phosphorylation and fatty acid metabolism pathways identified in mouse ATM as being affected by *Abcg1* expression were also significantly modulated in ATM from obese individuals in comparison to those from lean subjects (Fig. 3A). More precisely, the inflammatory response pathway was upregulated in all ATM populations (Inflammatory macrophages (IM), LAM and PVM) from obese individuals (Fig 3B), whereas the oxidative phosphorylation and fatty acid metabolism pathways were both reduced in LAM (Fig. 3C-D). We then proceed to characterize ATM transcriptome according to ABCG1 expression levels and obtained two distinct populations positive and negative for ABCG1. Coherent with our previous findings in mice, ABCG1-expressing ATM revealed that pathways for inflammatory response, oxidative phosphorylation and fatty acid metabolism were significantly enriched in comparison to ABCG1-negative ATM (Fig. 3E).

To validate these results with our in-house data and confirm the role of ABCG1 in the activation of these pathways, we then sorted ATM from omental WAT from 14 obese individuals (gating strategy in fig. S3). Clinical parameters of these subjects are described in Table 1. RNA sequencing was performed and ABCG1 transcript levels were used to define two groups in which ATM either presented a high (ABCG1^{high}) or a low expression of ABCG1 (ABCG1^{low}) expression (Fig. 3F-G). We observed that ABCG1^{low} ATM exhibited a similar transcriptomic signature (Fig. 3H) that the one observed in ABCG1-negative ATM by Hildreth *et al.*³¹ and in our mice model of macrophage *Abcg1* deficiency, thus confirming the contribution of this transporter in the activation of inflammatory and oxidative phosphorylation pathways.

***Abcg1*-deficient macrophages present a low bioenergetic profile and rely less on fatty acid oxidation.**

Intracellular energy metabolism is intimately linked to ATM inflammatory response. To understand how *Abcg1* deficiency guides ATM bioenergetics and functional responses, we switched to bone marrow-derived macrophages (BMDM). BMDM from *Abcg1*^{MACKO} and Wt mice were treated for 24h with insulin and glucose as described by Coats *et al.*⁵. Such molecules were reported to mimic the metabolic state of ATM during DIO. In our experimental conditions, TRL were added as a source of fatty acids (Fig. 4A) in order assess the contribution of the Lpl-mediated hydrolysis of TG and the *Abcg1*/Lpl pathway in ATM activation. First, we confirmed that our *in vitro* model was able to recapitulate the inflammatory phenotype observed in *Abcg1*^{MACKO} mice as RNAseq analysis of *Abcg1*-

deficient BMDM (Fig. 4B) revealed several downregulated GO terms related to inflammation (Fig. 4C). We then focused on ATM bioenergetics and observed that *Abcg1*-deficient BMDM exhibited an impaired respiratory and glycolytic capacity, assessed by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively (Fig. 4D-E). This *Abcg1*-dependent respiratory phenotype mirrors the transcriptomic signature evidenced in ATM from *Abcg1*^{MACKO} mice (Fig. 2H) and ABCG1^{low} human ATM (Fig. 3I). ATP production relying on glycolysis as well as glucose uptake were also significantly reduced upon *Abcg1* deficiency (Fig. 4F-G). We analyzed the capacity of ATM to use FA as an energy substrate and showed that fatty acid oxidation (FAO) was also reduced in *Abcg1*-deficient BMDM (Fig. 4H). Coherently, the mRNA levels of FAO-related genes such as *Cpt1a*, *Acox1* and *Lcad* were reduced in macrophages from *Abcg1*^{MACKO} mice (Fig. 4I). Taken together, these results suggest that the anti-inflammatory phenotype of *Abcg1*-deficient macrophages is intimately linked to their low bioenergetic metabolism and their reduced capacity to catabolize fatty acids.

Fatty acid handling by Abcg1 controls membrane lipid remodeling and inflammation in macrophages.

Because fatty acid metabolism is impaired in ATM from *Abcg1*^{MACKO} mice and ABCG1^{low} human ATM, we hypothesized that *Abcg1* deficiency might alter fatty acid handling and contribute to the anti-inflammatory phenotype. To gain a broad view of macrophage lipidome under *Abcg1* deficiency, we first performed lipidomic analysis by LC-MS/MS of metabolically active BMDM from Wt and *Abcg1*^{MACKO} mice. Impaired phosphatidylethanolamine (PE) metabolism (PE, plasmalogen PE (PEp) and lysophosphatidylethanolamine (LPE)) and reduced abundance of phosphatidylcholine (PC) were detected in *Abcg1*-deficient BMDM (Fig. 5A). Although the total amount of TG was not altered (Fig. 5A), we observed an elevated abundance of several TG species in *Abcg1*-deficient BMDM in comparison to Wt cells (Figure 5B). A closer look on these lipid species revealed a major shift in the nature of FA stored within TG, as they were enriched in proinflammatory SFA (C16:0 and C18:0) (Fig. 5C). This increase in the content of fatty acids containing 16 and 18 carbon chains in TG was paralleled with a reduction of the same species in membrane phospholipids (Fig. 5D), thereby resulting in an unchanged total amount of these SFA (C16 and C18) in both BMDM from Wt and *Abcg1*^{MACKO} mice (Figure 5E). As a consequence, the ratio of phospholipids over triglycerides containing C16:0 and C18:0 and more largely saturated fatty acids was markedly reduced in *Abcg1*-deficient macrophages (Figure 5F-G). Similar results were observed in sorted ATM from Wt and *Abcg1*^{MACKO} mice fed a HFD for 8 weeks (Fig. S4A-E). In addition, the amount of C16:0 and C18:0 FA in TG was strongly inversely correlated with the same FA in phospholipids in ATM ($r=-0.74$, $p=0.0004$, $n=18$ mice). Altogether, these results suggested that *Abcg1* deficiency led to a redistribution of SFA from membranes to lipid droplets for storage and neutralization, and could partly explain the low inflammatory profile following *Abcg1* deletion.

It is well described that SFA content in membranes affects parameters such as rigidity/fluidity which drive inflammatory signaling and activation in macrophages^{33,34}. Coherent with the reduction of SFA content detected in membranes upon *Abcg1* deficiency, membrane rigidity was also diminished in *Abcg1*-deficient BMDM compared to controls as assessed by the quantification of membrane general polarization using the fluorescent probe Laurdan (Fig. 5H).

Our previous work showed that *Abcg1* expression controls Lpl bioavailability at the membrane surface²³. In accordance to this, we observed that Lpl activity was significantly reduced in *Abcg1*^{MACKO} BMDM (Fig. 5I). Since Lpl is bound to the plasma membrane by glycoproteins such as heparan sulfate proteoglycans (HSPG), glycomic analysis on BMDM revealed that *Abcg1* deficiency resulted on a modulation of 28 membrane glycoproteins (Fig. 5J), further supporting the proposed mechanism through which *Abcg1*-mediated modification of membrane order is necessary for Lpl release from the plasma membrane and subsequent activity. Finally, to evaluate if the anti-inflammatory effects of *Abcg1* deficiency were Lpl-dependent, we treated BMDM from *Abcg1*^{MACKO} and WT mice with tetrahydropipstatin (THL), an Lpl inhibitor, and analyzed cytokine and chemokine expression levels. This treatment was able to completely reverse the anti-inflammatory effect of *Abcg1* deletion by increasing *Ccl2*, *Ccl5*, *Il1b* and *Il10* mRNA levels (Fig. 5K), therefore underlying the importance of the *Abcg1*/Lpl axis in ATM inflammatory polarization.

Altogether, these results showed that the redistribution of the fatty acid pools orchestrated by *Abcg1* deficiency modulates membrane lipid composition and structure and contributes to the anti-inflammatory response in macrophages through a mechanism involving lipoprotein lipase.

Discussion

In adipose tissue from obese individuals LAM handle large quantities of lipids, and while the capacity of ATM to metabolize these lipids is expected to be beneficial, the fate of different lipid pools as well as their impact on ATM function remains largely undescribed. The present study provides new important information on the understanding of the mechanisms altering ATM lipid metabolism and macrophage plasticity in response to excess nutrients. By targeting membrane Abcg1 transporter, we revealed that ATM activation depends on their capacity to mobilize different FA pools partitioned between membranes and lipid droplets. As such, inhibition of Abcg1 diverted the membrane SFA pool towards a neutralization pathway, thus rendering these FA unavailable for activation of inflammatory signaling. This contributed to a less metabolically activated ATM phenotype, which globally improved adipose tissue health and attenuated insulin resistance.

Rewiring of fatty acid metabolism following Abcg1 deficiency involved a reduction of the relative abundance of several SFA-containing phospholipid classes such as PE and PC. The saturation degree and the length of the carbon chain of FA incorporated in membrane phospholipids are important modulators of the physiochemical properties of the membrane, including lipid packing and fluidity, which can contribute to the recruitment of inflammatory signaling proteins³⁵. The importance of membrane lipid composition in diet-induced inflammation and diabetes has been highlighted in a model of myeloid FAS deficiency, in which the absence of endogenous FA synthesis led to a disruption of membrane order and prevented the assembly of inflammatory signaling networks and the development of IR. This study also revealed that disruption of endogenous FA synthesis mainly altered phospholipid content on membranes microdomains, with no effect on whole-cell membranes³³. Our study however suggests that exogenous SFA issued from TRL have a major impact on whole-membrane lipid composition, more precisely the nature of FA incorporated into phospholipids, and are accompanied by a modulation of membrane fluidity and inflammation through a mechanism controlled by Abcg1. Similarly, inflammatory signaling has been found to support remodeling of membrane lipidome in lipid-loaded BMDM³⁶, thus underlying the reciprocal relationship between membrane lipid composition and inflammation.

The data in this study also showed that Abcg1-mediated depletion of SFA in membrane phospholipids was inversely associated to an enrichment of SFA-containing TG. We propose that this diversion of SFA from membranes to lipid droplets contributed to the robust reduction of the inflammatory phenotype observed in both Abcg1-deficient and low Abcg1 ATM from obese mice and patients, respectively. Indeed, the mobilization of inflammatory SFA into TG in lipid droplets allows for the neutralization of their inflammatory action through a mechanism of enzyme-catalyzed

esterification. Koliwad et al. demonstrated that the overexpression of diacylglycerol acyltransferase-1 (DGAT1), one of the main enzymes involved in FA esterification, was beneficial in ATM-mediated inflammation and IR as it increased TG storage capacity³⁷. Altogether, our study indicates that activation of the intracellular flux of SFA from membranes to lipid droplets upon Abcg1 deficiency may represent an anti-inflammatory pathway in LAM. However, this pathway is likely inefficient upon DIO as ATM Abcg1 expression is upregulated in this metabolic context.

We also evidenced that macrophage Abcg1 deficiency was accompanied by an alteration of ATM bioenergetic capacity, as illustrated by the impairment of glycolysis-dependent ATP production likely resulting from the reduction of macrophage glucose uptake. FAO was also decreased, suggesting an impairment of FA catabolism and a potential contribution of this mechanism to the increased SFA storage in lipid droplets. Moreover, this alteration of FAO did not reveal to affect ATP production associated with respiration, consistent with previous studies indicating that stimulation of macrophages with TRL inhibits the respiratory chain by promoting cytochrome dysfunction³⁸. The modulation of ATM energetics could also account for the inflammatory shift between Abcg1^{MACKO} and Wt ATM. Indeed, it is proposed that in a basal state macrophage glycolytic and respiratory capacities are associated with pro-inflammatory and anti-inflammatory polarization, respectively. However, in obesity, LAM present mixed inflammatory traits together with a hypermetabolic profile characterized by both high OXPHO and glycolysis rates⁸. In this context, some have shown that the inflammatory response of ATM is mainly driven by glycolysis, and that inhibition of this metabolic route significantly decreases ATM inflammatory cytokine release¹¹. Another recent study has nonetheless reported that OXPHO, rather than glycolysis, drives ATM activation and that its dysfunction ameliorates obesity-induced inflammation³⁹, thereby suggesting a pro-inflammatory effect of this pathway. Then, the reduction of both glycolysis and respiratory capacity observed in Abcg1-deficient ATM is likely to contribute to attenuation of their proinflammatory phenotype. This also supports the notion that successful metabolic adaptation (in regards of lipid metabolism and also energy metabolism) by ATM guides their functional response in the context of obesity.

In addition, our study provides a new, competent model of ATM activation by lipids that reconciles findings in mice and humans under DIO. As alluded to before, ATM are subject to a particular tissue microenvironment in which FFA originate from adipocytes, but also from lipolysis of circulating TRL. Recent models have proposed that *in vitro* stimulation of macrophages with glucose, insulin and palmitate is able to mimic the metabolic activation of ATM in obesity given that it induces the activation of lipid-specific programs⁶. This approach, however, bypasses the major role of TRL as a significant source of FFA in the context of DIO. Our findings suggest that addition of VLDL, together with glucose and insulin, is a relevant model for studying ATM plasticity in obesity as we were able to

validate the proinflammatory role of Abcg1 observed under these conditions in ATM isolated from both obese mice and humans. Moreover, the effect of TRL on ATM activation is highly dependent of FA/TG lipolysis because it allows for the intracellular uptake of remnant TRL. We here demonstrate the importance of the Abcg1 / Lpl axis in the inflammatory activation of ATM. Indeed, the use of an LPL inhibitor and the consequential inhibition of TRL hydrolysis abolished the anti-inflammatory action associated to Abcg1 deficiency. Our previous work has shown that Abcg1 expression is linked to Lpl bioavailability in macrophages, and that its inhibition results in the trapping of the enzyme at the cell surface as a consequence of the disrupted membrane structure^{23,40}. We here provided more information about the underlying mechanism through which Abcg1 controls Lpl activity. Indeed, Abcg1 deficiency in BMDM is accompanied by an alteration of several membrane glycoproteins, likely affecting Lpl binding to the cell surface and therefore its activity. This observation not only reinforces the major role of Abcg1 in membrane remodeling but uncover for the first time the contribution of Abcg1 in the distribution of membrane glycoproteins.

Globally, this pathway emphasizes the notion that Abcg1 function is highly dependent of the metabolic context⁴⁰. In a context of high cholesterol (i.e. in atherosclerotic plaques), macrophage Abcg1 main role is cholesterol transport⁴¹, and thus its inhibition is pro-inflammatory⁴². By contrast, in a high FA context (i.e. metabolic diseases such as obesity), macrophage Abcg1 predominantly governs FA metabolism through different pathways and its inhibition is anti-inflammatory as evidenced in the present study. Such a role is however mostly attributed to macrophages in adipose tissue, given that Abcg1-deficiency in liver macrophages was without effect on diet-induced liver inflammation.

We propose a model in which the lack of Abcg1 in ATM is accompanied by a reduced Lpl activity and a decrease of TRL lipolysis and SFA release. Pro-inflammatory SFA are then less available to activate inflammatory pathways through the CD36/TLR axis. The lower abundance of SFA together with the decreased expression of genes involved in FA metabolism (uptake, transport and catabolism) impair the use of SFA for phospholipid synthesis and FAO. As a consequence, membrane phospholipids are impoverished in SFA content, leading to a reduction of membrane rigidity. Because of the impaired Lpl activity, we also see that ATM internalize a higher content of SFA-enriched TRL through specialized receptors including VLDLR, leading to increased SFA storage and neutralization in lipid droplets. Taken together, this rewiring of FA metabolism upon Abcg1 deficiency attenuates the inflammatory phenotype of LAM and alleviates AT inflammation and IR in DIO (Figure 6).

In summary, our study helps to decipher the complex function of ATM during high energy diets and its impact on obesity-induced adipose tissue inflammation and IR, by delineating the contribution of macrophage Abcg1 in FA handling. Our data support the notion that the nature of dietary FA plays

a major role in the development of metabolic disorders and that diets enriched in unsaturated FA, preferentially in omega-3 PUFAs which possess potent anti-inflammatory properties and insulin-sensitizing effects, might be protective. The use of omega-3 PUFAS (icosapent ethyl) has been indeed proposed as a potential treatment in diabetic and statin-treated patients in the ANCHOR trial, as it was able to reduce inflammatory parameters after a 12-week therapy⁴³.

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Acknowledgments

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Contributors.

VDD, PK, CM, CR, EF, HD, LMH, IG, GL performed experiments and collected data. VDD, EF, AK, MG, OB, FD and WLG designed the research. AS recruited the patients. VDD, PK, ML, MP, MD, NV, OB and WLG analyzed the data. IG and MG provided key resources. All authors gave critical comments on the manuscript. VDD and WLG wrote and edited the manuscript. WLG funded and supervised the research.

Declaration of interests.

The authors declare no competing financial interests.

	All (n=14)	Low <i>ABCG1</i> (n=7)	High <i>ABCG1</i> (n=7)	<i>P</i> value
Age (years)	42.37 ± 4.14	37.19 ± 4.42	45.07 ± 6.67	0.224
BMI (kg/m ²)	41.02 ± 1.25	39.62 ± 1.30	42.43 ± 2.09	0.277
Weight (kg)	115.79 ± 4.89	114.86 ± 5.95	116.71 ± 8.24	0.858
HbA1c (%)	5.92 ± 0.31	5.80 ± 0.50	5.97 ± 0.41	0.828
TC (g/L)	2.03 ± 0.13	1.81 ± 0.27	2.14 ± 0.13	0.265
HDL-C (g/L)	0.57 ± 0.11	0.81 ± 0.26	0.46 ± 0.06	0.128
LDL-C (g/L)	1.58 ± 0.20	1.91 ± 0.62	1.41 ± 0.10	0.287

Table 1. Clinical and biological parameters of patients included in the study. Values are means ± S.E.M. BMI: body mass index, HDL-C: high-density lipoprotein-cholesterol, LDL: low-density lipoprotein-cholesterol, TC: total cholesterol.

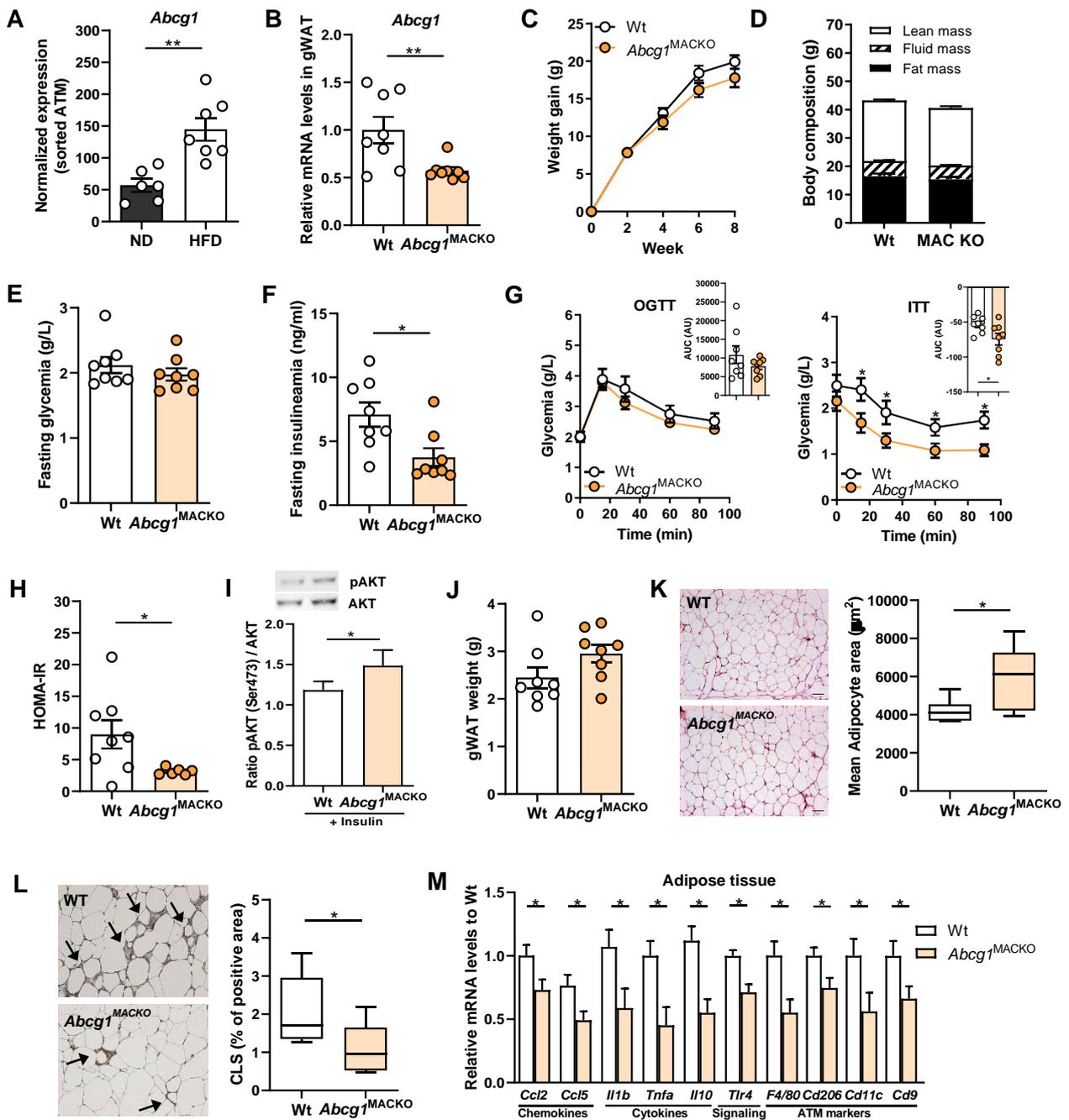


Figure 1. *Abcg1* deficiency in ATM alleviates adipose tissue inflammation and insulin resistance.

(A) *Abcg1* mRNA levels in sorted gonadal adipose tissue macrophages (ATM) from mice fed a normal (ND) or a high-fat diet (HFD). (B) Relative mRNA levels of *Abcg1* in whole gonadal white AT (gWAT) from Wt and *Abcg1*^{MACKO} mice under HFD for 8 weeks. Body weight gain (C), body composition (D), fasting glycemia (E), fasting plasma insulin levels (F), oral glucose tolerance test (OGTT) (G, left panel) and insulin tolerance test (ITT) (G, right panel) (Area under the curve (AUC) in insert) and HOMA-IR score (H) in Wt and *Abcg1*^{MACKO} mice fed a HFD. Phosphorylated AKT (Ser473) (pAKT) to total AKT ratio in response to insulin (I), total weight (J), hematoxylin & eosin staining (scale bar, 100µm) (K, left panel) and quantification of mean adipocyte area (K, right panel), F4/80⁺ macrophage staining (L, left panel) and quantification of crown-like structures (CLS) (L, right panel) and relative mRNA levels of inflammatory genes and ATM markers (M) in total gWAT from Wt or *Abcg1*^{MACKO} mice fed a HFD. Results are expressed as mean ± SEM. n = 6-8 mice per group. *p < 0.05. **p < 0.01 versus Wt.

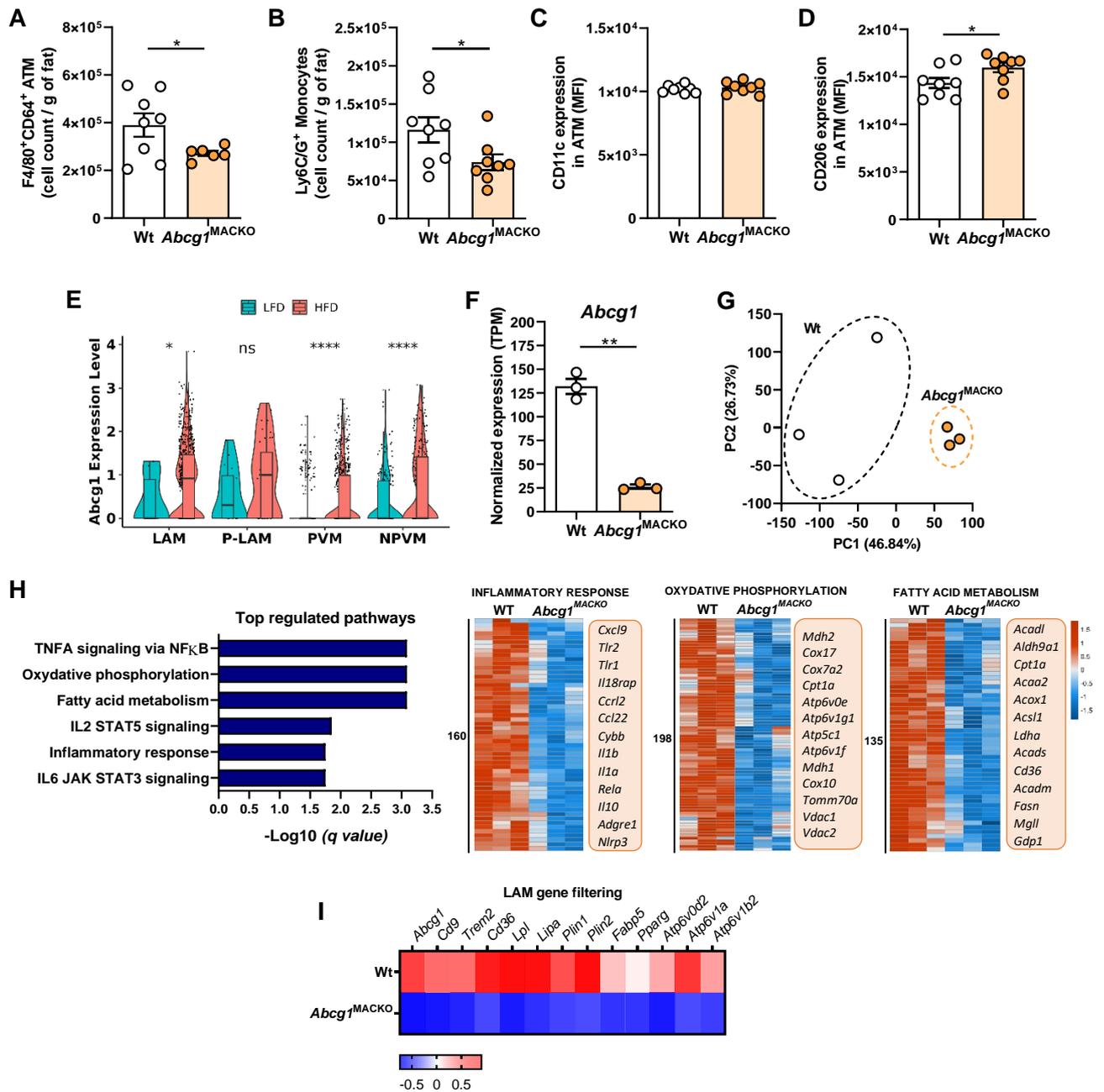


Figure 2. *Abcg1* deficiency reduces inflammatory status and metabolic activation of ATM. Wild-type (Wt) and *Abcg1*^{MACKO} mice were fed a high-fat diet (60%) for 8 weeks. Absolute macrophage content (defined as viable CD45⁺CD11b⁺CMHII⁺F4/80⁺CD64⁺ cells) (A), percentage of monocytes (defined as viable CD45⁺Ly6C/G⁺) (B), pro-inflammatory Cd11c⁺ (C) and anti-inflammatory Cd206⁺ (D) adipose tissue macrophages (ATM) (mean fluorescence intensity) in gonadal adipose tissue. (E) *Abcg1* expression in ATM subpopulations by single cell RNA-seq analysis from mice fed a Low (LFD) or High-fat diet (HFD). (F) *Abcg1* TPM values in sorted ATM (n=3 mice per group). (G) Principal component analysis of the transcriptome from bulk-RNAseq of sorted ATM. (H) Bar plot of the top modified pathways and corresponding heatmaps. (I) Heatmap of the differentially expressed genes (DEG) signing lipid-associated macrophages (LAM) between Wt and *Abcg1* deficient ATM. Results are expressed as mean \pm SEM. n = 6-10 mice per group. *p < 0.05. **p < 0.01. LAM = Lipid-associated macrophages. P-LAM = Proliferating lipid-associated macrophages. PVM = Perivascular-like macrophages. NPVM = Non-perivascular-like macrophages.

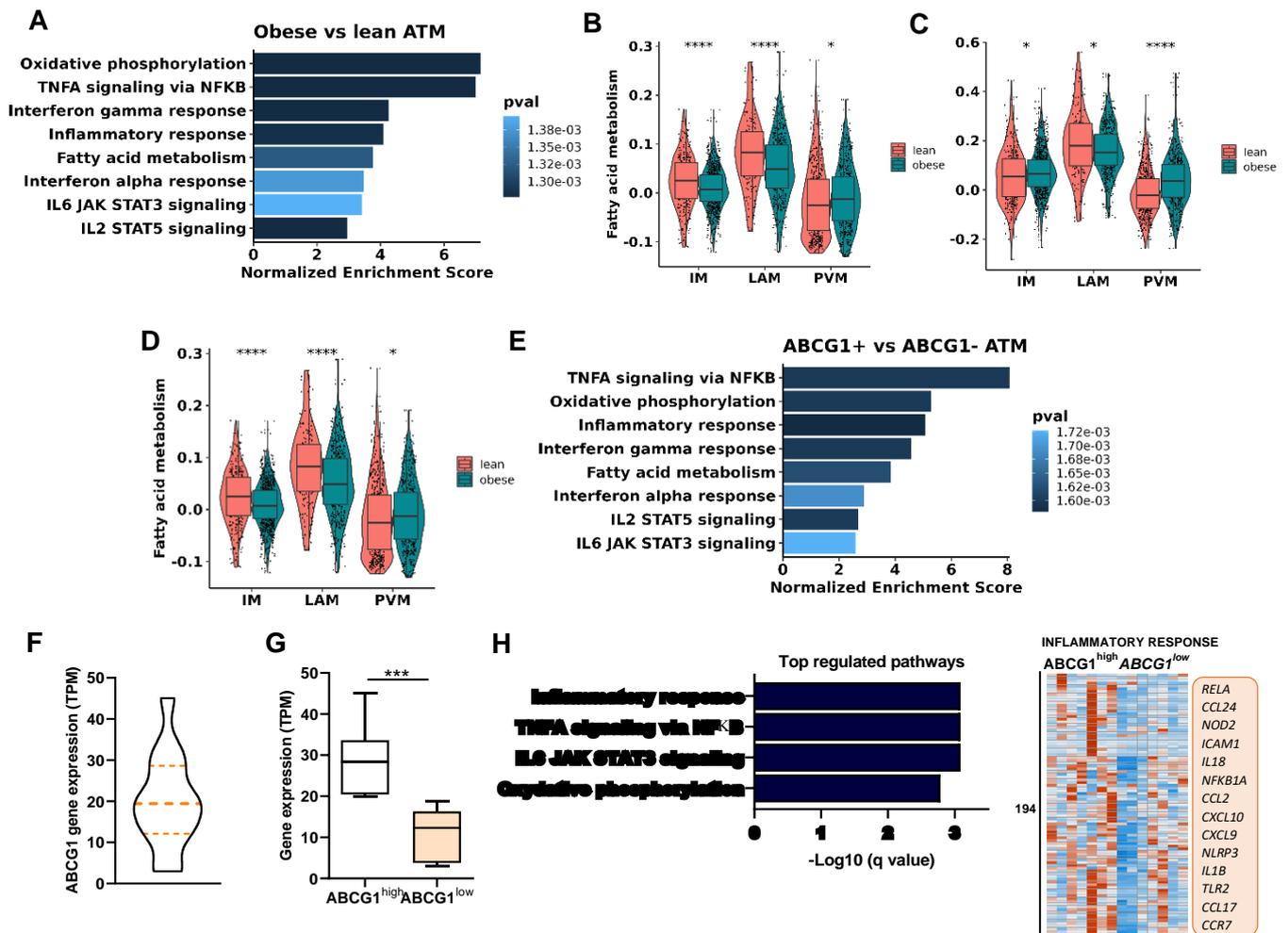


Figure 3. ABCG1 expression is associated with pro-inflammatory pathways and oxidative phosphorylation in human ATM from obese individuals. (A) Bar plot of Normalized Enrichment Score between ATM from obese vs lean individuals for inflammatory pathways. (B) Violin plot comparing scores in human ATM subtype according to lean or obese status, for inflammatory response, (C) oxidative / phosphorylation and (D) fatty acid metabolism. (E) Bar plot of Normalized Enrichment Score between ABCG1-positive vs ABCG1 negative ATM for inflammatory pathways (F) Violin plot showing ABCG1 expression (Transcript per million, TPM) by RNA-Seq analysis in sorted omental ATM from 14 obese subjects. (G) ABCG1 expression in ABCG1^{high} (n=7) and ABCG1^{low} (n=7) ATM groups. (H) Bar plot of the top modified pathways (left panel) and corresponding heatmap for inflammatory response (right panel). Results are expressed as mean \pm SEM. n = 6-10. ***p < 0.001. IM = Inflammatory macrophages. LAM = Lipid-associated macrophages. PVM = Perivascular-like macrophages

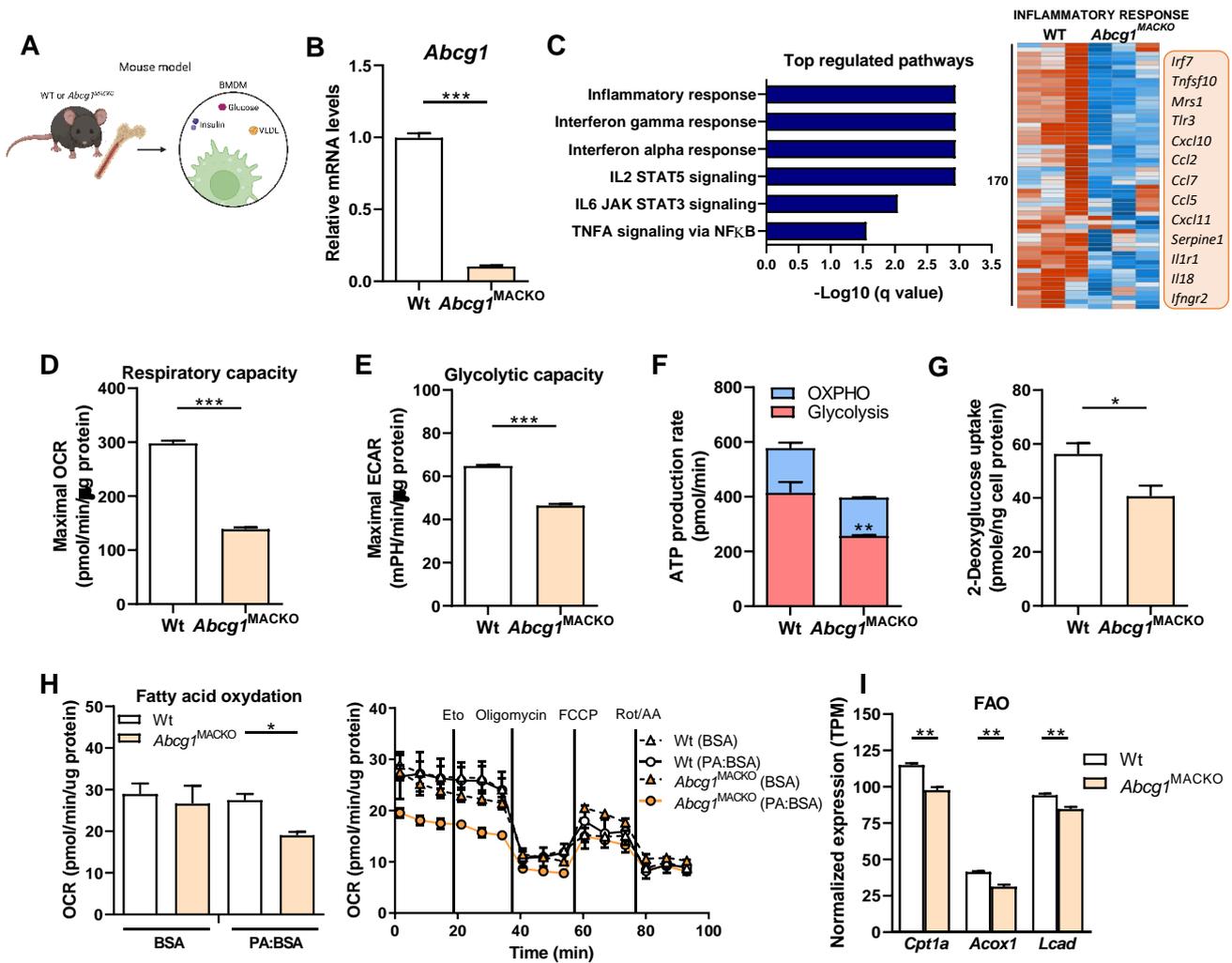


Figure 4. *Abcg1*-deficient macrophages present a low bioenergetic profile and a reduced fatty acid oxidation capacity. Bone marrow-derived macrophages (BMDM) isolated from Wt and *Abcg1*^{MACKO} mice were metabolically activated with very-low density lipoproteins (VLDL) (50 µg protein/ml), insulin (10mM) and glucose (30mM) for 24h. (A) Transcript per million (TPM) values for *Abcg1* in RNA-Seq analysis. (B) Bar plot of the top modified pathways in *Abcg1*^{MACKO} BMDM (left panel) and corresponding heatmaps (right panel). Maximal respiration rate defined as the maximal oxygen consumption rate (OCR) (D) and maximal glycolytic rate defined as the maximal extracellular acidification rate (ECAR) (E) in Wt and *Abcg1*^{MACKO} BMDM. (F) ATP production rate from glycolysis and mitochondria oxydative phosphorylation (OXPHO) in Wt and *Abcg1*^{MACKO} BMDM. 2-deoxyglucose uptake (G) and fatty acid oxydation (FAO) capacity (H) in Wt and *Abcg1*^{MACKO} BMDM. (I) TPM values for genes involved in fatty acid oxydation. BMDM were plated from a pool of 4-6 mice of each genotype. Results are expressed as mean ± SEM. *p < 0.05. **p < 0.01. ***p < 0.001 versus Wt. BSA: bovine serum albumin, PA: palmitic acid, Eto: etoxomir, FCCP: Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, Rot/AA: Rotenone/Antimycin.

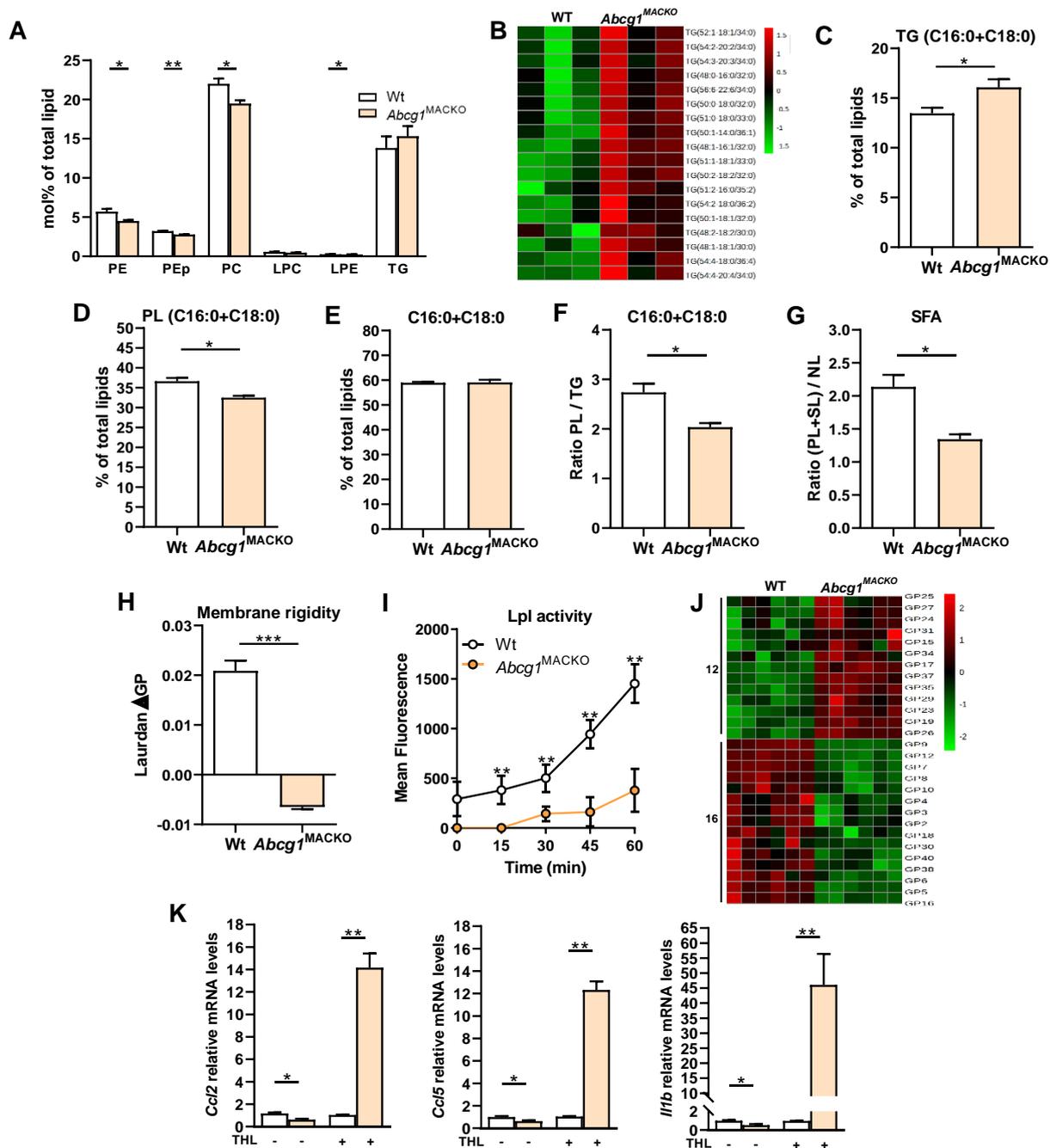


Figure 5. Redistribution of saturated fatty acid pools by *Abcg1* governs membrane lipid remodeling and inflammation in macrophages. Bone marrow-derived macrophages (BMDM) from Wt and *Abcg1*^{MACKO} mice were metabolically activated with very-low density lipoproteins (VLDL) (50 μ g protein/ml), insulin (10mM) and glucose (30mM) for 24h. Concentrations of lipid classes by lipidomic analysis (A) and heatmap of modified triglyceride (TG) species in BMDM from Wt and *Abcg1*^{MACKO} mice (n=3) (B). Abundance of triglycerides (TG) (C), phospholipids (PL) (D) and total lipids (E) containing saturated fatty acids (SFA) with 16 (C16:0) and 18 (C18:0) carbon chain length. (F) Ratio of PL over TG containing C16:0 and C18:0 fatty acids. (G) Ratio of PL and sphingolipids (SL) over neutral lipids (NL) containing SFA. (H) Differential generalized polarization (GP) of membranes corresponding to membrane rigidity in BMDM from Wt and *Abcg1*^{MACKO} mice. Measurement of lipoprotein lipase activity (I) and heatmap corresponding to the top modified membrane glycoproteins (J) in Wt and *Abcg1*^{MACKO} BMDM. (K) Relative mRNA levels of chemokines and cytokines in Wt and *Abcg1*^{MACKO} BMDM treated with VLDL \pm tetrahydrolipstatin (THL, a LPL inhibitor). BMDM were plated from a pool of 4-6 mice of each genotype. Results are expressed as mean \pm SEM. *p < 0.05. **p < 0.01. ***p < 0.001. LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PEp: plasmalogen PE.

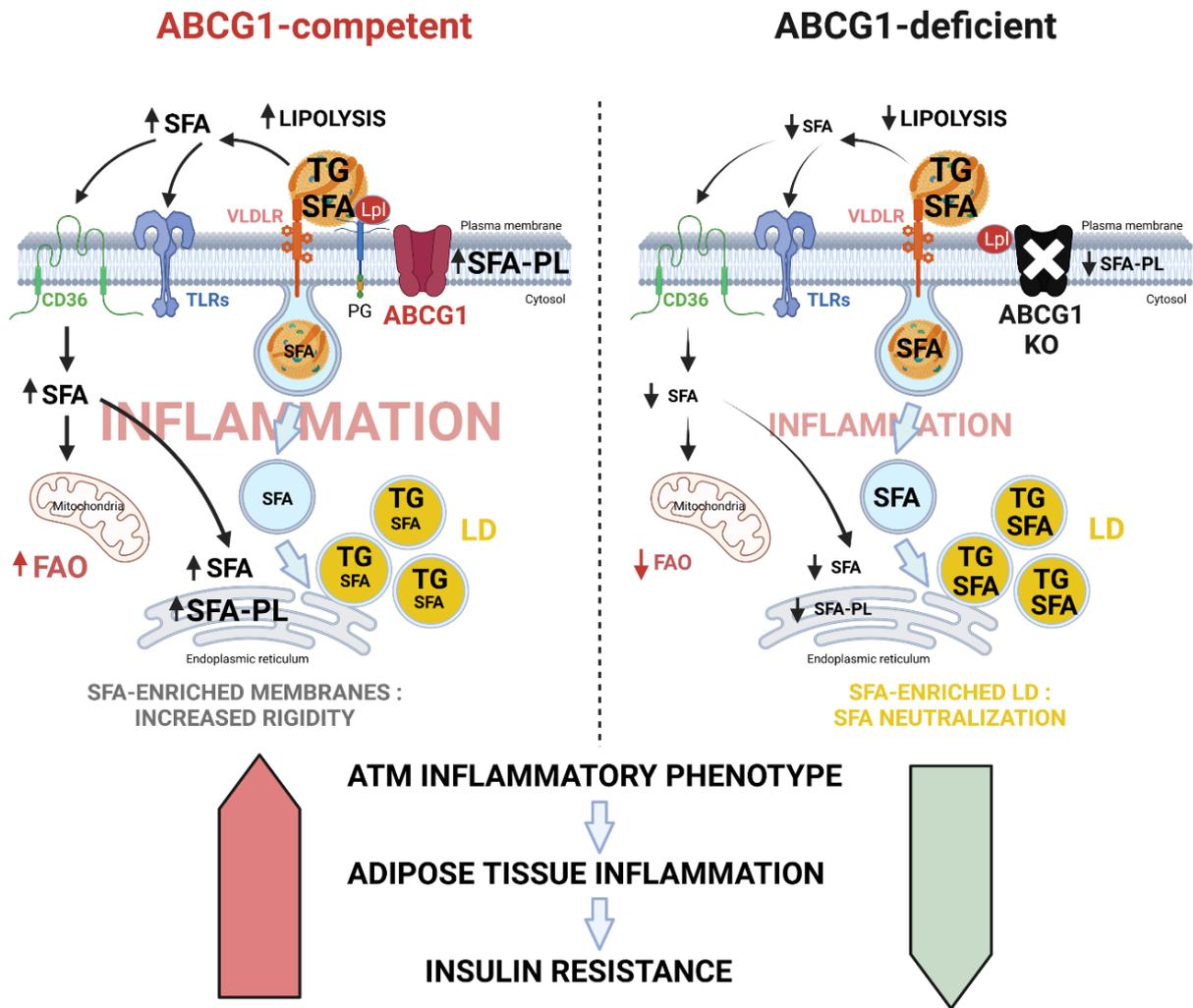
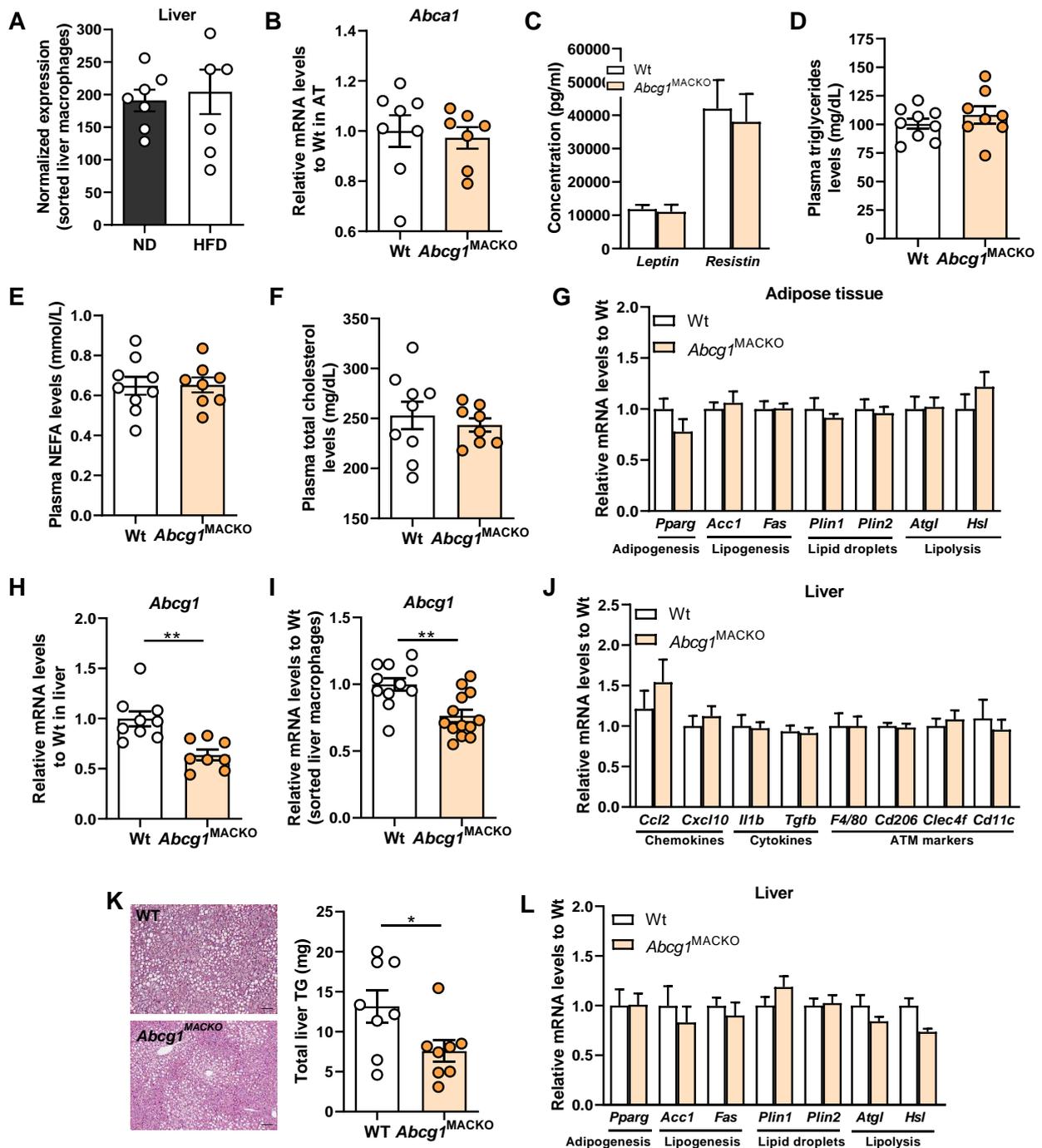
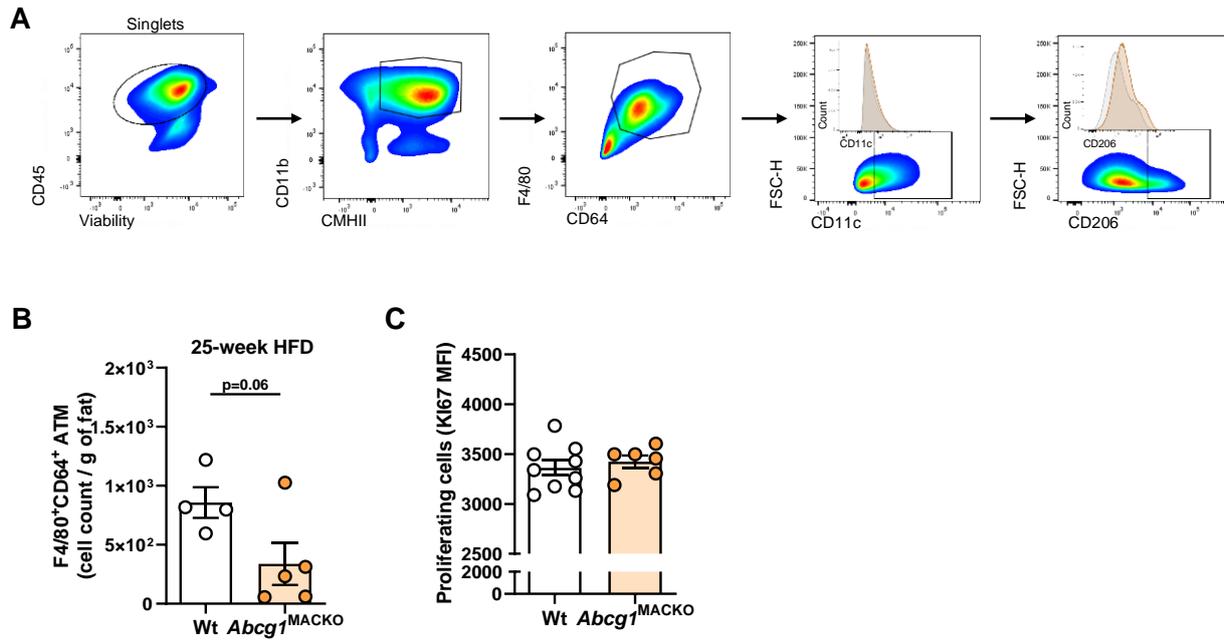


Figure 6. Proposed mechanism for the role of Abcg1 in ATM plasticity and insulin resistance in DIO.

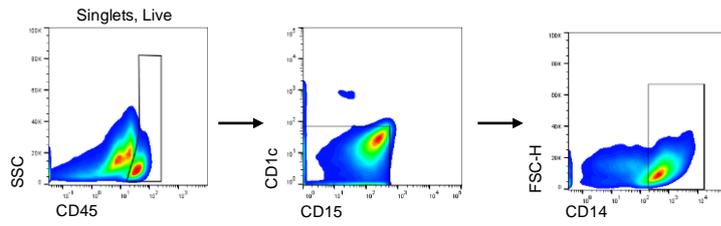
Supplemental data



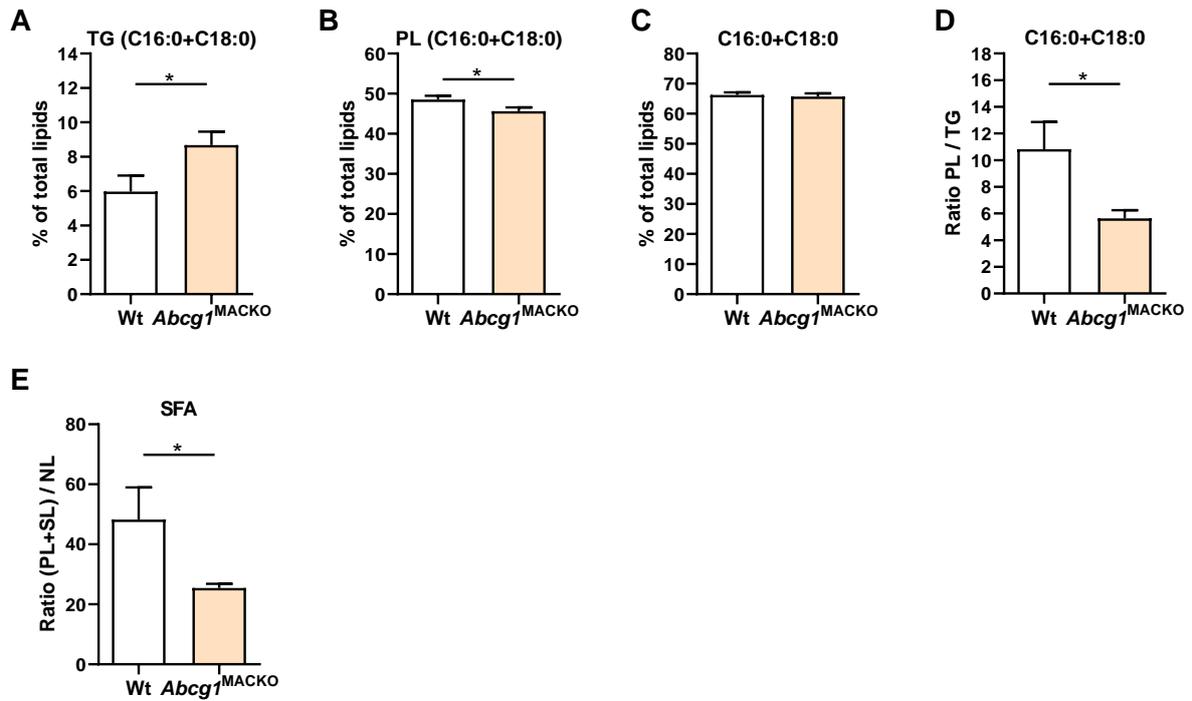
Supplemental Figure 1. No effect of macrophage *Abcg1*-deficiency in liver inflammation. (A) *Abcg1* mRNA levels in sorted liver macrophages from mice fed a normal (ND) or a high-fat diet (HFD). (B) Quantification of *Abca1* mRNA levels in whole gonadal adipose tissue (AT) from Wt and *Abcg1*^{MACKO} mice under high-fat diet for 8 weeks. Plasma leptin and resistin (C), triglycerides (D), non-esterified fatty acids (E) and total cholesterol (F) concentrations. Relative mRNA levels of genes involved in lipid metabolism in whole gonadal AT. Relative mRNA levels of *Abcg1* in (H) total liver and (I) sorted liver macrophages. (J) Relative mRNA expression of inflammatory genes and macrophage markers in total liver of Wt or *Abcg1*^{MACKO} mice. (K) Hematoxylin & eosin staining of liver (scale bar. 100µm) (left panel) and quantification of total TG content (right panel). (L) Relative mRNA levels of genes involved in lipid metabolism in whole liver. Results are expressed as mean ± SEM. n = 6-13 mice per group. *p < 0.05 and ** p < 0.005 versus Wt.



Supplemental Figure 2. Analysis of mouse gonadal ATM by flow cytometry. (A) Gating strategy for adipose tissue macrophages (ATM) from Wt and *Abcg1*^{MACKO} mice under high-fat diet (HFD). Total (defined as viable CD45⁺CD11b⁺CMHII⁺F4/80⁺CD64⁺ cells) (B) and proliferating (defined as Ki67⁺ cells, mean fluorescence intensity (MFI)) (C) ATM in gonadal adipose tissue from mice under 25- and 8-week HFD, respectively. Results are expressed as mean \pm SEM. $n = 4-9$ mice per group. * $p < 0.05$.



Supplemental Figure 3. Analysis of human omental ATM by flow cytometry. Gating strategy for adipose tissue macrophages (ATM) sorting in omental adipose tissue from obese subjects undergoing bariatric surgery.



Supplemental Figure 4. Redistribution and neutralization of pro-inflammatory saturated fatty acids in *Abcg1*-deficient ATM. Gonadal adipose tissue macrophages (ATM) were sorted from Wt and *Abcg1*^{MACKO} mice fed a high-fat diet (60%) for 8 weeks. Abundance of triglycerides (TG) (A), phospholipids (PL) (B) and total lipids (C) containing saturated fatty acids (SFA) with 16 (C16:0) and 18 (C18:0) carbon chain length. (D) Ratio of PL over TG containing C16:0 and C18:0 fatty acids. (E) Ratio of PL and sphingolipids (SL) over neutral lipids (NL) containing SFA. Results are expressed as mean \pm SEM. * $p < 0.05$. $n=8-10$ mice per group.

Antibody	Dye	Species	Clone	Reference
Fixable viability dye	eF506	N/A	N/A	eBiosciences, 65-0866-14
Fixable viability dye	eF520	N/A	N/A	eBiosciences, 65-0867-18
CD45	BUV395	Mouse	30-F11	BD Biosciences, 564279
CD19	FITC	Mouse	1D3	BD Biosciences, 553785
Ly6C/G	PerCP Cy5	Mouse	RB6-8C5	eBioscience, 14-5931-82
CD11b	eF450	Mouse	M1/70	eBiosciences, 48-0112-82
CMHII	AF700	Mouse	M5/114.15.2	eBiosciences, 56-5321-82
F4/80	APC	Mouse	BM8	eBiosciences, 17-4801-82
CD64	PE	Mouse	X54-5/7.1	eBiosciences, 12-0641-82
CD11c	APC Cy7	Mouse	HL3	BD Biosciences, 561241
CD206	PE Cy7	Mouse	MR6F3	eBiosciences, 25-2061-82
CD45	BV711	Human	HI30	Biologend, 304050
CD3	FITC	Human	UCHT1	BD Biosciences, 555332
CD19	FITC	Human	HIB19	BD Biosciences, 555412
CD15	Pacific Blue	Human	W6D3	Biologend, 323022
CD1c	APC Cy7	Human	L161	Biologend, 331520
CD11b	APC	Human	M1/70	Biologend, 101212
CD14	PE	Human	M5E2	Biologend, 301806
Bodipy	FITC	N/A	N/A	Invitrogen, D3821
Ki67	AF700	N/A	N/A	BD Biosciences, 561277

Supplemental Table 1. Surface markers for FACS analysis and cell sorting

Gene	Forward	Reverse
Ccl2	GCCTGCTGTTACAGTGGC	CAGGTGAGTGGGGCGTTA
Ccl5	TGCAGAGGACTCTGAGACAGC	GAGTGGTGTCCGAGCCATA
Cxcl10	GCTGCCGTCATTTTCTGC	TCTCACTGGCCCCTCATC
Il1b	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
Tnfa	TCTTCTCATTCTGCTTGTGG	GGTCTGGGCCATAGAACTGA
Tgfb	TGGAGCAACATGTGGAAGTC	CAGCAGCCGGTTACCAAG
Il10	CAGAGCCACATGCTCCTAGA	GTCCAGCTGGTCCTTTGTTT
F4/80	GGAGGACTTCTCCAAGCCTATT	AGGCCTCTCAGACTTCTGCTT
Cd206	CCACAGCATTGAGGAGTTTG	ACAGCTCATCATTTGGCTCA
Cd11c	GAGCCAGAAGTTCCCAACTG	TCAGGAACACGATGTCTTGG
Cd9	CTGTGGCATAGCTGGTCCTTTG	AGACCTCACTGATGGCTTCAG
Clec4f	GAACAAGCTCTGGACACGACAA	AGCCTCTGGATAGCCACTTC
Plin1	GAGAAGGTGGTAGAGTTCCTCC	GTGTGTCGAGAAAGAGTGTGGC
Plin2	CTCCACTCCACTGTCCACCT	GCTTATCCTGAGCACCTGA
Pparg	AGGCCGAGAAGGAGAAGCTGTTG	TGGCCACCTCTTTGCTCTGCTC
Acc1	AGAACCCGAAACTCCCAGAA	TGCAATCTTATCCCCCAAAG
Fasn	CCAAGCAGGCACACACAA	CACTCACACCCACCCAGA
Atgl	TGACCATCTGCCTTCCAGA	TGTAGGTGGCGCAAGACA
Hsl	TCCTCATGGCTCAACTCCTT	GCTGCCTCAGACACACTCCT
Nono	GCTCGTGAGAAGCTGGAGAT	TTCTTGACGTCTCATCAAATCC
CycloA	TGGAGAGCACCAAGACAGA	TGCCGGAGTCGACAATGAT
Hsp90	TACTCGGCTTTCCCGTCA	TGAAAGGCAAAGGTCTCCA
Hprt	TGACACTGGTAAACAATGC	AACACTTCGAGAGGTCCTTT

Supplemental Table 2. Mouse Primers for quantitative RT-PCR

		Lipid species	Structural elucidation	Structural elucidation method
NEUTRAL LIPIDS	Cholesteryl esters	CE(16:0)	16:0	[M+NH4] ⁺ PIS 369m/z
		CE(16:1)	16:1	
		CE(18:0)	18:0	
		CE(18:1)	18:1	
		CE(18:2)	18:2	
		CE(18:3)	18:3	
		CE(20:3)	20:3	
		CE(20:4)	20:4	
		CE(22:5)	22:5	
		CE(22:6)	22:6	
	Diacylglycerides	DG(30:0)	14:0_16:0	[M+NH4] ⁺ NL(RCOO+NH3)
		DG(32:0)	16:0_16:0	
		DG(32:1)	14:0_18:1	
		DG(34:0)	16:0_18:0	
		DG(34:1)	16:0_18:1	
		DG(34:2)	16:0_18:2	
		DG(34:2)	16:1_18:1	
		DG(34:4)	16:1_18:3	
		DG(36:1)	18:0_18:1	
		DG(36:2)	18:0_18:2	
		DG(36:2)	18:1_18:1	
		DG(36:3)	16:0_20:3	
		DG(36:3)	18:1_18:2	
		DG(36:4)	16:0_20:4	
		DG(36:4)	18:1_18:3	
		DG(36:4)	18:2_18:2	
		DG(38:3)	18:0_20:3	
		DG(38:3)	18:1_20:2	
		DG(38:4)	18:0_20:4	
		DG(38:4)	18:1_20:3	
	DG(38:5)	16:0_22:5		
	DG(38:5)	18:1_20:4		
	DG(38:6)	16:0_22:6		
DG(40:6)	18:0_22:6			
DG(40:7)	18:1_22:6			
Triacylglycerides	TG(48:0-16:0/32:0)	16:0_16:0_16:0	[M+NH4] ⁺ NL(RCOO+NH3)	
	TG(48:1-16:1/32:0)	16:0_16:0_16:1		
	TG(48:1-18:1/30:0)	14:0_16:0_18:1		
	TG(48:2-14:1/34:1)	14:1_16:0_18:1		
	TG(48:2-16:0/32:2)	16:0_16:1_16:1		
	TG(48:2-18:1/30:1)	14:0_16:1_18:1		
	TG(48:2-18:2/30:0)	14:0_16:0_18:2		
	TG(48:3-14:0/34:3)	14:0_16:1_18:2		
	TG(50:0-18:0/32:0)	16:0_16:0_18:0		

		TG(50:1-14:0/36:1)	14:0_18:0_18:1	
		TG(18:1_32:0)	16:0_16:0_18:1	
		TG(18:0_32:2)	16:1_16:1_18:0	
		TG(18:1_32:1)	16:0_16:1_18:1	
		TG(18:2_32:0)	16:0_16:0_18:2	
		TG(14:1_36:2)	14:1_18:1_18:1	
		TG(18:1_32:2)	16:1_16:1_18:1	
		TG(18:2_32:1)	16:1_16:0_18:2	
		TG(14:0_36:4)	14:0_16:0_20:4	
		TG(18:0_33:0)	18:0_16:0_17:0	
		TG(18:1_33:0)	16:0_17:0_18:1	
		TG(15:0_36:2)	15:0_18:1_18:1	
		TG(16:0_35:2)	16:0_17:0_18:2	
		TG(18:1_34:0)	16:0_18:0_18:1	
		TG(16:0_36:2)	16:0_18:1_18:1	
		TG(16:1_36:2)	16:1_18:1_18:1	
		TG(18:2_34:1)	16:0_18:1_18:2	
		TG(16:1_36:3)	16:1_16:0_20:3	
		TG(20:4_32:0)	16:0_16:0_20:4	
		TG(17:0_36:2)	17:0_18:1_18:1	
		TG(18:1_36:0)	18:0_18:0_18:1	
		TG(18:0_36:2)	18:0_18:1_18:1	
		TG(20:2_34:0)	16:0_18:0_20:2	
		TG(18:1_36:2)	18:1_18:1_18:1	
		TG(20:3_34:0)	16:0_18:0_20:3	
		TG(18:0_36:4)	18:0_16:0_20:4	
		TG(18:2_36:2)	18:1_18:1_18:2	
		TG(20:4_34:0)	16:0_18:0_20:4	
		TG(20:4_34:1)	16:0_18:1_20:4	
		TG(22:5_32:0)	16:0_16:0_22:5	
		TG(20:4_36:2)	18:1_18:1_20:4	
		TG(22:5_34:1)	16:0_18:1_22:5	
		TG(22:6_34:0)	16:0_18:0_22:6	
		TG(20:4_36:4)	16:0_20:4_20:4	
GLYCEROPHOSPHOLIPIDS	Lysophosphatidylcholine	LPC(16:0)	16:0	[M+H] ⁺ PIS 184m/z
		LPC(16:1)	16:1	
		LPC(16:2)	16:2	
		LPC(18:0)	18:0	
		LPC(18:1)	18:1	
		LPC(18:2)	18:2	
		LPC(20:0)	20:0	
		LPC(20:1)	20:1	
		LPC(20:2)	20:2	
		LPC(20:3)	20:3	
		LPC(20:4)	20:4	
		LPC(20:5)	20:5	
		LPC(22:3)	22:3	

		LPC(22:4)	22:4	
		LPC(22:5)	22:5	
		LPC(22:6)	22:6	
Lysophosphatidylethanolamine		LPE(16:0)	16:0	[M+H] ⁺ NL 141
		LPE(16:1)	16:1	
		LPE(18:0)	18:0	
		LPE(18:1)	18:1	
		LPE(18:2)	18:2	
		LPE(20:3)	20:3	
		LPE(20:4)	20:4	
		LPE(20:5)	20:5	
		LPE(22:1)	22:1	
		LPE(22:2)	22:2	
		LPE(22:3)	22:3	
		LPE(22:4)	22:4	
		LPE(22:5)	22:5	
		LPE(22:6)	22:6	
Phosphatidic acid		PA(34:0)	16:0_18:0	extrapolated from literature
		PA(34:1)	16:0_18:1	
		PA(34:2)	16:0_18:2	
		PA(36:1)	18:0_18:1	
		PA(36:2)	18:0_18:2	
		PA(36:4)	16:0_20:4	
		PA(38:3)	18:0_20:3	
		PA(38:4)	18:0_20:4	
Phosphatidylcholine		PC(28:0)	14:0_14:0	extrapolated from literature
		PC(30:0)	14:0_16:0	
		PC(30:1)	14:0_16:1	
		PC(30:2)	14:1_16:2	
		PC(32:0)	16:0_16:0	
		PC(32:1)	16:0_16:1	
		PC(34:0)	16:0_18:0	
		PC(34:1)	16:0_18:1	
		PC(34:2)	16:0_18:2	
		PC(34:3)	16:1_18:2	
		PC(36:0)	18:0_18:0	
		PC(36:1)	18:0_18:1	
		PC(36:2)	18:0_18:2	
		PC(36:3)	18:1_18:2	
		PC(36:4)	16:0_20:4	
		PC(36:5)	16:1_20:4	
		PC(36:6)	14:0_22:6	
		PC(38:2)	18:0_20:2	
		PC(38:3)	18:0_20:3	
		PC(38:4)	18:0_20:4	
	PC(38:5)	16:0_22:5		

		PC(38:6)	16:0_22:6		
		PC(40:4)	20:0_20:4		
		PC(40:5)	18:0_22:5		
		PC(40:6)	18:1_22:5		
		PC(40:7)	18:1_22:6		
		PC(40:8)	18:2_22:6		
	Phosphatidylethanolamine	PE(28:0)	14:0_14:0		extrapolated from literature
		PE(30:0)	14:0_16:0		
		PE(30:1)	14:0_16:1		
		PE(32:0)	16:0_16:0		
		PE(32:1)	16:0_16:1		
		PE(34:0)	16:0_18:0		
		PE(34:1)	16:0_18:1		
		PE(34:2)	16:0_18:2		
		PE(36:0)	18:0_18:0		
		PE(36:1)	18:0_18:1		
		PE(36:2)	18:0_18:2		
		PE(36:3)	18:1_18:2		
		PE(36:4)	16:0_20:4		
		PE(36:5)	16:1_20:4		
		PE(38:2)	18:0_20:2		
		PE(38:3)	18:0_20:3		
		PE(38:4)	18:0_20:4		
		PE(38:5)	16:0_22:5		
		PE(38:6)	16:0_22:6		
		PE(40:4)	20:0_20:4		
		PE(40:5)	18:0_22:5		
		PE(40:6)	18:0_22:6		
		PE(40:7)	18:1_22:6		
		PE(40:8)	18:2_22:6		
	PE plasmalogen	PE(16:0p/18:2)	18:2		[M+H] ⁺ PIS(RCO+PE)
		PE(16:0p/20:1)	20:1		
		PE(16:0p/20:2)	20:2		
PE(16:0p/20:3)		20:3			
PE(16:0p/20:4)		20:4			
PE(16:0p/22:4)		22:4			
PE(16:0p/22:5)		22:5			
PE(16:0p/22:6)		22:6			
PE(18:0p/18:1)		18:1			
PE(18:0p/18:2)		18:2			
PE(18:0p/20:3)		20:3			
PE(18:0p/20:4)		20:4			
PE(18:0p/20:5)		20:5			
PE(18:0p/22:6)		22:6			
PE(18:0p/24:5)	24:5				
Phosphatidylglycerol	PG(32:0)	16:0_16:0	extrapolated from literature		
	PG(34:1)	16:0_18:1			

SPHINGOLIPIDS		PG(34:2)	16:0_18:2	
		PG(36:1)	18:0_18:1	
		PG(36:2)	18:0_18:2	
		PG(36:3)	18:1_18:2	
		PG(36:4)	16:0_20:4	
		PG(38:2)	18:0_20:2	
		PG(38:3)	18:0_20:3	
		PG(38:4)	18:0_20:4	
		PG(38:6)	16:0_22:6	
	Phosphatidylinositol	PI(32:1)	16:0_16:1	extrapolated from literature
		PI(34:1)	16:0_18:1	
		PI(34:2)	16:0_18:2	
		PI(36:1)	18:0_18:1	
		PI(36:2)	18:0_18:2	
		PI(36:3)	18:1_18:2	
		PI(36:4)	16:0_20:4	
		PI(38:2)	18:0_20:2	
		PI(38:3)	18:0_20:3	
		PI(38:4)	18:0_20:4	
		PI(38:5)	16:0_22:5	
		PI(38:6)	16:0_22:6	
		PI(40:4)	20:0_20:4	
		PI(40:5)	18:0_22:5	
	PI(40:6)	18:0_22:6		
	PI(40:7)	18:1_22:6		
	Phosphatidylserine	PS(34:0)	16:0_18:0	extrapolated from literature
		PS(34:1)	16:0_18:1	
		PS(34:2)	16:0_18:2	
		PS(36:1)	18:0_18:1	
		PS(36:2)	18:0_18:2	
		PS(36:3)	18:1_18:2	
		PS(36:4)	16:0_20:4	
		PS(38:3)	18:0_20:3	
PS(38:4)		18:0_20:4		
PS(38:6)		16:0_22:6		
PS(40:4)		20:0_20:4		
PS(40:5)		18:0_22:5		
PS(40:6)	18:0_22:6			
Dihydroceramides	Cer 18:0;O2/16:0	16:0	[M+H] ⁺ PIS 266m/z	
	Cer 18:0;O2/18:0	18:0		
	Cer 18:0;O2/18:1	18:1		
	Cer 18:0;O2/22:0	22:0		
	Cer 18:0;O2/23:0	23:0		
	Cer 18:0;O2/24:0	24:0		
	Cer 18:0;O2/24:1	24:1		
	Cer 18:1;O2/14:0	14:0		[M+H] ⁺ PIS 264m/z
Cer 18:1;O2/15:0	15:0			

		Cer 18:1;O2/16:0	16:0	
		Cer 18:1;O2/18:0	18:0	
		Cer 18:1;O2/18:1	18:1	
		Cer 18:1;O2/19:0	19:0	
		Cer 18:1;O2/20:0	20:0	
		Cer 18:1;O2/22:0	22:0	
		Cer 18:1;O2/23:0	23:0	
		Cer 18:1;O2/24:0	24:0	
		Cer 18:1;O2/24:1	24:1	
		Cer 18:1;O2/25:0	25:0	
		Cer 18:1;O2/26:0	26:0	
		Cer 18:1;O2/26:1	26:1	
	Sphingadienines	Cer 18:2;O2/14:0	14:0	[M+H] ⁺ PIS 262m/z
		Cer 18:2;O2/16:0	16:0	
		Cer 18:2;O2/18:0	18:0	
		Cer 18:2;O2/18:1	18:1	
		Cer 18:2;O2/19:0	19:0	
		Cer 18:2;O2/20:0	20:0	
		Cer 18:2;O2/20:1	20:1	
		Cer 18:2;O2/21:0	21:0	
		Cer 18:2;O2/22:0	22:0	
		Cer 18:2;O2/23:0	23:0	
		Cer 18:2;O2/23:1	23:1	
		Cer 18:2;O2/24:0	24:0	
		Cer 18:2;O2/24:1	24:1	
		Cer 18:2;O2/24:2	24:2	
	Cer 18:2;O2/26:0	26:0		
	Cer 18:2;O2/26:1	26:1		
	Sphingomyelin	SM 30:1;O2	14:0	extrapolated from literature
		SM 32:1;O2	16:0	
		SM 34:1;O2	16:0	
		SM 34:2;O2	16:1	
		SM 36:1;O2	18:0	
SM 36:2;O2		18:0		
SM 38:1;O2		20:0		
SM 38:2;O2		20:0		
SM 40:1;O2		22:0		
SM 40:2;O2		22:0		
SM 41:1;O2		23:0		
SM 41:2;O2		23:0		
SM 42:1;O2		24:0		
SM 42:2;O2		24:1		
SM 42:3;O2		24:1		
SM 42:4;O2	24:2			

Supplemental Table 3. Structural elucidation of lipid species.

GENERAL DISCUSSION AND PERSPECTIVES

Obesity is characterized by an accumulation of dietary fat in metabolic tissues resulting in chronic inflammation. Excessive storage of fat in adipose tissue triggers an inflammatory response orchestrated by ATM, who are major effectors for the development of diet-induced insulin resistance and T2D (Hotamisligil et al. 1993, Saltiel and Olefsky 2017). In DIO, high amounts of FFA are present in the circulation and contribute directly to AT inflammation and insulin resistance. Indeed, these lipids promote the metabolic activation of ATM by inducing transcriptional and phenotypic changes consistent with a high metabolic rate (Kratz et al. 2014, Hill et al. 2018, Jaitin et al. 2019), which ultimately influence the fate (healthy versus unhealthy) of AT via the production of inflammatory cytokines, death adipocyte clearance, lipid catabolism and the subsequent instalment of fibrosis and hypoxia (Karczewski et al. 2018, Maniyadath et al. 2023). Sensing of these dietary FA by ATM is therefore a key event at governing AT successful adaptation to energy surplus. Once in the ATM, captured FA can be stored and neutralized into TG (Shapiro et al. 2013), catabolized through the lysosomal pathway (Xu et al. 2013) and/or metabolized into a wide range of lipid species including membrane phospholipids and sphingolipids which exert distinct effects on ATM activation (van der Veen et al. 2017, Petkevicius et al. 2019, Raichur et al. 2019). The capacity of ATM to handle FA plays a major role in its activation status and the progression towards an inflammatory, insulin-resistant phenotype, although the mechanisms participating in this handling of lipids are still not yet fully understood.

The membrane ATP-binding cassette G1 (ABCG1) transporter has been identified as an active player in controlling lipid homeostasis in macrophages. Beyond its well described role in cholesterol transport (Vaughan and Oram 2005, Tarling and Edwards 2011), our team previously demonstrated that ABCG1 plays an important role in FA release from TRL and reuptake by macrophages by controlling the bioavailability of LPL through plasma membrane lipid remodeling (Olivier et al. 2012). Such a role could have major implications on FA uptake and metabolism/distribution on ATM and thus participate to the development of obesity-associated comorbidities. Consistent with this hypothesis, numerous studies have found an association between the expression of ABCG1, T2D and obesity (Mauldin et al. 2006, Hardy et al. 2017).

The main goal of my project was therefore to assess the contribution of ABCG1 to ATM activation and lipid rewiring in a context of DIO. The originality of the project stems from the notion that ABCG1 functions as a Janus-faced metabolic switch depending on the lipid environment, with opposite actions in a cholesterol- versus a fat-rich context. Such a mechanism is reinforced by our data uncovering a

new, unexpected anti-inflammatory role of Abcg1-deficiency in macrophages in a high-fat environment, whereas the inhibition of Abcg1 was reported to induce inflammation in cholesterol-loaded macrophages (Yvan-Charvet et al. 2007, Lammers et al. 2009). Indeed, our results unveiled that Abcg1 deficiency in myeloid cells led to a marked reduction in AT inflammation and ATM content in mice fed a high-fat diet (60% kcal fat) for 8 and 25 weeks, despite inducing no changes in overall body composition and fat mass. This phenotype was associated with a complete remodeling of ATM lipidome, which consisted of decreased inflammatory SFA on membrane phospholipids through their redirection to lipid droplets. This neutralization pathway compromised FA-mediated inflammatory signaling activation, reflected by a switch of ATM transcriptome and energy metabolism towards a less activated profile, thereby ameliorating adipocyte health, liver histology and systemic insulin resistance.

Our data argue that in the context of obesity, ABCG1 expression in ATM rather promotes inflammation. In adipocytes though, ABCG1 has been attributed an anti-inflammatory role. Indeed, previous findings have shown that ABCG1 presence and activity (Chol efflux to HDL) in adipocytes is necessary to generate an anti-inflammatory phenotype in ApoA1 transgenic mice fed a high-fat, high-sucrose, high-Chol diet, which mainly consisted on decreased inflammatory cytokine and chemokine expression, but also reduced macrophage presence (Umemoto et al. 2013). This dual role of ABCG1 on AT global health highlights that the beneficial or deleterious actions of this transporter are highly dependent on the cell type that is targeted. The ATM-dependent effect of ABCG1 seems nonetheless to be predominant in obese states, as, in line with our data, total knockout of this transporter in mice fed a high-fat, high-Chol diet was protective against DIO (Buchmann et al. 2007).

These observations open up several questions regarding AT healthy expansion. For instance, while it has been established that adequate adipogenesis, adipocyte maturation and lipid storage is necessary for proper function of the cell (Vishvanath and Gupta 2019), at what point does adipocyte hypertrophy becomes deleterious and leads to significant ATM infiltration? This has been tackled in a recent study where it was determined that the critical threshold size for classical efferocytosis by ATM was up to 25 μm . Medium-sized (< 50 μm) lipid droplets were fragmented into smaller portions and then efferocytized; beyond this size, however, fragmentation nor efferocytosis were possible, adipocytes thus engaged in an apoptotic process which led to CLS formation and inflammation before lipid uptake. It is interesting to note that ATM in this CLS were metabolically activated (Lindhorst et al. 2021). We could then propose that in our mice model of myeloid Abcg1 deficiency, adipocyte expandability capacity is enhanced, in part, as a consequence of reduced ATM-mediated inflammation. This reduction of inflammation contributes to maintain insulin signaling and adipocyte storage capacity. Adipocyte apoptosis is therefore reduced, preventing CLS formation. In coherence with this statement, we also observed significantly less CLS in AT from Abcg1 myeloid-deficient mice. To further assess the

consequences of macrophage ABCG1 deficiency on AT homeostasis, ATM efferocytosis capacity as well as adipocyte death rate and metabolism (i.e. lipolysis, lipogenesis, glucose uptake) should be investigated.

As ATM accumulate in CLS during DIO, they form multiple intracellular lipid droplets and display a transcriptomic phenotype specialized in lipid handling. This reflects an attempt by ATM to buffer excess lipids and limit maladaptive adipocyte hypertrophy, as seen above, but becomes inefficient as ATM are overwhelmed by the continuous lipid flux. While lipids follow several different metabolic pathways within ATM, it has been suggested that according to the lipid charge (large vs small lipid droplets) ATM either direct lipids to one or two types of vesicles: a classic vesicle filled with neutral lipids (the LD), found only in ATM that contain the largest amounts of lipids, and another one characterized by an acidic ring (lysosomes) and found in ATM with both large and small amounts of lipids, reminiscent of the LAM phenotype (Xu et al. 2013, Grijalva et al. 2016, Hill et al. 2018). Activation of the lysosomal pathway could therefore represent a protective mechanism through which ATM catabolize excess fat to reduce their lipid load and maintain their function. Accordingly, we observed an increase of ATM lysosomal activity following *Abcg1* inhibition (data not shown), but no difference of neutral lipid content. In our model, we believe that this is explained by the concomitant reduction of fatty acid oxidation, another non-negligible form of lipid catabolism.

While a bit astonishing, the authors of the aforementioned study also claimed that intracellular lipid content in ATM was not associated to any direct changes to their inflammatory profile (Xu et al. 2013). This uncoupling between inflammation and LD accumulation has also been evidenced in a mice model of myeloid HILPDA (hypoxia inducible lipid droplet associated protein) inhibition, which showed that despite reduced ATM lipid storage there was no significant modulation of AT inflammation (van Dierendonck et al. 2020). Similarly, stimulation of THP-1 macrophages with conditioned media from adipocytes that had undergone lipolysis did enhance storage of neutral lipids, but did not translate into increased macrophage-dependent inflammatory phenotype (Caspar-Bauguil et al. 2015). There are several potential explanations for this uncoupling in ATM. First, the degree to which ATM-derived inflammatory cytokines increases AT inflammation and worsens the overall metabolic profile could be dominated by quantitative increases in ATM numbers rather than changes in the classically defined inflammatory state. This does not mean that obesity does not induce changes in ATM phenotype, as has been extensively studied in recent literature (Hildreth et al. 2021), but can be supported by the fact that impairing ATM recruitment and migration into AT is sufficient to reduce AT inflammation and insulin resistance (Weisberg et al. 2006, Ito et al. 2008). In this same line of evidence, our protective phenotype following *Abcg1* deficiency was accompanied by decreased chemokine expression and decreased monocyte recruitment into AT without changing total intracellular TG levels in ATM LD.

More importantly, it showed that instead of total TG amount, the nature of FA is crucial in governing ATM inflammatory phenotype.

Second, it highlights the notion that intracellular lipid accumulation in the form of LD in macrophages is a mechanism of lipid management that does not lead to the expression of inflammatory genes. This postulate has been widely studied in obesity settings, where it was demonstrated that the lipid-laden ATM phenotype is characterized by the induction of lipid metabolism pathways rather than inflammatory ones (Jaitin et al. 2019). It can be further validated by recent findings on murine and human atherosclerotic plaque macrophages showing that inflammatory and foamy macrophages are part of two distinct subsets identified by different metabolic and gene signatures (Depuydt et al. 2020, Willemsen and de Winther 2020). These macrophage-like foam cells have been observed to express the Trem2 and CD9 markers also seen on lipid-laden ATM, as well as high levels of the Abcg1 transporter, and have been suggested to mitigate vascular inflammation and promote plaque calcification and stabilization (Kiss et al. 2019, Roy et al. 2022). Finally, in NAFLD, a similar Trem2⁺CD9⁺ macrophage population has also been found and has been associated to NASH progression and liver fibrosis beyond any inflammatory role (Barreby et al. 2022). Overall, it is safe to say that metabolic disease gives rise to a particular macrophage subset specialized in lipid handling. However, whether it exerts beneficial or deleterious actions has not yet been fully elucidated. Our findings for instance, show that Abcg1 inhibition is accompanied by a decreased expression of the abovementioned markers, but the resulting ATM phenotype is protective. More research is therefore needed to better understand the complex role of these lipid-associated macrophages in metabolic disease.

Third, it brings out the contribution of other metabolic pathways more directly implicated with inflammatory signaling. Besides storage in lipid droplets and lysosomal catabolism, FA can be incorporated into membrane phospholipids and modify membrane properties, which ultimately contributes to the assembly of complex machinery that drives inflammatory signaling (Ruysschaert and Lonez 2015, Sezgin et al. 2017). Indeed, alteration of the lipid and protein (such as Rho-GTPases) content of resistant microdomains by fatty acids is a critical event in the triggering of the inflammatory signaling cascade in ATM (Wei et al. 2016). We demonstrate that Abcg1 myeloid deficiency entails a remodeling of membrane lipidome by depletion of pro-inflammatory C16 and C18 SFA in phospholipids and neutralization of these same lipid species inside LD, thereby providing a mechanistic link between ATM inflammation and lipid uptake in the absence of LD enhancement. To further validate these differences in lipid distribution within the lysosomal, LD or membrane compartment in ATM, we believe it could be interesting to trace specific FA, particularly SFA in TLR, by isotopic labeling.

Further clarification of the actual functional consequences of membrane lipid remodeling are also needed and could rely on the characterization of membrane-anchored inflammatory proteins by membrane proteomic analysis. We are particularly interested in TLR4, whose inflammatory signaling has been found to be altered following modulation of lipid raft composition (Sun et al. 2009, Ito et al. 2015). The pertinence for the investigation of this pathway could be substantiated by the recent evidence showing that just as lipidome remodeling influences inflammatory signaling, pro-inflammatory stimuli (i.e. TLR4 ligands) can also reshape macrophages membrane and intracellular lipid composition (Hsieh et al. 2020).

We were thus driven to replicate our findings in an *in vitro* model of bone marrow-derived macrophages (BMDM) that resemble the pathophysiological context of obesity. Existing literature has suggested a cocktail of insulin, glucose and palmitate as able to recapitulate the transcriptional changes observed in ATM from obese mice (Coats et al. 2017, Hill et al. 2018). In DIO, however, high amounts of FFA are present in the circulation as a consequence of the LPL-mediated lipolysis of TRG such as VLDL. LPL activity is therefore necessary for FFA and remnant VLDL uptake into the cell. More importantly, VLDL signaling in ATM has been reported to promote pro-inflammatory M1 phenotype and to aggravate adipose tissue inflammation and insulin resistance in DIO (Shin et al. 2017). The aforementioned approach of using free palmitate therefore neglects to take into account the key role of LPL to FFA release and lipid uptake by ATM. As such, we observed that stimulation of *Abcg1*-deficient BMDM with VLDL along with glucose and insulin successfully replicated the transcriptomic changes observed in murine ATM following *Abcg1* inhibition, as well as that in human ATM with low *Abcg1* expression. The definitive contribution of macrophage LPL to ATM metabolic activation was then validated by the use of an LPL inhibitor which abolished the anti-inflammatory effects of *Abcg1* deficiency.

Just as choosing the appropriate effectors is necessary to maintain biological relevance, the mice model used for assessing the contribution of a ubiquitously-expressed gene such as *Abcg1* is also important, and could account for the potential divergences observed between our work and existing literature. Buchmann and coll. first reported that global *Abcg1* deficiency decreased body weight and fat mass, ameliorated adipocyte health, glucose tolerance and insulin sensitivity as well as decreased liver steatosis in mice fed a HFD (Buchmann et al. 2007). Wei and coll. instead published that BM transplantation of *Abcg1*-deficient mice to Wt littermates did not influence glucose metabolism or body composition in mice fed a high-fat, high-carbohydrate diet. Nevertheless, it increased ATM total Chol content and, surprisingly, the number of M2 macrophages (Wei et al. 2015). Our work overlaps with some of the findings in both these studies, showing that mice deficient for *Abcg1* on the myeloid

lineage and under a HFD had similar weight gain, decreased insulin resistance, presented less intrahepatic fat and promoted anti-inflammatory ATM polarization.

BM transplantation is more specific of the immune cell compartment, as it targets the hematopoietic cell compartment and by extension macrophages, but also other immune cells. One might argue that even if other immune types do contribute to AT inflammation during DIO, ATM are still the most important drivers of this inflammatory phenotype and above all, of AT insulin resistance. Still, the principal inconvenient with this model though comes from the fact that the prior lethal irradiation required for BM transplant significantly impairs HFD-induced weight gain (up to -33% fat mass, -45% adipocyte volume and -38% preadipocytes) as compared to non-irradiated, HFD-fed mice. More importantly, it also seems to decrease ATM numbers in scWAT and vWAT in conjunction with plasma insulin levels (-62% and -68%, respectively)(Katiraei et al. 2017). In addition, it has recently been observed that during HFD, BM adipocytes prime monocytes towards an invasive Ly6C^{high} phenotype, which favors their infiltration in AT and therefore ATM content (Boroumand et al. 2022). This mechanism could be abrogated with BM irradiation and could explain, along with the difficulty to gain weight and the targeting of other immune types, why liver histology and insulin metabolism are not altered following *Abcg1* deletion according to the study of Wei et al (Wei et al. 2015).

To bypass some of these biases, the *LysMCre* mouse model was used for deletion of *Abcg1* on macrophages, although targeting of other immune cells such as dendritic cells, monocytes and granulocytes is to be expected. While there could be indeed consequences of *Abcg1* inhibition on these other immune types, characterization of the deletion efficiency of the *LysMCre* strain was revealed to be almost at its highest in mature macrophages (98% vs 16% in dendritic cells, for instance)(Clausen et al. 1999), arguing in favor of the specificity of our targeting. We could then suggest that the positive effect of macrophage *Abcg1* deficiency on insulin resistance and liver lipid accumulation is a consequence of a metabolically healthier AT, therefore preventing lipid spill over into other tissues and disturbances in insulin signaling. With that in mind, full phenotyping of *Abcg1*-deficient liver macrophages needs to be performed to establish the different metabolic and transcriptomic adaptations of these cells following *ABCG1* inhibition and HFD. It is nonetheless important to mention that, in contrast to what observed in ATM, *Abcg1* deficiency in liver macrophages was without effect on their inflammatory phenotype as well as in liver inflammation, highlighting the specific action of *Abcg1* in ATM during DIO. Assessing the consequences of myeloid *Abcg1* deficiency of pancreatic macrophages could also be of interest to further corroborate the selectivity of our model and its repercussion towards ATM-mediated insulin resistance, especially given that basal insulin levels, and therefore insulin secretion, were also different between genotypes.

It is important to note that the association between macrophage *Abcg1* expression and the transcriptomic and metabolic signature of mouse ATM during HFD was also validated by our own human data obtained from omental ATM of morbidly obese subjects (mean BMI = 41 ± 1.25), as well as public data on obese mice (60% kcal fat for 18 weeks) and humans (mean BMI = 31 ± 0.4) (Hildreth et al. 2021, Sárvári et al. 2021).

Given the wide, systemic protective effects of macrophage *Abcg1* deficiency, we believe the therapeutic targeting of this transporter in ATM could be of particular interest for the prevention of obesity-associated insulin resistance, T2D and other comorbidities. It is important to know that up to this day, there are no known inhibitors of the *Abcg1* transporter. However, gene targeting in macrophages has already been considered as an option to treat metabolic disease. For instance, nanoparticles targeting NLRP3 inflammasome with gene editing have been developed and confirmed to alleviate AT inflammation of high-fat diet-induced type 2 diabetes mice by delivering CRISP/Cas9 into macrophages of liver, spleen and adipose tissue (Xu et al. 2018). Although more research is needed to design nanoobjects specifically targeting ATM, it is noteworthy that RNAi-based methods to selectively silence genes in epididymal ATM, and not macrophages from other tissues, have been reported. These are based on the intrinsic phagocytic function of ATM as well as the specificity of the administration method (i.e. intraperitoneal, which targets ATM, vs. oral gavage, which targets all macrophages) (Soto et al. 2012, Aouadi et al. 2013). As such, intraperitoneal injection of siTNF α encapsulated in glucan shells has shown to ameliorate whole-body homeostasis in *ob/ob* mice via inhibitory effects on the TNF signaling pathways on ATM alone (Aouadi et al. 2013). Of note, the use of this technology could be of pertinence to further confirm the specificity of our model.

Overall, our study demonstrates the complex role of ATM on regulating whole-body homeostasis and puts forward an unexpected contribution of the membrane *Abcg1* transporter in this specific context. Our data also highlights the importance of the intracellular distribution of dietary FA (SFA vs PUFA) in the activation of ATM. In that sense, we could propose that the well-known beneficial effects of omega-3 PUFA supplementation on inflammatory parameters in diabetic and hyperlipidemic individuals (Ballantyne et al. 2012) might stem, in part, from their capacity to inhibit ABCG1 expression in macrophages (Ku et al. 2012).

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APPENDICES

Review

Rewiring of Lipid Metabolism in Adipose Tissue Macrophages in Obesity: Impact on Insulin Resistance and Type 2 Diabetes

Veronica D. Dahik, Eric Frisdal, Wilfried Le Goff

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Review

Rewiring of Lipid Metabolism in Adipose Tissue Macrophages in Obesity: Impact on Insulin Resistance and Type 2 Diabetes

Veronica D. Dahik, Eric Frisdal and Wilfried Le Goff *

Institute of Cardiometabolism and Nutrition (ICAN), Hôpital de la Pitié, Sorbonne Université, Inserm, UMR_S1166, 75013 Paris, France; veronica.dahik@etu.upmc.fr (V.D.D.); eric.frisdal@sorbonne-universite.fr (E.F.)

* Correspondence: wilfried.le_goff@sorbonne-universite.fr

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Abstract: Obesity and its two major comorbidities, insulin resistance and type 2 diabetes, represent worldwide health issues whose incidence is predicted to steadily rise in the coming years. Obesity is characterized by an accumulation of fat in metabolic tissues resulting in chronic inflammation. It is now largely accepted that adipose tissue inflammation underlies the etiology of these disorders. Adipose tissue macrophages (ATMs) represent the most enriched immune fraction in hypertrophic, chronically inflamed adipose tissue, and these cells play a key role in diet-induced type 2 diabetes and insulin resistance. ATMs are triggered by the continuous influx of dietary lipids, among other stimuli; however, how these lipids metabolically activate ATM depends on their nature, composition and localization. This review will discuss the fate and molecular programs elicited within obese ATMs by both exogenous and endogenous lipids, as they mediate the inflammatory response and promote or hamper the development of obesity-associated insulin resistance and type 2 diabetes.

Keywords: adipose tissue macrophages; metabolic activation; obesity; lipid; inflammation; insulin resistance; type 2 diabetes

1. Introduction

Once considered a high-income nation problem, obesity has all but tripled in the last 50 years, reaching pandemic proportions. Estimates indicate that about 641 million adults worldwide are obese (BMI ≥ 30), presenting an alarming mean increase in BMI of 0,61 kg/m² per decade [1]. Obesity is characterized by the accumulation of excess fat arising from a disproportionate energy intake and expenditure. It is the primary risk factor for several pathologic conditions, including nonalcoholic fatty liver disease (NAFLD), insulin resistance (IR), type 2 diabetes (T2D) and cardiovascular disease (CVD), which have also seen their prevalence dramatically rise [2], concomitant with the global rise in the prevalence of obesity.

T2D is a disease met with increased fasting glycaemia due to glucose intolerance and ineffective insulin secretion or signaling. The mechanisms of insulin deficiency are beyond the scope of this review; however, the resulting insulin-resistant state has consequences both systemically and at the tissue level, thus altering the subject's metabolic status [3]. IR is a feature characteristic of early-onset T2D and may occur in the principal calory repositories including the adipose tissue (AT), skeletal muscle and liver. The central role of AT in maintaining whole-body homeostasis is evidenced during obesity, as ectopic fatty acid (FA) and cholesterol (Chol) accumulation in cell types other than adipocytes leads to metabolic dysfunction. Among the cells capable of ectopic lipid storage, adipose tissue macrophages (ATMs) forming crown-like structures (CLS) have been found to accumulate lipids in obese states [4–6]. Indeed, the most salient features of diet-induced AT metabolic inflammation (metaflammation) can be

resumed as follows: I) enhanced ATM proliferation and metabolic activation promoting inflammatory cytokine production succeeded by II) monocyte-derived ATM recruitment and foam-like cell formation around apoptotic adipocytes and altered metabolism and III) increased pro-inflammatory signaling creating a vicious cycle of metabolic dysfunction.

Metabolically activated (MMe) ATMs are part of a rather new concept of an activation profile that was introduced by Kratz et al., and distinguishes them both functionally and phenotypically from the M1 and M2 phenotypes [7,8]. More importantly, MMe ATMs present significant changes in their intrinsic metabolism, including lipid mobilization (i.e., lysosomal catabolism, storage within lipid droplets (LDs) and efflux) and utilization (i.e., free fatty acids (FFA)/Chol synthesis and oxidation for energy production or membrane incorporation) [9–11]. On this account, the emerging fields of lipidomics and metabolomics have proven key in the study of ATM function in regard to individual lipid species. Particularly, it has brought back interest on the relationship between ATM cellular/membrane lipid composition and inflammatory profile, as it has been evidenced that the inflammatory and adaptive properties of immune cells, including macrophages, are dependent of cellular lipid status [12]. The object of this review is therefore to discuss the current knowledge regarding ATM metabolic activation by dietary lipids.

2. ATM Heterogeneity in Obesity: Activation and Function

The permanent crosstalk that exists between adipocytes and immune cells is of the utmost importance, as several studies have established a causal link between increased excess calory intake and chronic low-grade AT inflammation. Indeed, the activation of the innate immune response during obesity, and more importantly that of ATMs, has proven to be the primary mechanism responsible for the development of insulin resistance (IR) since the discovery that the main source of pro-inflammatory cytokines in AT is in fact ATMs [13,14]. Coherent with this observation, it was shown that ATMs account for the most enriched immune cell in obese AT (from 10% in lean states up to 40% when obese) [15]. Specific depletion of phagocytic cells by intraperitoneal injection of clodronate liposomes protected mice from high fat diet-induced obesity, notably by improving insulin sensitivity, glucose homeostasis and inflammatory status, including decreased plasma tumor necrosis factor- α (TNF α) and increased plasma adiponectin (Ad) [16,17]. Further research selectively targeting ATM genes confirmed their definitive role in obesity-associated metabolic disorders and inflammation by displaying an altered lipid and glucose metabolism as evidenced in lipoprotein lipase (LPL) and TNF α -depleted ATMs [18,19].

Multiple macrophage activation and polarization states have been described in the literature and vary along a continuum, from the classically activated, pro-inflammatory (M1) profile to the alternatively activated, anti-inflammatory (M2) state [20,21]. M1 and M2 macrophages have roles in diet-induced obesity (DIO) and IR, with M2 macrophages being associated with lean, insulin-sensitive states, and M1 macrophages being the predominant subset present in insulin resistant, obese subjects [22,23]. Indeed, reduced bone marrow-derived M1 ATM recruitment, either by monocyte chemoattractant protein-1 (MCP-1) or its receptor C-C chemokine receptor type 2 (CCR2) depletion has been shown to be protective against diet-induced inflammation and IR [24,25]. However, while the M1/M2 dichotomy has been often employed under obese settings thanks to its obvious advantage of reflecting the Th1/Th2 response, it poorly portrays the various challenges associated with DIO. Beyond cytokine production, AT expansion during DIO is accompanied by the appearance of hypoxic zones. Hypoxia provides a means for explaining several metabolic changes seen during obesity, as the hypoxia-inducible transcription factor-1 α (*Hif-1 α*) is implicated in glucose metabolism, inflammatory cytokine expression, adipocyte hypertrophy and death, among other gene programs [26,27]. Most importantly, it has a role in ATM activation, as myeloid-specific deletion of this transcription factor reduces ATM accumulation and IL-1 β production [28].

Failure to characterize ATMs from obese subjects, plus shared characteristics between the M1 and M2 phenotype [20] highlighted the possibility of the presence of a new ATM phenotype exclusive to the metabolic disorders seen in obesity. On this account, the treatment of human monocyte-derived

macrophages (HMMDM) with insulin, glucose and palmitate (PA), all of which are readily available in DIO, generated a polarization state different from the M1 and M2 states that have been referred to as MMe ATMs [7]. Although expressing some canonical M1 and M2 markers, MMe ATMs are not driven by the LPS/TLR4 pathway that ensues in interferon (IFN) production, but are rather induced by the peroxisome proliferator activated receptor- γ (PPAR γ) and the autophagy-associated protein p62 [7]. It is interesting to note that PPAR γ agonist, rosiglitazone [29] or IL-33 [30] treatment increases M2-like ATM content and reduces macrophage lipid accumulation. This, plus ulterior studies determining that MMe ATMs indeed heighten inflammation, but are also involved in mechanisms for dead adipocyte clearance, such as lysosomal exocytosis [8], would lead us to believe the metabolically active phenotype is most resembling to the M2 state. Accordingly, Silva et al., reported a monocyte-derived Cd11c⁺Cd64⁺ ATM population which significantly increased under a high fat diet (HFD), and was associated with a protective, anti-inflammatory response [31].

Henceforth, numerous researchers have depicted different ATM populations with different metabolic and transcriptomic profiles to the M1 and M2 macrophages, though still all falling under the MMe subtype. For instance, ATMs isolated from visceral AT and epididymal white AT (WAT) from obese individuals and mice, respectively, were shown to express high levels of CD9, as well as genes involved in lipid metabolism and lysosomal functions such as *Cd63*, *Lpl*, *Plin2*, and *Lamp2*—the latter two incidentally also upregulated in both landmark studies abovementioned [7,8]. They were also found to accumulate intracellular lipids through a lysosomal-dependent pathway [32]. Coherent with the fact that lysosomal activity is met with sturdy changes in energy metabolism, metabolic activation resulted in the alteration of several metabolic routes. In contrast to LPS-activated M1 macrophages, MMe ATMs revealed increased OXPHOS and a persistent glycolytic rate [27]. Similar observations were made in ATMs (Cd11b⁺ cells in the stromal vascular fraction (SVF)) issued from obese subjects, which displayed a high energy metabolic phenotype with significantly high OXPHOS and glycolysis. These parameters were, however, reduced in ATMs from lean patients, indicative of a low bioenergetic state [33]. Furthermore, Jaitin et al., reported that these lipid-associated ATMs present a large, highly conserved gene expression signature characterized by expression of, among others, *Cd9* and *Cd63*, *Lipa* (lysosomal function), *Cd36*, *Lpl*, *Fabp4*, *Fabp5* (for lipid metabolism) and *Atp6v0b*, *Atp6v1b2* (involved in OXPHOS). This program was revealed to be under the control of Trem2 and necessary to counteract obesity-related metabolic disorders, as Trem2 deficiency enhanced HFD-induced weight gain, hypercholesterolemia, glucose intolerance and IR [5]. These assessments prompt us to believe that MMe ATMs do not undergo the binary switch associated with the M1/M2 phenotypes, but are hosts to a more dynamic regulation of lipid metabolism which should be taken into consideration for future research.

Lastly, it is interesting to note the spatio-temporal differences of ATM localization and recruitment during lean and obese states. Indeed, M1-like macrophages have been reported to be located in CLS and increased during a HFD, while M2-like ATMs are most often found in interstitial spaces within AT [34]. Recent reports also show the existence of vasculature associated macrophages (VAMs), which seem to be issued from tissue-resident macrophages and tightly associated with blood vessels. Their numbers are, however, more important under lean states [31]. The question as to whether MMe ATMs localize in cluster around dying adipocytes or not is still to be studied; nonetheless, certain similarities with the M2 phenotype, as seen above, suggest they are present in a more scattered pattern.

3. Lipid Metabolism in Healthy and Obese AT

For quite some time now, macrophages have been recognized as capable stocking lipids intracellularly, although most studies were initially conceived in the context of atherosclerosis. A growing body of evidence has nevertheless been able to ascertain that ATMs can also uptake lipids, primarily by buffering circulating Chol and free FA (FFA) (at both early and late stages of overweight/obesity) and dying necrotic-like adipocytes (when advanced obesity). One of the first accounts of lipid laden ATMs in human obesity was observed by Shapiro and coll., who managed to

associate “AT foam cell” presence with fasting glucose and insulin levels [4]. This increase in lipid-laden ATMs was maximal at peak adipocyte size [6]. Given that the origin (i.e., circulating vs. adipocyte-issued) of these lipids is determinant of ATM activation, the following section will concentrate on the mechanisms of lipid handling by obese ATMs and its implication in macrophage pro- or anti-inflammatory signaling (Figure 1).

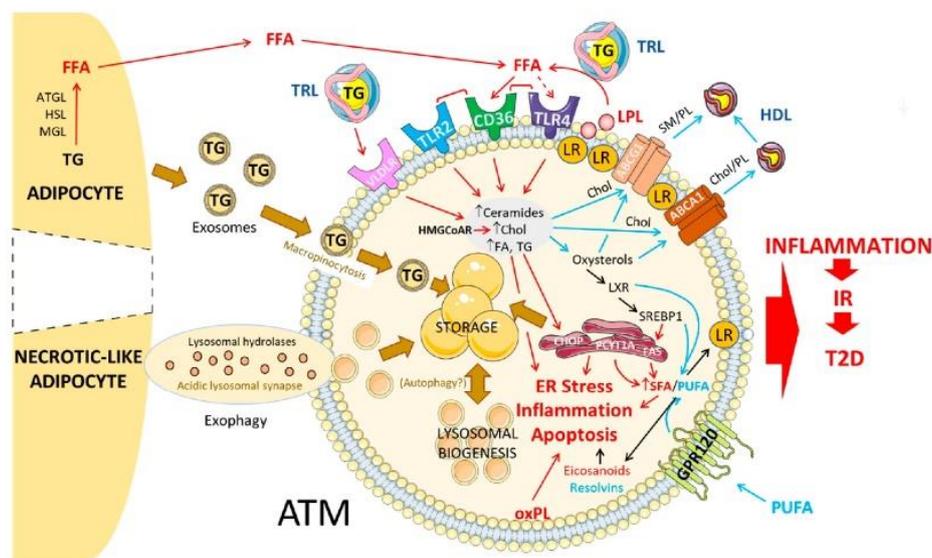


Figure 1. Lipid handling in adipose tissue macrophages in diet-induced obesity. In diet-induced obesity, adipose tissue macrophages are metabolically activated by free fatty acids (FFA) released by lipolysis of triglycerides from adipocytes or from triglyceride-rich lipoproteins by lipoprotein lipase, through Cd36 and toll-like receptors. Accumulation and storage of lipids in adipose tissue macrophages (ATMs) under the form of lipid droplets occur through the uptake of triglyceride-rich lipoproteins by specific receptors such as very low-density lipoprotein (VLDLR) or through the internalization of adipocyte-derived exosomes by macropinocytosis. In crown-like structures, necrotic-like adipocyte clearance by ATM in acidic lysosomal synapses through a mechanism of exophagy also contributes to foam cell formation. As a result, ATMs are characterized by an increased content of triglycerides, free cholesterol, ceramides and fatty acids, which can either be synthesized into other lipids (See Figure 2), be stored in lipid droplets or catabolized through the lysosomal pathway. The accumulation of free cholesterol and sphingolipids such as sphingomyelin into membrane lipid rafts promotes the recruitment of TLR4 at the cell surface and contributes to the activation of inflammatory signaling pathways. ABCA1 and ABCG1 transporters promote the efflux of lipids from ATM to high-density lipoproteins allowing the reduction in lipid raft formation, thus ensuring an optimal lipoprotein lipase activity which participates in foam-like cell formation. Fatty acid synthesis, elongation and desaturation are under the control of liver X receptor (LXR) and sterol regulatory element-binding protein (SREBP1) thus exerting an important role in the relative cellular content of saturated and polyunsaturated fatty acids. Increased cellular levels of free cholesterol and saturated fatty acids trigger endoplasmic reticulum stress, inflammation and apoptosis, which can be alleviated by an enrichment in polyunsaturated fatty acids through mechanisms dependent or independent of the GPR120 receptor. Production of eicosanoids and resolvins from ω -6 and ω -3 polyunsaturated fatty acids, respectively, induces or represses inflammation in ATM. Increased turnover of phosphatidylcholine as well as oxidation of phospholipids also participate in the inflammatory status of ATM. As a whole, the rewiring of the lipid metabolism in ATM promotes adipose tissue inflammation and contributes to the establishment of insulin resistance and type 2 diabetes. ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; ATGL, Adipose triglyceride lipase; Chol, cholesterol; CHOP, CCAAT-enhancer-binding protein homologous protein; FAS, fatty acid synthase; FFA, free fatty acids; GPR120, G protein-coupled receptor 120; HMGCoAR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HSL, hormone-sensitive lipase;

IR, insulin resistance; LPL, lipoprotein lipase; LR, lipid rafts; LXR, liver X receptor; MGL, monoacylglycerol lipase; oxPL, oxidized PL; PCYT1A, phosphate cytidyltransferase 1; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelin; SREBP1, sterol regulatory element-binding protein 1; T2D, type 2 diabetes; TLR, toll-like receptor; TG, triglyceride; TRL, triglyceride-rich lipoproteins; VLDLR, very low-density lipoprotein receptor.

3.1. Circulating Lipids: Mechanisms of Uptake into ATMs

3.1.1. FFA

The main tissues for fatty acid (FA) release are muscle and AT, with AT being the preferential target. For this, triglyceride-rich lipoproteins (TRL), including chylomicrons and very low-density lipoproteins (VLDL) are hydrolyzed by tissue-bound LPL and release FFA. These can then be captured by adipocytes and macrophages within the AT for subsequent storage within lipid droplets (LD) (by both adipocytes and ATMs) [32,35] and other intracellular compartments, such as lysosomes (by ATMs) [36]. The primary source of LPL is macrophages, and both its retention at the plasma membrane thereby reducing its bioavailability, or its inhibition by tetrahydrolipstatin treatment has been shown to reduce triglyceride (TG) accumulation in HMDM [37]. Consistently, specific ATM LPL genetic deletion, as performed by Aouadi et al., resulted in reduced lipid storage in macrophages, increased FFA serum levels and glucose intolerance [19].

3.1.2. VLDL/VLDLR

Obesity and obesity-related metabolic disorders are associated with elevated plasma TG levels, high low-density lipoprotein (LDL)-Chol and low high-density lipoprotein (HDL)-Chol contents, with non-HDL levels positively associated with pro-inflammatory ATMs even in healthy subjects [38,39]. Within blood vessel, TG is transported in the form of lipoproteins, including VLDL, which can bind to the VLDL receptor (VLDLR) at the surface of adipocytes and macrophages for TG uptake [40,41]. The expression and proper function of this receptor therefore represent an important target in DIO and diet-induced inflammation, and SNPs of this gene have been found to be associated with BMI and cardiovascular risk in humans [40]. In accordance with this observation, VLDLR overexpression has been shown to increase TG content within ATMs in a DIO mouse model. This was also accompanied by increased M1-like ATM polarization and IR. Improved AT inflammation and insulinemia were, however, restored after the adoptive transfer of VLDLR KO BM to Wt mice even under a HFD [41].

3.2. Adipocyte-Released Lipids: Mechanism of Uptake into ATMs

3.2.1. Lipolysis-Dependent Lipid Release

Hypertrophic adipocytes are able to release FFA by a classic, lipase-dependent mechanism as an effort to limit further enlargement of the LD that may compromise cell viability. TG hydrolysis and the release of FFA from adipocytes to the circulation or other cells, such as ATMs, are mediated by intracellular lipases adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL). Adipocyte ATGL deficiency in mice has been shown to decrease ATM infiltration in response to acute lipolysis upon treatment with the β 3-adrenergic agonist CL 316,243, as assessed by Cd11c and F4/80 markers. However, a higher ATM infiltration and pro-inflammatory gene expression was detected in AT from adipocyte ATGL-deficient mice upon DIO [42]. Similarly, subjects homozygous for a deletion of the HSL gene display increased MCP-1 levels and ATM infiltration, along with systemic IR and T2D [43].

3.2.2. Exosomes

An alternative lipid source for macrophages has been described by numerous authors, whereby lipid-filled exosomes are released by adipocytes and contribute to the pro-inflammatory phenotype of ATM [44–46]. This mechanism is independent of lipase activation, as inhibition of ATGL in adipocytes did not alter the distribution of these exosomes, which were incidentally found to

increase macrophage TG content by 8-fold in BM-ATMs independently of diglyceride acyltransferase (DGAT)1/2. Moreover, the exosomes were not only able to induce ATM activation and foam-like cell formation, but also lead to ATM-like differentiation from BM progenitors by activating a gene program characteristic of macrophages that reside exclusively in AT [44]. This very same role in ATM differentiation has been found to link exosome release with glucose intolerance and IR [47]. Curiously, a link between the lysosome and exosome pathways has been established, as exosomes were found to deliver TG to lysosomes for FFA release [44].

3.2.3. Exophagy

An active mechanism for lipid release specifically targeting dying, necrotic-like adipocytes around CLS has also recently been described. It entails the formation of a tightly sealed acidic lytic compartment where lysosomal hydrolases, delivered by exocytosis, allow the hydrolysis and subsequent internalization of large amounts of lipids, as well as dead adipocyte fragments [48,49]. This process has been termed exophagy and shares some similarities with autophagy, a widely studied mechanism of lipid clearance. This form of lipid uptake has been shown to lead to foam-like cell formation, as evidenced by LipidTOX labelling [48].

3.3. Fate of Lipids in ATM

3.3.1. Storage in the Form of LD

Adipocytes and ATMs, among other cells, store fat in the form of LD. LDs are typically composed of a neutral lipid core and sealed off to the cytosol by a set of molecules including membrane phospholipids (PL), perilipins (PLIN), and free Chol [50]. Despite slight differences in the disposition and constitution of the droplet—one large unilocular vesicle formed primarily of TG in adipocytes vs. various smaller vesicles composed of both TG and cholesteryl esters (CE) in ATMs—LD biogenesis and lipid uptake are increased in obesity and contribute to the dysfunctional phenotype observed in both these cell types and associated with DIO [35]. While it was often believed that LDs were the primary—if not the only—form of lipid capture in ATMs, notably those organized in CLS [51], this has been contested by results showing that MMe ATMs present reduced LD formation, as lipids undergo catabolism via a lysosome-related pathway. Most importantly, the inhibition of this pathway increased LD content in macrophages [36]. This form of storage is therefore likely to be preferentially present in CLS ATMs. Likewise, it is mostly found in omental fat rather than subcutaneous AT [4].

3.3.2. Lysosome-Mediated Lipid Handling

The contribution of the lysosomal pathway to inflammatory signaling and lipid handling has been the subject of numerous studies. Lipid catabolism, as it occurs in lysosomes, is mainly achieved by the action of LIPA, whose deficiency and overexpression have been shown to alter immune (efferocytosis, T cell and macrophage function) and metabolic (insulin signaling, VLDL metabolism, ATM lipid content, polyunsaturated fatty acid (PUFA) release and lipid mediator synthesis) homeostasis [52–54]. In ATMs, the lysosomal program has been associated with the MMe phenotype, as evidenced by the enrichment of lysosome-related genes *Lipa* and *Lamp2*, among others, in both obese mice [5,32] and humans [36]. As such, its inhibition by chloroquine is accompanied by significantly enhanced lipid accumulation within macrophages and reduced AT lipolysis [36]. Defective lysosomal exocytosis by NADPH oxidase 2 (Nox2) inhibition also leads to less effective dead adipocyte clearance by MMe and thus contributes to metabolic disorders in DIO [8]. Lysosomal lipolysis has also been associated with the induction of the M2 phenotype in bone marrow derived macrophages (BMDM) and peritoneal macrophages in response to FFA, as assessed by oxygen consumption rate and M2 markers Cd206, Relm α and Cd301 [55]. Interestingly though, while short hairpin RNA treatment targeting *Lipa* did result in impaired M2 polarization [55], hindering lysosomal function has failed to be associated with the classic inflammatory M1 profile [36]. Cell-intrinsic lysosomal lipid handling may therefore

represent a means for ATM to counteract classic activation by FFA and would lead to the “mixed” MMe phenotype, which is incidentally characterized by both M1 and M2 markers [7,36].

3.3.3. Autophagy

A complementary mechanism to the lysosomal pathways is thought to be autophagy (also termed lipophagy). This form of lipid mobilization into lysosomes does not conform to a simple role in energy balance, but is also involved in major cellular responses. In AT, for instance, autophagy is necessary for adipogenesis and adipocyte differentiation [56]. In liver and atherosclerotic plaque macrophages, autophagy is required for LD clearance and normal macrophage function [57,58]. In obesity and obesity-associated ATMs, however, autophagy's role is less clear. In vitro experiments on BM-ATMs and mice under a HFD confirmed autophagy upregulation in DIO; it was nonetheless found dispensable for ATM activation and lipid uptake as evidenced by both *Atg7* deletion and 3-methyladenine treatment [53]. Contradicting results were found using a model of unpolarized RAW macrophages exposed to AT conditioned media plus oleate (OA) as a lipid source. Interrupted autophagy was indeed found to either decrease LD biogenesis and lipid uptake by ATMs when targeted at the early steps, or to support LD formation when targeted at a later stage [35]. These discrepancies could be partly explained by the different lipid substrates (whole AT or OA) to which the macrophages were exposed to. In addition, the inflammatory phenotype of ATMs could also alter lipid efflux efforts by autophagy. It was indeed demonstrated that LPS + IFN γ stimulated macrophages are incapable of further forming and enlarging existing LDs [59]. Lastly, another argument that might allow us to explain these differences has been evoked by Flaherty et al., and involves the existence of a lipid storage site other than LDs that can nonetheless fuse with lysosomes, but does not follow the autophagic route [44].

3.3.4. Lipid Efflux/Export

Along with the influx of lipids, their proper clearance is equally important for macrophage function and turns out to be compromised during obesity. Dietary Chol alone is able to further promote ATM accumulation in mice fed a diabetogenic diet, and is positively associated with chronic systemic inflammation, IR and atherosclerosis [60]. This effect is attributed in part to its ability to induce *Mcp-1* and *Saa3* production by adipocytes, which actively contribute to macrophage recruitment into AT [61]. HDL and Apolipoprotein A-1 (ApoA-1) have also been attributed anti-inflammatory properties in AT [39,61], and while it is worthy to acknowledge their capacity to suppress inflammatory gene expression, HDL themselves can be undergo regulation by inflammatory genes as revealed by identification of SNPs *cis* variants associated with low-HDL [62].

Reducing Chol content in AT through stimulation of Chol efflux may therefore represent an efficient way to alleviate inflammation. For instance, ApoA-1 and HDL exert some of their anti-inflammatory effects in AT in an *Abca1/g1*-dependent manner by removing Chol from adipocyte membranes in vitro [61]. Intracellular Chol levels are linked to impaired glucose tolerance and IR and as such, *Abca1* hematopoietic deletion not only induced monocytes and increased inflammatory cytokine and macrophage content in both AT and liver, but also enhanced ATM response to saturated FA (SFA). Hematopoietic *Abca1*^{-/-} mice fed a diabetogenic diet supplemented with Chol were thus more insulin resistant [63]. While seemingly logic, these results have been contested by experiments conducted in myeloid *Abca1* KO and Wt mice who developed similar IR, hypercholesterolemia and hepatic steatosis. Although ATM in the SVF from myeloid *Abca1*^{-/-} mice did accumulate more Chol than their Wt counterparts, neither macrophage infiltration nor inflammatory *Tnf α* , *Mcp-1* and *Il-1 β* cytokine expression was different [64]. These differences could be attributed to a number of factors (i.e., hematopoietic vs. myeloid targeting, content and length of the diet, *Abca1/g1* levels in AT only vs. in ATM). Regarding *Abcg1*, its expression has been shown to be significantly enhanced under obese and caloric restriction settings [65,66]. This increase has been revealed to be associated with ATMs, as evidenced by the isolation of F4/80⁺ cells from the SVF of AT obese mice [65]. Particularly, M2 ATM content, assessed by Cd11c⁻Cd206⁺ marker expression, was found to be positively associated with

Abcg1 expression. Indeed, its deletion in the myeloid lineage resulted in a 2-fold decrease in M2 macrophages and enhanced total Chol content. This deficiency also hindered macrophage migration in response to Chol loading [66]. Other landmark studies agree that Abca1/g1 macrophage expression is dependent on the ATM phenotype. For instance, Abca1/g1 expression is enhanced in lipid-laden, MMe ATM vs. lean ATM [7]. This is coherent with the observation that while Chol efflux capacity to ApoA-1 was significantly reduced in M1 ATM, it was surprisingly enhanced in primary obese and in vitro polarized MMe ATM [9]. This could be associated with a protective mechanism in which lipid-laden ATMs promote Chol efflux to lower inflammation, but are ultimately overwhelmed by the lipid charge.

3.4. Regulation of Intracellular Lipid Metabolism in ATM

Lipid mobilization both within and outside the cell is a mechanism that strongly depends on three major processes—lipid uptake (either at whole-body level or within the cell), synthesis and clearance or conversion into other metabolites—as these participate in determining the cellular metabolic status. Macrophages, and most importantly ATMs, are capable of regulating their own lipid metabolism essentially through two major families of transcription factors, the sterol regulatory element-binding protein (SREBP) and the liver X receptor (LXR), that tightly control Chol and FA synthesis. We will focus on the mechanisms that drive ATM metabolic dysfunction in obesity.

Interference with any of the steps involved in Chol metabolism has been associated with altered ATM function. For instance, myeloid deletion of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis, has been shown to significantly reduce ATM accumulation by 77% in *Hmgcr*^{-/-} mice vs. Wt, despite similar fat mass composition and body weight. This was accompanied by a reduction in Tnf α , Il-1 β and Mcp-1 expression, which consistently translated into decreased macrophage chemotaxis and migration into AT, improved glucose tolerance and insulin sensitivity [67]. Similarly, treatment of obese mice with the synthetic LXR agonist GW3965 not only decreased neutrophil migration, but most importantly reduced ATM infiltration—both F4/80⁺Cd11b⁺Cd206⁺ and F4/80⁺Cd11b⁺Cd11c⁺ populations—into visceral AT. Moreover, these changes were associated with enhanced insulin-related gene expression and improved insulin sensitivity in AT and muscle [68]. LXR therefore exerts multiple, non-exclusive roles in inflammation, Chol metabolism and IR.

In contrast to sterol synthesis, FA synthesis or de novo lipogenesis (DNL) is driven by both LXRs and SREBPs, notably the SREBP-1a isoform in macrophages [69]. For this, citrate issued from the TCA cycle is successively transformed into acetyl-CoA and malonyl-CoA, the latter of which can then act as a substrate for the so-called multi-enzymatic complex FA synthase (FAS). The main DNL end product is long chain SFA palmitate (PA; C16:0), which is intrinsically associated with inflammatory pathways that will be detailed later on in this review. Elongation and desaturation steps can then follow for generation of other FA. This process is achieved by different families of enzymes (for details, see Section 4.3.2), which are also under the control of SREBP and LXR. As for Chol synthesis, hindering any of the steps involved in DNL leads to an altered ATM phenotype and lipid metabolism. TCA cycle disruption and corresponding citrate accumulation in LPS-challenged BMDM drives M1-like polarization by overproduction of oxidative stress-associated metabolites such as NO [70]. Myeloid deletion of FAS has shown beneficial effects on glucose tolerance and IR by reducing ATM recruitment to obese AT and pro-inflammatory cytokine expression [71]. In addition, DNL has been found to be required for monocyte differentiation into macrophages [72].

DNL allows the transformation of excess glucose in FA, and as such is upregulated by both insulin and SFA. In regard to insulin, it is able to act at the transcriptional and post-transcriptional levels, the latter consisting mainly of inhibiting proteolytic cleavage and translocation of SREBP1 to the nucleus [73]. At the transcriptional level, insulin has been found to activate mTORC, which is incidentally involved in SREBP-1a cleavage and allows proper phagocytosis downstream of the TLR4 pathway in BMDMs [69]. On this account, TLR4 has been revealed to alter FA synthesis in a biphasic, time-dependent regulation of SREBP-1 expression. Activation of the toll-like receptor TLR resulted in an early (T0 to T6 h) activation phase with enhanced inflammatory cytokine production and impaired FA

synthesis—mainly PUFA—by inhibition of LXR and LXR-dependent genes; while the late (T12–T24 h) resolution phase was characterized by upregulation of SREBP-1 and its target genes, which contributed to anti-inflammatory mediator production and counteracted NF- κ B signaling [74]. LXR is equally involved in monounsaturated fatty acids (MUFA) and PUFA synthesis, as it upregulates the expression of enzymes necessary for UFA production, such as ACSL3, FADS 1 and 2 and ELOVL5, both in vivo and in vitro in human macrophages and murine foam cells. This effect is achieved in a SREBP-1-dependent manner, as well as by LXR itself in a more direct manner [75]. This lipogenic action on PUFA provides another link, besides Chol efflux and inhibition of its synthesis, for LXR anti-inflammatory, insulin sensitizing effects. In fact, LXR derepression upon NCoR deletion specifically in macrophages contributed to an insulin-sensitive phenotype in all major insulin-target tissues, reduced FFA serum levels and decreased macrophage Tnf α production by impairing TLR response and increasing PUFA production. This was achieved without changes in body weight [76]. These and other mechanisms of Chol and FA metabolism regulation by LXRs have been reviewed in [77,78]

4. Membrane Dynamics and Inflammatory Signaling in ATM

A distinct process by which FA and Chol, either captured or newly synthesized, alter ATM inflammatory response in the context of obesity is through their major role as membrane components. Although Chol does not enter the biosynthesis of other lipid classes, FA can be metabolized into a large range of lipid molecules involved in the metabolic activation of ATM with consequences on AT inflammation and IR (Figure 2). Membrane composition and optimal membrane properties (i.e., rigidity vs. fluidity) are crucial for a number of cellular processes such as glucose transport [79], immune cell function and inflammatory signaling [11,71,80,81], AT expansion [82] and even insulin signaling [83,84], all of which are compromised during DIO. Membrane dynamics are therefore of particular interest in the study of diabetes-promoting IR and contribute to this “membrane-centric” view of T2D [85].

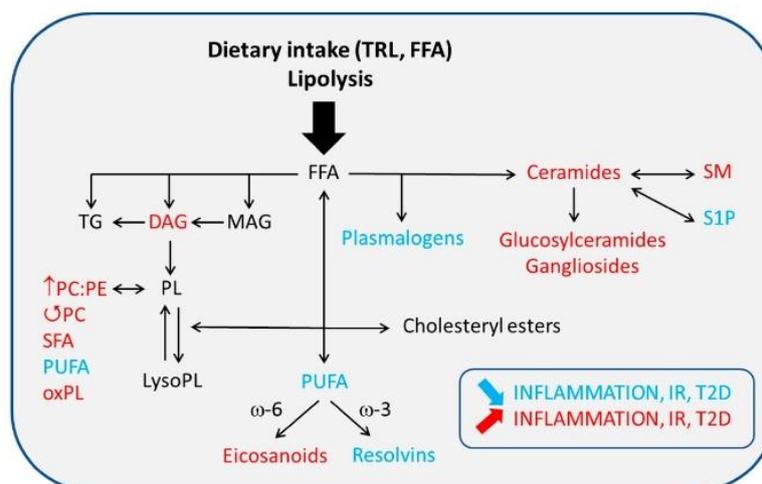


Figure 2. Fate of fatty acids in adipose tissue macrophages. The association of fatty acid (FA)-derived lipids with inflammation, IR and T2D is indicated in blue (protective) or red (deleterious). DAG, diacylglycerol; FFA, free fatty acids; IR, insulin resistance; MAG, monoacylglycerol; oxPL, oxidized PL; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PUFA, polyunsaturated fatty acid; S1P, sphingosine-1-phosphate; SFA, saturated fatty acid; SM, sphingomyelin; T2D, type 2 diabetes; TG, triacylglycerol; TRL, triglyceride-rich lipoproteins; \updownarrow turnover.

4.1. Altered Chol ATM Content and Membrane Signaling

ATMs from obese mice are characterized by an accumulation of free Chol, which was proposed to contribute to lipid-induced cytotoxicity during DIO [29]. Chol implication in inflammatory

processes as portrayed above is in part due to its key role in the formation of lipid rafts which are dynamic microdomains (10–200µm) that allow selective recruitment of other lipids and proteins necessary for intracellular signaling [86]. These rafts have been found to favor the clustering and stabilization of the TLR machinery (i.e., co-receptors CD14 and CD36 and co-adaptor protein for MyD88, TIRAP) [81]. Moreover, given the presence of a Chol recognition amino-acid consensus sequence in the structure of TLR, TLR localization at the membrane and downstream signaling seems dependent on this sterol. Chol loading of both plasma and endosomal membranes in macrophages enhanced TLR4-dependent p38 activation [87]. This effect on inflammation was abrogated by Chol depletion of detergent-resistant membrane domains by Abca1 and β-cyclodextrin, as it disrupts MyD88 recruitment to the membrane; such a mechanism being stimulated by LXR agonists [88]. Similarly, Abcg1-deficient human macrophages and myeloid cells present increased Chol plasma membrane content, which correlates with abnormal LPL retention within lipid rafts and hindered activity, thereby abolishing TG accumulation under VLDL stimulation [37]. Furthermore, Wei et al., demonstrated that changes in membrane Chol and lipid raft content through macrophage FAS inhibition alter global membrane order and composition, thus disrupting Rho GTPase-dependent signaling. These changes were associated with reduced inflammation and IR, a phenotype which was rescued by exogenous sterols but not exogenous FA [71]. These observations further validate Chol involvement in ATM metabolic activation; it is nonetheless of key importance that future research clearly discriminates plasma membrane Chol content vs. other organelles (i.e., ER, endosomes membranes), as well as from that stocked in LD, as this distinction is not always (easily) made. Indeed, accumulation of Chol in ER was reported to induce cytotoxicity and apoptosis in macrophages through the activation of the cell death effector CHOP of the unfolded protein response [89].

4.2. Altered PL/FA ATM Content and Membrane Signaling

4.2.1. Glycerophospholipids (GPL)

An ever-increasing number of studies has linked altered membrane glycerophospholipid GPL species—mostly phosphatidylcholine (PC) and phosphatidylethanolamine (PE)—and SFA/PUFA content to metabolic disorders. PC:PE ratio disturbance has been implicated in obesity-associated ER stress, inflammation, decreased insulin signaling and steatohepatitis [90–92]. Although most of these studies have focused on the effects of different GPL amounts on hepatocyte membranes, more recent data have found a similar relation between altered PC/PE levels and ATM activation. For instance, PC synthesis, assessed by *Pcyt1a* expression, and lysoPC content is increased in ATMs from genetically obese, insulin-resistant mice. Myeloid-specific inhibition of endogenous PC synthesis improves the metabolic phenotype and lowers macrophage inflammatory response to palmitate, without affecting macrophage number. Instead, this protective effect is mediated by reduced PC turnover at the plasma membrane thus allowing the incorporation of anti-inflammatory, membrane-fluidifying PUFAs, which also contribute to reduced ER stress [11]. This remodeling could be mediated by lysophosphatidylcholine acyltransferase 3 (Lpcat3), although no conclusive evidence was found in the study previously mentioned. LPCAT3 is part of the LPLAT family, and its expression is incidentally high in macrophages. Its role for the re-esterification of lysoPC into PC has been associated with altered inflammatory signaling at the plasma membrane, as activation of this enzyme in macrophages decreased activation of the c-Src/JNK pathway in membrane microdomains [93]. This further highlights the importance of membrane saturation degree on macrophage activation in obesity.

In AT, LXR agonist-mediated insulin sensitizing and anti-inflammatory effects in obese mice were correlated with decreased SFA- and MUFA-containing PCs, while an upregulation of PC and some PE species with high PUFA content was seen. TG species containing C16:0 were equally reduced [68]. Significantly increased expression (+80%) of phosphatidylglycerol (PldG) synthase, the principal enzyme involved in PldG synthesis, was also observed in whole AT depots from HFD-fed mice and positively correlated with high plasma PldG levels and *Tlr4* mRNA expression in WAT. Importantly, daily PldG treatment of obese mice seemed to promote M2-like polarization, as evidenced

by increases in M2 markers such as CD163 and CD301. PldGs were also able to inhibit AT lipolysis, albeit through indirect mechanisms [94]. Intriguingly, some adipocyte-derived PL species have been revealed to act in a paracrine manner and influence ATM activation state. Saturated lysoPC generated by phospholipase A₂ (PLA₂) activity were able to promote NLRP3 activation in LPS-primed macrophages and contribute to the general insulin resistant profile in adipocyte-specific *Hif-1α* knockdown mice [95]. Lastly, overweight and obese subjects also display increased serum PldG and PE, both of which are correlated with BMI, IL-6 and Ad expression in a positive and negative manner, respectively, suggesting a link between these PL and low grade, metabolic inflammation [94]. These results, along with those observed in mice, suggest that the obesity-associated increase in PldG levels may reflect a protective mechanism by which ATM-mediated inflammation is (attempted to be) controlled.

Obesity is characterized by an increased oxidative stress in adipose tissue in both humans and mice which was associated with the development of metabolic disorders [96]. Interestingly, Nox2, a key enzyme in the production of ROS, is a driver of the inflammatory and adipocyte-clearing properties of MME ATM, and ablating Nox2 both improves and worsens DIO-induced metabolic disorders depending on the duration of high-fat feeding [8]. As for FFA and free Chol, PL-bound FAs are subject to oxidation. Oxidized phospholipids (PLs) (oxPL) are recognized as danger-associated molecules and are therefore linked to certain inflammatory pathways. OxPLs derived from PC have been found to induce an Il-1β-driven hyperinflammatory state in macrophages by promoting both OXPHOS and glycolysis; a phenotype also displayed in vivo in hypercholesterolemic mice after LPS administration [97]. OxPL have thus been implicated in steatohepatitis [98], atherosclerosis [99,100] and more recently, in obesity and diet-induced ATM activation [33]. Indeed, Cd11b⁺ cells isolated from the SVF of HFD-fed mice saw their oxPL content double at 9 weeks of HFD. CLS-localized ATM (marked Cx3cr1⁺F4/80^{hi}Cd11c⁺Cd206⁺) were abundant in different oxPLs, specifically full-length species which were found to promote Il-1β and Il-6 expression. In contrast, ATM from lean mice (marked Cx3cr1⁻F4/80^{lo}Txnrd1⁺HO1⁺) presented higher truncated oxPLs, which were revealed to have antioxidant properties and inhibit mitochondrial respiration in BMDM [33]. Evidence linking oxPL and lipotoxic ceramide production through CerS activation has also been reported [101]. No evidence of oxPL capacity to induce lipid accumulation within ATMs has been observed. Of note, oxPL can equally be found in oxLDL or oxVLDL particles and also accumulate intracellularly in LDs [102].

4.2.2. Sphingolipids (SL)

DIO has also been associated with dysfunctional sphingolipid (SL) metabolism. Among them, ceramides have highly inflammatory, insulin resistant properties that eventually lead to T2D. Ceramide accumulation is observed in plasma and visceral AT from obese women with metabolic syndrome and is accompanied by inflammatory marker expression [103]. Pharmacological inhibition of short chain C16:0 ceramide synthesis correlated with lower weight gain in mice fed a HFD, as well as improved glucose tolerance and insulin sensitivity [104]. The mechanisms guiding ceramide effects are numerous. De novo ceramide synthesis is upregulated by adipose mTORC1 deficiency, which incidentally increases ATM recruitment to CLS, inflammatory cytokine production through NLRP3 activation and IR [105]. Accordingly, ceramide accumulation by VLDLR overexpression in ATM is able to induce M1-like polarization and IR [41]. However, studies conducted with obese mice unable to de novo synthesize ceramides in ATM found contradicting results, as no differences regarding the insulin resistant and NLRP3-dependent inflammatory state were observed. Moreover, this deficiency did not affect M1-like or M2-like polarization, nor adiposity [106]. These discrepancies might be due to cell types—adipocytes vs. myeloid cells—or genes targeted—mTORC1 vs. VLDLR vs. serine palmitoyltransferase long chain 2 (SPTLC2)—and convey the importance of the alternative pathways that might act in a compensatory manner to assure ceramide production.

It is nonetheless unexpected that SPT deficiency did not aggravate the phenotype, as this enzyme also allows the production of sphingomyelin (SM). While the role of SM in ATM-driven inflammation and IR has been less studied, its major contribution to the formation of lipid rafts and LPL bioavailability [107] is expected to induce major changes in inflammatory signaling and

lipid storage. This has notably been observed in macrophages where SM synthases 1 and 2 and SPT have been inhibited, and that fail to recruit TLR4 to the membrane, rendering them less sensitive to LPS stimulation [81]. Lastly, altering SM turnover at the membrane by phospholipid transfer protein (PLTP) inhibition in mice has found to be protective against diet-induced weight gain and IR. Since PLTP mediates lipid transfer—mostly SM and Chol—from cell membranes to plasma and vice versa, its deficiency was proven to decrease the levels of these two lipid types in lipid rafts and enhance GLUT4 expression and insulin receptor activity in adipocytes [108]. Given its potential as a target in metabolic disorders, this mechanism might be interesting to explore in ATMs.

Globally, ATM metabolic activation in DIO is associated with a switch to more lipotoxic lipids. It is important to note that we cannot rule out the contribution of other membrane lipids, such as phosphatidylinositol, phosphatidylserine, phosphatidic acid and plasmalogens. Due to their low abundance in membranes, their effects might be masked by the other lipids. It is thus preferable to study membrane dynamics in relation to individual PL species and nature of the acyl chain. The diet content of ω -3 and ω -6 PUFAs has been found to be of particular importance in determining membrane composition [109] and can thus significantly influence membrane signaling in ATMs.

4.3. FA Nature and ATM-Mediated Inflammation

4.3.1. SFA

The inflammatory phenotype of human ATMs is correlated with the FA spectrum of membrane PL, and SFA and PUFA content share a positive and negative association, respectively, with the proportion of these macrophages in obese AT [110]. The SFA to PUFA ratio has also been implicated in ER stress response, and decreased ER saturation and rigidification by inhibition of the de novo PC synthesis pathway, which preferentially selects and binds SFA to PC, has been shown to ameliorate ER stress in BMDMs despite palmitate exposure [11]. Fatty acid-binding protein-4 (Fabp4) acts as an essential lipid chaperone in the enrichment of GPL in SFA and in lipid-induced ER stress in macrophages [111]. Beyond a role in rigidity and fluidity, FAs from membrane PL attain their inflammatory properties by their ability to directly activate inflammatory pathways in macrophages and other cells, or act as precursors for lipid mediators once hydrolyzed from the PL backbone.

SFAs are known for their pro-inflammatory capacity and presence of only single bonds within their acyl chain. SFA with acyl chains up to 16 carbons can be synthesized endogenously, while those containing longer chains are obtained by elongation, with the starting product of stearate (ST; C18:0). They can also be brought by diet, typically animal fats. SFAs are associated with NLRP3 activation; indeed, both PA and ST treatment of human macrophages is able to induce IL-1 β secretion. Interestingly, this effect is mediated by rigidification of the plasma membrane, as endogenous SFA induces a significant increase in saturated PC content, which disrupts Na, K-ATPase activity and favors K⁺ efflux, a mechanism that has been long associated with inflammasome activation. Unsaturated oleate or linoleate co-treatment prevent this outcome [112]. NLRP3 activation is linked to the pro-inflammatory JNK pathway, and JNK activation has been found to exert a role in ATM-mediated IR and development of T2D [113,114], but only under an obesity setting. Mice presenting a macrophage-specific double knockout of *Jnk1* and *Jnk2* displayed a similar metabolic status when fed a normal chow diet. However, when under a HFD, JNK-deficient mice were protected against glucose intolerance and IR, showed decreased ATM infiltration and M1-like polarization [115]. PA can also activate JNK through a mechanism involving the pro-inflammatory p62/NBR1 adaptor proteins in the MAPK pathway, and NBR1 inhibition results in decreased Il-6 and Tnf α production by BMDM. Furthermore, it impairs M1-like polarization in ATMs isolated from HFD-fed obese mice [116]. PA has also been found to induce NF- κ B activation in a TLR4 dependent manner, and macrophages from mice lacking Tlr4 revealed blunted inflammatory cytokine production and increased insulin sensitivity [117]. PA has also been shown to induce Tlr4 translocation into lipid rafts in adipocytes [118].

Whether PA acts as a direct TLR4 ligand has been extensively challenged in recent years [119]. The first piece of evidence showing PA is not a direct agonist of this receptor was brought in by

Lancaster et al., who demonstrated that PA treatment does not induce the conformational changes, dimerization nor endocytosis that characterize TLR4 active state. The absence of TLR4 in murine macrophages, although protective against PA inflammatory effects when used alone, is not sufficient to inhibit PA effects when cells had been previously primed with other TLR agonists [120]. It is interesting to note that this priming of *Tlr4*^{-/-} macrophages was associated with a metabolic reprogramming that rendered them similar to the WT phenotype. These modifications included the upregulation of genes typical of M1-like metabolism and alteration of its lipidome, most notably ceramides, SM, PE plasmalogens and lysoPC/PE plus CE with PA and ST on their acyl chains [120]. Similar observations have been made in studies for PA effect on ceramide production. Indeed, while PA alone was able to induce C16:0 ceramide synthesis and promote Tnf α and Il-1 β secretion, this effect was independent of the TLR. In contrast, co-treatment with LPS not only potentiated PA effects, but also turned out to require the TLR4/MyD88 axis. Curiously though, this upregulation of SL murine macrophage content did not involve changes in SPT expression, again hinting at the importance of alternative pathways for ceramide production [121]. In this context, adipose FA binding protein (FABP) was reported to play an important role in ceramide production and cell death in macrophages upon incubation with SFA (PA and stearic acid) [122]. Finally, PA is able to induce the expression of *Hif-1 α* in macrophages even under normoxic conditions [28] and to promote pro-inflammatory cytokine expression. This might therefore provide another mechanism, independent of TLR4, for ATM activation during obesity.

Other SFAs such as lauric acid (LU, C12:0) act through similar mechanisms. LU treatment on RAW macrophages was shown to promote TLR4 recruitment to lipid rafts and the subsequent homodimerization of this receptor, as evidence by GFP, FLAG and FITC labeled constructs of the TLR4/MyD88 pathway [123]. Stearic acid (SA, C10:0) was also shown to induce TLR4 activation, although to a minor degree [124]. This and other mechanisms (i.e., CD36/TLR2 activation in adipocytes and macrophages, IRS-1 phosphorylation, PCG-1 activation) [125,126] contribute to the overall inflammatory and insulin resistant profile seen in DIO and ATM.

4.3.2. UFA

UFAs, on the other hand, present at least one double bond in their acyl chain and promote an anti-inflammatory environment by inhibiting leukocyte chemotaxis, adhesion molecule expression and production of pro-inflammatory eicosanoids while upregulating that of specialized pro-resolving lipid mediators (SPMs) [127]. When it comes to PUFA, the most prominent species are ω -3 and ω -6 PUFAs. The common precursors for longer, more complex PUFAs are linoleic acid (LA, C18:2n-6) and α -linoleic acid (ALA, C18:3n-3), which have the particularity of being essential FAs that can only be obtained through diet, mostly vegetable fats. Once absorbed, they can undergo a series of elongation and desaturation steps which take place in the ER and involve 3 different enzyme families: ACSL3-4 for FA activation, the ELOVL family for the rate limiting step in elongation and FASD1-2 for desaturation. These enzymes are under the control of SREPB and LXR, as stated above, and thus their transcription is modulated by agonist treatment or derepression of these transcription factors [78]. Their deficiency would lead to impaired long-chain PUFA synthesis, as observed in BMDM from LXR deficient mice, who present decreased levels of ELOVL5, ACSL3 and FASD1 and altered PUFA metabolism—diminution of arachidonic acid and docosahexaenoic acid levels [75]. *Elovl5* knockdown mice also presented reductions in ω -3 and ω -6 PUFA levels, while they accumulated C18 PUFAs [128].

The most prominent and commonly studied PUFAs are γ -linoleic acid (γ -LA; C18:3n-6), arachidonic acid (AA; C20:4n-6), eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), the latter two sharing strong anti-inflammatory properties. While γ -LA is decreased in the visceral AT of type 2 diabetic, obese women, AA, EPA and DHA are all increased in subcutaneous AT. Most interestingly, AA levels correlate to CD68 expression [129]. Although most AA functions stem from its ability to produce bioactive lipid mediators, AA holds properties innate to itself. AA is the most prevalent PUFA and has been found to disrupt LPS- and SFA-mediated TLR4 activation in macrophages

by directly binding to its co-receptor MD2 and decreasing the stimulation of its downstream target NF- κ B and MAPK [130]. Contradicting results have, however, arisen from the observation that free AA treatment is able to induce p38 and JNK signaling in human monocytes, as well as to generate a foam-like phenotype by promoting the accumulation of TG, CE and AA itself in LDs [131]. This pool is, however, not used for eicosanoid production [132], but still represents a mechanism that may favor the ATM phenotype distinctive of obesity as lipid-laden monocytes migrate into AT. JNK signaling is associated with apoptotic mechanisms and accordingly, AA treatment of RAW cells caused cell cycle arrest and apoptosis in a JNK-dependent manner [133]. Increased intracellular AA content by altering the Lands cycle also resulted in apoptosis of monocyte-derived macrophages [134].

When it comes to ω -3 PUFA, *in vitro* and *in vivo* models display different results. DHA treatment of model membranes resulted in increased membrane fluidity but also an increase in Chol domains, suggesting it is capable of forming distinct Chol-rich or poor domains. On the contrary, EPA, AA and ALA all increased fluidity by reducing Chol domains. EPA notably did so despite temperature and Chol content changes, revealing more consistent effects [135]. However, these results have been contested by observations that EPA treatment of Chol-loaded HMDM does not induce significant changes of the free Chol:GPL ratio. Instead, it participates in foam-like cell formation by reducing ABCA1/G1-mediated Chol efflux and increasing TG content stored in LD [136]. In mice, ω -3 PUFA macrophage production by NCoR depression has been associated with hypoinflammation [76]. Anti-inflammatory M2 polarization and lipid catabolism is promoted in mice under a DHA/EPA-rich diet, where shorter chain FAs were used for β -oxidation while PUFAs were oriented to GPL and SPM production [137]. EPA alone also displays protective, insulin sensitizing effects, as supplementation of this PUFA prevented and even reversed AT inflammation and adipocyte hypertrophy, accounted for by adipocyte size, adipogenesis-related genes, ATM infiltration and FA oxidation (FAO) [138]. Likewise, DHA shares similar effects by promoting M2-like polarization in the AT of obese mice [139]. These protective actions are most likely due to its ability to inhibit NF- κ B and NLRP3 inflammasome activation, as well as to target *de novo* ceramide synthesis and TLR4 recruitment into lipid rafts [123,140,141]. Evidence also suggests that these FAs work in concert with the insulin sensitizing adipokine adiponectin, given that its neutralization augments M1 markers *Il-6*, *Ccl2*, *Nos2*, among others, and increases neutral lipid content within ATMs [142]. The discovery of the G protein-coupled receptor (GPCR) GPR120, recently renamed fatty acid receptor 4 (FFA4), as well as DHA and EPA capacity to interact and activate PPAR α , represent another means for the anti-inflammatory character of ω -3 PUFA [125,139]. Gpr120 is highly expressed in adipocytes and macrophages, and its deficiency abrogates the anti-diabetic effects that come with ω -3 PUFA supplementation [143,144]. Indeed, DHA and EPA exposure of Gpr120 null mice had no effect on monocyte/macrophage chemotaxis, M1-like and M2-like gene expression as well as overall and tissue-specific insulin sensitivity [143].

Although less studied, MUFAs also display anti-inflammatory properties that can oppose SFA action. A MUFA-enriched diet favored a more insulin sensitive phenotype in mice and humans, as well as reduced IL-1 β and NO secretion by macrophages, decreased the number of M1-like ATMs and limited adipocyte hypertrophy [145,146]. In BM-derived macrophages, the ω -9 oleic acid (OA, C18:1n-9) was able to inhibit NLRP3 signaling through the induction of AMPK [145]. This inhibition is consistent with results showing that OA is capable of promoting M2-like polarization in RAW macrophages and mesenteric AT, but not epididymal AT [106]. The anti-inflammatory ω -7 palmitoleic acid (PM, C16:1n-7) acts independently of AMPK and also PPARs, but is able to lower M1 polarization, cytokine production and ameliorate IR in muscle [114,147]. In accordance with these observations, Cao and et al., showed that PM enrichment in the AT of FABP deficient mice allowed for a significant flux of this FA to the muscle and liver, improving metabolic status and whole-body insulin sensitivity [148].

4.3.3. Eicosanoids and Other Bioactive Lipid Mediators

In addition to directly modifying cytokine production and inflammatory signaling, ω -3 and ω -6 PUFAs are direct precursors for the production of potent bioactive lipid mediators such as eicosanoids and resolvins. AA, being the most abundant ω -6 PUFA, is responsible for the production of

prostaglandins (PG), which are generated by the action of cyclooxygenases (COXs), and thromboxanes (TX), leukotrienes (LT) and lipoxins (LX) which are issued by lipoxygenases (LOXs). These metabolites are responsible for most of the bioactivity attributed to AA. EPA and DHA are equal substrates for COXs and LOXs, but also for Cytochrome P450 (CYPs) enzymes which mediate their conversion into protectins (PD), maresins (for DHA) and resolvins (D-series when issued from DHA, E-series when issued from EPA). These 3 families are referred to as SPMs.

Their production is mainly mediated through the action of PLA₂, which allows the release of different PUFAs from membrane PL [109]. The PLA₂ superfamily is composed of numerous lipolytic enzymes that are classified according to their induction profile (i.e., cytosolic, secretory, Ca²⁺ independent, among others) [109]. Their implication in the development of metabolic disorders and inflammation has been analyzed in plasma, adipocytes, pancreatic islets and macrophages in both mice and humans. Group X sPLA₂ has been found to promote PGE₂ secretion and inhibit glucose stimulated insulin secretion [149]. Similarly, it inhibits LXR activation in macrophages, decreases ABCA1/G1 expression, augments intracellular Chol content in macrophages and promotes inflammatory signaling dependent on TLR4 [149]. PLA₂ group V is upregulated in plasma from diabetic subjects and associated SNPs correlate to LDL levels [150]. Moreover, its secretion by hypertrophic adipocytes favors M2-like polarization through PGs and other lipid-mediated processes, limits IR in DIO, promotes phagocytosis and intriguingly promotes atherosclerosis [151–153]. Other members of the PLA₂ superfamily also warrant research, notably regarding their possible role in diet-induced ATM metabolic activation.

LPLATs can also contribute to eicosanoid production as they assure PUFA—preferably AA and EPA—turnover at the *sn*-2 position of membrane PL. This has been demonstrated in *in vitro* and mouse models by altering the expression or activity of the LPCAT3 member. Indeed, while Lpcat3 inhibition in RAW macrophages and shLpcat3-treated mice showed reduced LA- and AA-containing membrane PC and a pro-inflammatory phenotype, its activation reduced the levels of lysoPC and thus limited the amount of free AA that could be used for PGE₂ production in LPS-primed macrophages [93]. For complementary roles of the PLA₂ and LPLAT enzymes in membrane dynamics, obesity and obesity-associated metabolic disorders, see [109,154,155]. Lastly, SFA and UFA also control eicosanoid production by regulating the expression of the enzymes mentioned above. Such is notably the case of LU and PA, which were the most potent inducers of COX expression in RAW macrophages. This effect was inhibited by PUFAs such as DHA [124]. Conjugated linoleic acid, on the contrary, displayed an inhibitory effect on COX expression in BMDM and thus resulted in reduced TXA₄ and PGE₂ synthesis [156]. DHA- and EPA-derived SPMs are characterized by their anti-inflammatory features, while AA-derived eicosanoids are pro- or anti-inflammatory, depending on the context (Table 1). Such is the case of PGE₂, which has antilipolytic, antifibrogenic, proresolving properties when used as an exogenous stimulus on human WAT explants from obese subjects [157]. It has also been shown to promote an anti-inflammatory ATM phenotype [158]. In contrast, PGE₂ production or signaling inhibition has also been shown to be protective against obesity-associated inflammation, as it decreased TNF α , MCP-1 and IL-6 expression by macrophages and AT lipolysis in HFD-fed rodent models and human AT [159]. These discrepancies may notably be due to the specificity of the response, as whole-body inhibition of COX enzymes also downregulates the expression of other prostaglandins (PGs) which might otherwise be responsible for the inflammatory state observed in obesity. This might, however, not be true for cell specific COX inhibition, as obese mice subject to bone marrow transplantation from *Cox1*^{-/-} donors actually showed increased AT inflammation, whereas hepatic inflammation was diminished [160]. Moreover, while PGE₂ is the primary ligand for EP3, we cannot rule out the binding of this PG to other receptors, or even other PGs to EP3. Lastly, while PGE₂ indeed seemed to promote ATM migration in a model of increased lipolysis after fasting, this was done so without increasing inflammation in AT [161].

Table 1. Principal PUFA-derived lipid mediators in adipose tissue (AT) inflammation and ATM activation during obesity. H: human, M: mouse, R: rat, En: endogenous, Ex: exogenous.

MEDIATOR	PRECURSOR	SPECIES: MODEL	SOURCE	CELL TYPE/TISSUE	REPORTED ACTIONS	REFERENCES
PRORESOLVING MEDIATORS						
RVD1	DHA	M: DIO	En, Ex	ATMs, Peritoneal macrophages	<ul style="list-style-type: none"> ■ Reduced pro-inflammatory markers (TNFα, IL-6, leptin) and ROS ■ Induces phagocytosis 	[139,162]
		M: <i>db/db</i> mice	Ex	ATMs	<ul style="list-style-type: none"> ■ Improved glucose homeostasis and insulin sensitivity ■ Increased adiponectin secretion ■ Decreased CLS formation and ATM M1 polarization 	[163]
		M: DIO H: lean and obese	En, Ex	Epididymal AT, BMDM, adipocytes, PBMC	<ul style="list-style-type: none"> ■ Reduced monocyte/macrophage transadipose migration 	[162]
		H: lean and obese \pm T2D and THP-1 cells	En, Ex	Omental AT and THP-1 macrophages	<ul style="list-style-type: none"> ■ Promotes the IL-10/STAT3 pathway at low concentrations ■ Increases p38 MAPK phosphorylation and IL-1α production 	[164]
RVD2	DHA	M: DIO H: Lean and obese	Ex	Epididymal AT	<ul style="list-style-type: none"> ■ Reduced pro-inflammatory markers (TNFα, IL-6, IL-12, leptin) ■ Induced adiponectin secretion ■ Decreased leptin secretion 	[162]
RVE1	EPA	M: <i>ob/ob</i> mice	Ex	Epididymal AT	<ul style="list-style-type: none"> ■ Induced expression of adiponectin, GLUT4, IRS-1 and PPARγ in AT ■ Reduced monocyte/macrophage transadipose migration 	[165]

Table 1. Cont.

MEDIATOR	PRECURSOR	SPECIES: MODEL	SOURCE	CELL TYPE/TISSUE	REPORTED ACTIONS	REFERENCES
PD1	DHA	M: <i>ob/ob</i> mice	Ex	Epididymal AT	<ul style="list-style-type: none"> Induced expression of adiponectin 	[165]
MARESIN 1	DHA	M: DIO and <i>ob/ob</i> mice and H: lean subjects	Ex	Epididymal AT, subcutaneous AT	<ul style="list-style-type: none"> Ameliorated insulin signaling (AKT phosphorylation) and glucose homeostasis (GLUT4, AMPK expression, blood glucose levels) Reduced IL-1β, TNFα, MCP-1 expression in AT Attenuated ATM infiltration and reduced the M1:M2 ratio Induced adiponectin secretion 	[166]
LXA4	AA	M: DIO	Ex	Epididymal AT	<ul style="list-style-type: none"> Induced adiponectin secretion 	[162,167]
		M: DIO and 3T3-L1, J774 cells	Ex	ATMs, 3T3-L1 adipocytes, J774 macrophages	<ul style="list-style-type: none"> Promoted an anti-inflammatory ATM phenotype Downregulated diet-induced autophagy 	[167]
		M: Standard diet and 3T3-L1, J774 cells	Ex	Perigonadal AT and 3T3-L1 adipocytes, J774 macrophages	<ul style="list-style-type: none"> Decreased IL-6, TNFα and MCP-1 secretion by AT and macrophages Increased insulin sensitivity by promoting IL-10, GLUT-4 and IRS-1 expression 	[168]
PGD2	AA	M: DIO + aP2-Cre/L-PGDS ^{fllox/fllox} mice, DIO and <i>ob/ob</i> mice and H: obese subjects	En, Ex	ATM, epididymal AT, subcutaneous AT, BMDM	<ul style="list-style-type: none"> Decreased weight gain and promoted insulin sensitivity Altered FA metabolism: decreased SREBP-1, SCD1, FAS Increased ATGL- and MGL-mediated lipolysis Altered M1:M2 ratio in favor of anti-inflammatory M2 macrophages 	[169,170]

Table 1. Cont.

MEDIATOR	PRECURSOR	SPECIES: MODEL	SOURCE	CELL TYPE/TISSUE	REPORTED ACTIONS	REFERENCES
PGE2	AA	H: Lean and obese and 3T3-L1 cells	En, Ex	Omental AT, primary adipocytes, 3T3-L1 adipocytes	<ul style="list-style-type: none"> ■ Decreased inflammatory and fibrogenic gene expression ■ Inhibited adipocyte lipolysis ■ Promoted AT browning 	[157]
		M: DIO + CREB ^{LysM} KO mice	Ex	BMDM	<ul style="list-style-type: none"> ■ Mediated M2-like ATM polarization via CREB, thus maintaining insulin sensitivity 	[158]
PRO-INFLAMMATORY MEDIATORS						
PGE2	AA	H: lean and obese, M: <i>db/db</i> mice, R: DIO and 3T3-L1, RAW cells	En	SVF, Primary adipocytes, 3T3-L1 adipocytes, RAW macrophages	<ul style="list-style-type: none"> ■ Decreased M1-like ATM content and pro-inflammatory cytokine expression by COX or EP3 inhibition ■ Decreased macrophage migration and responsiveness to hypoxia by COX inhibition ■ Lower blood glucose levels and higher insulin sensitivity by COX or EP3 inhibition 	[159]
LTB4	AA	M: DIO and U937, 3T3-L1 cells	En, Ex	Peritoneal macrophages, 3T3-L1 adipocytes,	<ul style="list-style-type: none"> ■ Stimulated macrophage chemotaxis ■ Promoted activation of the NF-κB and JNK pathways in macrophages ■ Increased TNFα, IL-6, MCP-1, CXCL1 expression ■ Promoted IR in liver and muscle 	[171]
LTD4	AA	M: DIO and <i>ob/ob</i> mice	En, Ex	Epididymal AT	<ul style="list-style-type: none"> ■ Induced NF-κB activity 	[172]

The expression of these and other lipid mediators, as well as the enzymes needed for their synthesis is found in human visceral adipose tissue, although their ratio shifts during obesity in favor of AA-derived products [162,164]. Indeed, while the 5-LOX pathway is principally implicated in LT production and, to a lesser extent, resolvin synthesis, its upregulation during obesity is accompanied by increased conversion of AA into LTB4 and LTD4 in obese women with T2D while maintaining resolvin levels below the detection limit [129]. In addition, this pathway has been found to be correlated with LD formation within macrophages which also display higher LTB4 production levels [173]. It is interesting to note, however, that exercise and weight loss promotes the synthesis of other LOX-derived mediators, notably RvD1. This is accompanied by enhanced macrophage phagocytosis [174].

Proresolving and pro-inflammatory lipid mediators exert their actions notably through binding to specific GPCRs. RvD1 and LXA4 signal through GPR32/RDV1 and ALX/FPR2, the latter being expressed in murine ATMs and adipocytes as well as human cells [162,163,168]. Unexpectedly, myeloid deletion of *Fpr2* is associated with improved IR, lipid and glucose metabolism as well as lower inflammatory status due to reduced M1-like ATM infiltration [175], meaning other metabolites different to RvD1 and LXA4 are able to bind and more potently induce the FPR2 axis, whose properties seem to depend on the nature of the ligand. RvE1 signals through the receptor ERV1, and its overexpression in myeloid cells holds protective effects on DIO [176]. Interestingly, the chemerin receptor ChemR23 has also been found to bind RvE1 [162]. GPR18/DRV2, LGR6, GPR37 are receptors for RvD2, maresin and PD1, respectively [177,178]. PGE2 signaling is mediated by receptors EP1-4. Among them, EP3 has been shown to be increased in adipocytes of HFD-fed mice and increase adipocyte FFA release [159], while EP4 is induced in ATMs and seems to be implicated in ATM recruitment and lipid buffering [161]. BLT1, also called LTB4r1, is the receptor for LTB4 and is expressed in macrophages. Its deficiency results in higher glucose tolerance, lower blood insulin and FFA levels and improved insulin sensitivity in mice under a HFD. It also translated into lower M1-like ATM migration both in vitro and in vivo and ameliorated inflammatory profile [171].

5. Concluding Remarks

Obesity is first and foremost a metabolic disorder characterized by the excessive influx of dietary lipids which orchestrate ATM metabolic activation as a protective means for reducing extracellular and intracellular cytotoxic lipid levels. ATMs are at the crossroad of both lipid metabolism and lipid-mediated inflammation by adopting a specific program for buffering these lipids, a key feature for controlling AT inflammation and the development of IR and T2D. A better understanding of the mechanisms by which lipids shape the ATM phenotype during diet-induced obesity is thus necessary to counteract this pathology, and will most likely need to integrate multi-omic approaches in order to identify interaction networks through which single molecular lipid species are implicated in ATM dysfunction.

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Abbreviations

AA	Arachidonic acid
ABCA1/G1	ATP-binding cassette A1/G1
AT	Adipose tissue
ATM	Adipose tissue macrophages
Chol	Cholesterol
CLS	Crown-like structures
DHA	Docosahexaenoic acid

DIO	Diet-induced obesity
DNL	<i>De novo</i> lipogenesis
EPA	Eicosapentaenoic acid
FAS	Fatty acid synthase
FFA	Free Fatty Acid
HDL	High-density lipoprotein
HFD	High fat diet
IR	Insulin resistance
LD	Lipid droplet
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LXR	Liver X Receptor
MCP-1/CCL2	Monocyte chemoattractant protein-1/Chemokine C-C motif ligand 2
Mme	Metabolically activated
MUFA	Monounsaturated fatty acid
PA	Palmitic acid
PE	Phosphatidylethanolamine
PG	Prostaglandin
PL	Phospholipid
PldG	Phosphatidylglycerol
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid
SL	Sphingolipid
SREBP	Sterol Regulatory Element-Binding Protein
T2D	Type 2 Diabetes
TG	Triglyceride
TLR	Toll-like receptor
VLDL	Very low-density lipoprotein

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Collaborative article

**Phosphatidylserine enhances anti-inflammatory effects of reconstituted HDL in
macrophages via distinct intracellular pathways**

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RESEARCH ARTICLE

Phosphatidylserine enhances anti-inflammatory effects of reconstituted HDL in macrophages via distinct intracellular pathways

Maryam Darabi¹ | Marie Lhomme² | Veronica D. Dahik¹ | Isabelle Guillas¹ | Eric Frisdal¹ | Emilie Tubeuf¹ | Lucie Poupel¹ | Mili Patel³ | Emmanuel L. Gautier¹ | Thierry Huby¹ | Maryse Guerin¹ | Kerry-Anne Rye³ | Philippe Lesnik¹ | Wilfried Le Goff¹ | Anatol Kontush¹

¹INSERM, Institute of Cardiometabolism and Nutrition (ICAN), UMR_S1166, Sorbonne Université, Paris, France

²ICAN Analytics, Lipidomics Core, Foundation for Innovation in Cardiometabolism and Nutrition (IHU-ICAN, ANR-10-IAHU-05), Paris, France

³School of Medical Sciences, Faculty of Medicine, University of New South Wales, Sydney, New South Wales, Australia

Correspondence

Anatol Kontush, INSERM UMR-S 1166 ICAN, Sorbonne University, 91 boulevard de l'Hôpital, Paris 75013, France.
 Email: anatol.kontush@sorbonne-universite.fr

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Abstract

Phosphatidylserine (PS) is a minor phospholipid constituent of high-density lipoprotein (HDL) that exhibits potent anti-inflammatory activity. It remains indeterminate whether PS incorporation can enhance anti-inflammatory effects of reconstituted HDL (rHDL). Human macrophages were treated with rHDL containing phosphatidylcholine alone (PC-rHDL) or PC and PS (PC/PS-rHDL). Interleukin (IL)-6 secretion and expression was more strongly inhibited by PC/PS-rHDL than PC-rHDL in both tumor necrosis factor (TNF)- α - and lipopolysaccharide (LPS)-stimulated macrophages. siRNA experiments revealed that the enhanced anti-inflammatory effects of PC/PS-rHDL required scavenger receptor class B type I (SR-BI). Furthermore, PC/PS-rHDL induced a greater increase in Akt1/2/3 phosphorylation than PC-rHDL. In addition, PC/PS but not PC-rHDL decreased the abundance of plasma membrane lipid rafts and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation. Finally, when these rHDL formulations were administered to dyslipidemic low-density lipoprotein (LDL)-receptor knockout mice fed a high-cholesterol diet, circulating IL-6 levels were significantly reduced only in PC/PS-rHDL-treated mice. In parallel, enhanced Akt1/2/3 phosphorylation by PC/PS-rHDL was observed in the mouse aortic tissue using immunohistochemistry. We concluded that the incorporation of PS into rHDLs enhanced their anti-inflammatory activity by modulating Akt1/2/3- and p38 MAPK-mediated signaling through SR-BI in stimulated macrophages. These data identify PS as a potent anti-inflammatory component capable of enhancing therapeutic potential of rHDL-based therapy.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; ApoA-I, apolipoprotein A-I; ATF3, activating transcription factor 3; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; IL-6, Interleukin-6; LC/MS/MS, liquid chromatography-tandem mass spectrometry; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor kappa B; p38 MAPK, p38 mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; rHDL, reconstituted high-density lipoprotein; SR-BI, scavenger receptor class B type I; TGF- β , transforming growth factor β ; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor.

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KEYWORDS

atherosclerosis, inflammation, phospholipids, rHDL

1 | INTRODUCTION

Researchers have recently ramped up efforts to pharmacologically raise high-density lipoprotein (HDL) cholesterol (HDL-C) levels¹⁻⁴ or to increase the number of HDL particles through intravascular infusion of HDL mimetics containing apolipoprotein A-I (apoA-I)⁵⁻⁷ for the treatment of atherosclerotic cardiovascular disease. Nonetheless, contradictory data were obtained regarding atherosclerosis development and cardiovascular mortality, even while HDL-C levels were markedly raised.^{2,4,8} The modest cardiovascular benefit observed in only one of these trials was attributed to the moderate drop in plasma non-HDL cholesterol concentrations rather than to an elevation of HDL-C.⁴ Moreover, Mendelian randomization studies indicated no effect on the atherosclerotic cardiovascular disease risk from genetically elevated or reduced HDL-C levels.⁹⁻¹¹ These findings highlighted the importance of a functional improvement of HDL by HDL-targeting rather than just raising HDL-C levels. Indeed, HDL displays multiple anti-atherogenic activities, which may include the capacity to reduce inflammatory activation of endothelial cells and possibly macrophages.¹²⁻¹⁵

Emerging knowledge about the role of inflammation in atherosclerosis over the last decade has led to the development of a number of therapeutic approaches that target various inflammatory mediators.¹⁶ In this context, notably, the increasing evidence of the anti-inflammatory activity of HDL has stimulated interest in the investigation of molecules underlying this activity, which might prove useful as new therapeutic targets.

Lipidomic analyses show that phosphatidylserine (PS) is differentially distributed across HDL subclasses, being enriched in small, dense HDL particles possessing potent anti-atherosclerotic properties.¹⁷ The key role of PS in the resolution of inflammation was clearly delineated in the phenomenon of apoptosis.^{18,19} As a corollary, targeting of PS-containing liposomes to macrophages to mimic anti-inflammatory effects of apoptotic bodies was designed as a promising strategy for modulating these cells to an anti-inflammatory phenotype.²⁰⁻²³ In addition, specific receptors and transporters involved in HDL-mediated cholesterol efflux, such as ATP-binding cassette A1 (ABCA1) and G1 (ABCG1) transporters and scavenger receptor BI (SR-BI), were independently reported to be critical for the biological effects of PS-containing liposomes.²⁴⁻²⁶ Macrophages are key immune cells in chronic inflammation, and their activity plays a central role in atherosclerotic

plaque pathogenesis. Here, we propose for the first time that the key feature of PS in suppressing inflammation, together with its natural occurrence in HDL, provides a logical framework for developing PS-containing reconstituted HDL (rHDL) to enhance its anti-inflammatory properties as a novel therapeutic approach in cardiovascular and inflammatory diseases.

In this study, we characterized the anti-inflammatory effects exerted by PS-containing rHDL on primed pro-monocytic THP-1 cells and examined their underlying mechanisms. Our data revealed that PC/PS-rHDL inhibited proinflammatory cytokine secretion and expression to a greater extent than PC-rHDL and PC/PS-liposomes in tumor necrosis factor-alpha (TNF- α)- or lipopolysaccharide (LPS)-stimulated macrophages. Importantly, we determined that macrophage SR-BI was involved in the potent anti-inflammatory effects of PC/PS-rHDL coupled with Akt1/2/3 phosphorylation and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways. Finally, the potent anti-inflammatory activity of PS-containing rHDL was confirmed in vivo in a mouse model of dyslipidemia.

2 | MATERIALS AND METHODS

2.1 | Preparation of reconstituted HDL and liposomes

Discoidal rHDLs were prepared by cholate dialysis from a mixture of apoA-I, isolated from human ethylenediaminetetraacetic acid (EDTA) plasma, and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) 16:0/18:1 without or with 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) 16:0/18:1 (Avanti Polar Lipids, AL, USA)²⁷ at a molar ratio of 1:90 for PC-rHDL and 1:70:30 for PC/PS-rHDL. The rHDLs were dialyzed against 3 \times 5 L phosphate-buffered saline (PBS) before use and were stored at 4°C. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) (AB Sciex, Framingham, MA, USA) was used to characterize the phospholipidome of rHDL particles as described elsewhere.¹⁷ Liposomes were prepared as previously described.^{18,19} Briefly, POPC and POPS were dissolved in chloroform at a concentration of 20 mM, and a mixture of lipids at a molar ratio of 70:30 was prepared. Following evaporation under nitrogen gas, the lipid film was hydrated using PBS at a temperature above the gel-liquid

crystal transition temperature of the phospholipids and sonicated for 10 min. Next, the solution was centrifuged to remove any residual large particles.^{18,19}

2.2 | Human macrophage isolation and cell culture

Human THP-1 monocytic cells were obtained from the American Type Culture Collection. THP-1 macrophage cells that had been stably knocked down for ABCA1 and ABCG1 were kindly provided by Prof. Wendy Jessup (Sydney, Australia).^{28,29} All cell lines were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, and 100 U/ml penicillin/streptomycin at 37°C in 5% CO₂. Stable knock-downs of ABCA1 and ABCG1 in THP-1 macrophages were selected using puromycin or blasticidin, respectively.²⁸ The absence of ABCA1 and ABCG1 gene expressions was verified using quantitative real-time polymerase chain reaction (RT-PCR) and Western blot analysis.

Monocytes were isolated from human blood of healthy normolipidemic donors using Ficoll density gradient (Etablissement Français du Sang, Paris, France). Human monocyte-derived macrophages (HMDM) were obtained after 10 days of differentiation on plastic Primaria plates (Falcon) in RPMI 1640 medium supplemented with 10% heat-inactivated human serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 20 ng/ml human macrophage colony-stimulating factor.³⁰

2.3 | Cell treatment and measurement of proinflammatory cytokine production

THP-1 cells were plated at a density of 1.0×10^6 cells/well into 24-well plates and were differentiated into macrophage-like cells with 81 nmol/L phorbol 12-myristate 13-acetate (PMA) for 3 days. Cells were washed two times with PBS to remove PMA-supplemented media and rested for 24 h in fresh complete media. HMDM cells were seeded at a density of 2.5×10^5 cells/well into 24-well plates. Cell culture media containing serum and pre-existing cytokines was aspirated, and the cells were washed twice before adding fresh serum-free medium with or without LPS (100 ng/ml) or TNF- α (8.5 pmol/L) and rHDLs at different concentrations. All the rHDL concentrations in the cell and animal studies were based on the apoA-I content of rHDLs, which was measured using the BCA method.

For pre-incubation experiments, cells were first exposed to a medium supplemented with rHDL at 357 nmol apoA-I/L, equivalent to 10 µg protein/ml, or with PBS

(vehicle control) for 16 h and were extensively washed before LPS (100 ng/ml) challenging for 5 h. For post-incubation experiments following LPS (100 ng/ml) stimulation for 4 h, cells were washed two times with PBS, and a medium with or without rHDL at 357 nmol apoA-I/L, equivalent to 10 µg protein/ml, was added. The supernatants were collected and centrifuged at 200 g for 5 min. Levels of interleukin (IL)-6 in cell culture studies were measured using an enzyme-linked immunosorbent assay (ELISA) kit from R&D System (Abingdon, UK and eBioscience, CA, USA), while in vitro levels of IL-1 β , TNF- α , vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1) were evaluated by Multiplex technology using a semi-automated Biochip Array Technology analyzer (Evidence Investigator, Randox, UK) as described elsewhere.³⁰

2.4 | RNA extraction, reverse-transcription, and quantitative PCR

Total RNA from THP-1 macrophages cultured in 24-well plates was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, PA, USA) according to the manufacturer's instructions after double washing with 500 µl of cold PBS. Then, 500 ng of RNA was reverse-transcribed to cDNA (Applied Biosystems, CA, USA). Relative quantification of target mRNA was performed using specific primers on the LightCycler 480 real-time instrument (Roche, IN, USA) and was normalized to the mean expression of three different housekeeping genes, including 5'-aminolevulinic synthase (ALA), α -tubulin (TUBA), and human heat shock protein alpha (cytosolic), class B member 1 (HSP90AB1). Data were expressed as a relative change in mRNA expression or a fold change compared to control values.

2.5 | Cellular cholesterol efflux capacity of reconstituted HDL

Human THP-1 macrophages were incubated for 24 h with 50 µg protein/ml of [³H]cholesterol-labeled acetylated LDL (acLDL) (1 µCi/ml) in serum-free RPMI-1640 supplemented with 50 mM glucose, 2 mM glutamine, 0.2% bovine serum albumin (RGGG), 100 units/ml penicillin and 100 µg/ml streptomycin. Following removal of the labeling medium, cells were equilibrated in RGGG for additional 24 h.²⁸ Cellular cholesterol efflux to rHDL (178 nmol-1.7 µmol apoA-I/L rHDL, equivalent to 5–50 µg protein/ml) was assessed in a serum-free medium for a 4-h chase period. Finally, culture media were harvested and cleared of cellular debris by brief centrifugation. Cell radioactivity was determined by the extraction in hexane-isopropanol

(3:2), evaporation of the solvent, and liquid scintillation counting on a Wallac Trilux 1450 Microbeta scintillation counter (PerkinElmer, MA, USA). The percentage of cholesterol efflux was calculated as a percentage ratio of the radioactivity released in the culture media to the total radioactivity in the cells plus medium.²⁸

2.6 | Quantification of lipid rafts

Lipid rafts were identified using Alexa Fluor 594 conjugated cholera toxin B (Molecular Probes) against the raft marker ganglioside GM1, as previously described.³¹

2.7 | Macrophage efferocytosis capacity in the presence of reconstituted HDL

Apoptosis was induced in Jurkat cells with camptothecin (10 μ M) for 4 h. Apoptotic Jurkat cells were then added to THP-1 cells (at a ratio of 3:1) previously incubated with rHDLs (5 μ g/ml) and TNF- α (150 ng/ml) for 8 h. Following 30 min coincubation of Jurkat and THP-1 cells, efferocytosis was assessed in washed cells by flow cytometry (LSR II FORTRESSA SORP, BD Biosciences, NJ, USA) as previously described.³⁰

2.8 | Silencing of SR-BI gene transcription in macrophages using small interference RNA

For SR-BI silencing experiments, THP-1 cells were cultured on 6-well plates (4×10^6 cells/well). Following a 72-h incubation with PMA, cells were transfected with 50 nM of SR-BI siRNA or a negative control siRNA (scrambled sequence, Dharmacon, Thermo Fisher Scientific, MA, USA) using Lipofectamine RNAiMax (Invitrogen, CA, USA) according to the manufacturer's instructions. After 8 h incubation, the cells were washed with PBS and further cultured in fresh media for 24 h. siRNA-transfected cells were then exposed to rHDLs for 8 h before analyzing IL-6 secretion in the supernatant and total RNA isolation from the cells.

To further evaluate the role of SR-BI for the effect of rHDL, human SR-BI-blocking antibody or control non-blocking antibodies (5 μ g/ml, Novus Biologicals, CO, USA) were added to the cells 30 min prior to adding rHDL.^{28,29,32}

2.9 | Akt1/2/3, p38 MAPK, and NF- κ B activity assays

THP-1 macrophages (1×10^6 cells per well in a 24-well plate) were incubated with rHDLs or PBS and TNF- α

(8.5 pmol/L) for 8 h. The supernatant was collected for measuring inflammatory cytokines, and cell lysates were evaluated for kinase activation. The levels of phosphorylated and total Akt1/2/3, p38 MAPK, and NF- κ B were measured using an Abcam Simple Step kit (Abcam, Cambridge, MA) with antibodies directed against phosphates at pS473, pT180/pT182, and p65 (pS536) in the phosphorylated kinases. Both affinity tag-labeled capture antibody and HRP-conjugated detection antibody cocktails were diluted and loaded simultaneously into the assay microplate along with cell lysates or standards. Following 1 h incubation at 37°C at 400 rpm agitation and a wash step, tetramethylbenzidine substrate solution was added and incubated for 15 min in the dark. The reaction was then stopped, and the absorbance was measured at 450 nm in a standard ELISA plate reader. A lysis buffer alone was used as a negative control to verify binding specificity of the antibodies. The sample values were calculated from standard curves, and the results were expressed as the phosphorylated/total kinase ratios. To determine whether differential anti-inflammatory effects of rHDLs were associated with altered signaling pathways, we tested the effect of selective chemical inhibitors in combination with rHDLs.

Western blotting analysis was performed to confirm the results of ELISA. Cells were washed first with cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were separated by electrophoresis on NuPAGE 4–12% Bis-Tris Protein Gels (Thermo Fisher Scientific) and then transferred onto nitrocellulose membrane (iBlot; Thermo Fisher Scientific) using a semi-dry system. Subsequently, the membrane was blocked, incubated with primary antibodies overnight and revealed with IR dye 800 nm-conjugated secondary antibodies. Infrared fluorescence was acquired on a Li-Cor scanner (Odyssey system, LI-COR Biosciences, Germany). Antibodies against phosphorylated and non-phosphorylated Akt1/2/3 and p38 MAPK were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.10 | Animals

The C57BL/6J background mice backcrossed more than 6 times were obtained from Jackson Laboratories. In vivo, low-grade chronic inflammation in the context of dyslipidemia and atherosclerosis was induced by a 10-week high-cholesterol diet in LDL-receptor knockout female mice. All the mice were at 7 weeks of age with an average weight of 22 g and average plasma cholesterol concentration of 697 ± 52 mg/dl ($n = 6$ for each group)

at the beginning of the diet. PC/PS- or PC-containing rHDLs were administered as three weekly retro-orbital injections of 40 mg protein/kg of body weight. Control mice received buffered saline injections. Blood samples were collected from each mouse one week after the last injection following an overnight fast. No effects of any treatment on body weight and plasma cholesterol levels were observed (data not shown). All animal experiments were approved by the local ethics committee for animal care. Levels of cytokines IL-6 and IL-12 p40 were measured in mouse plasma using Bioplex™ multiplexing bead immunoassays (Bio-Rad Laboratories, Hercules, CA, USA).

2.11 | Atherosclerotic plaque and tissue immunofluorescence

The animals were sacrificed under isoflurane anesthesia and perfused with sterile ice-cold PBS. The aorta was carefully dissected, embedded in optimum cutting temperature medium (OCT, Sakura Finetek Europe, Zoeterwoude, The Netherlands), and snap-frozen on a liquid nitrogen-precooled metal plate. Cryosections 10 μm in thickness were prepared for histological and immunohistochemical analyses.³³ Atherosclerotic lipid lesions on the aortic root were quantified on serial cross-sections as previously described.³³ The plaque area was measured with the AxioVision Zeiss software on the captured images (Zeiss AxioImager M2 microscope, Oberkochen, Germany). In addition, aorta sections were stained with the DAPI nuclear dye as well as with rat anti-Mac-2 (CD68), rabbit anti-total/phosphorylated-Akt1/2/3, and anti-total/phosphorylated p38 MAPK antibodies. All primary antibodies were purchased from Cell Signaling Technology except Mac-2 (CD68), which was obtained from BioRad (Marnes-la-Coquette, France). Fluorescein isothiocyanate (FITC, green) and Texas Red (red) goat-conjugated secondary antibodies were purchased from Santa Cruz (Burlingame, CA, USA) Biotechnology and Vector Laboratories (Santa Cruz, CA, USA), respectively. The images were analyzed for relative fluorescence intensity using ImageJ software (NIH, Bethesda, MD, USA). The baseline fluorescence of the negative (no primary antibody) sections was subtracted from the total fluorescence before calculating the relative fluorescence. An oval-shaped selection was positioned over the area of interest, and the mean intensity was measured within this selection. Samples with excessive amounts of background or high signal intensity, as determined by >3-fold higher than average signal intensity of all samples, were excluded from further analysis. At least three areas of six thoracic aorta sections harvested from six animals in each treatment group were analyzed.

2.12 | Statistical analyses

Data are shown as mean ± SEM. Each experiment was performed in triplicate, and values are shown for at least three independent experiments unless otherwise indicated. The normality of the data distribution was verified using the D'Agostino-Pearson or Kolmogorov-Smirnov test when appropriate. Comparisons between two groups were performed by a two-tailed Student's *t* test for normally distributed or Mann-Whitney *U* test for nonparametric data. An analysis of variance (ANOVA) was conducted to compare three or more groups with a Newman-Keuls post hoc correction for multiple pairwise comparisons. The IC₅₀ values were calculated using a non-linear regression analysis. All statistical analyses were performed using Prism software from GraphPad Prism 6.0 (San Diego, CA, USA).

3 | RESULTS

3.1 | Composition of reconstituted HDLs

LC/MS/MS analyses revealed a POPC:apoA-I molar ratio of 90:1 and a POPC:POPS:apoA-I molar ratio of 70:30:1 for rHDLs (Figure 1A). This composition is typical for rHDL prepared in vitro and close to lipid/protein molar ratios measured in human plasma HDL.²⁹ As rHDL particles did not contain a hydrophobic core, they were enriched in phospholipids relative to plasma HDL.³⁴ In addition, the PS content of PC/PS-rHDL was elevated as compared to that detected in plasma HDL particles¹⁷; the former thereby represented a proof-of-principle model of native HDL.

3.2 | Phosphatidylserine incorporation enhances anti-inflammatory effects of rHDL

Both LPS and TNF-α treatment induced increases in several inflammatory biomarkers in differentiated THP-1 cells. To determine the influence of rHDL co-incubation with LPS or TNF-α on the inflammatory response of macrophages, concentrations of IL-6, which is known to contribute to atherosclerosis via atherosclerotic plaque formation, progression, and destabilization,^{35,36} were measured in the culture media of activated THP-1 and HMDM cells in the absence and presence of rHDLs.

As shown in Figure 1, both PC-rHDL and PC/PS-rHDL suppressed LPS- or TNF-α-induced IL-6 secretion from differentiated THP-1 cells in a dose-dependent manner.

The effect of PC/PS-rHDL on IL-6 secretion upon LPS stimulation was significantly stronger as compared with that of PC-rHDL (−54% vs. −42% and −67% vs. −58% at a

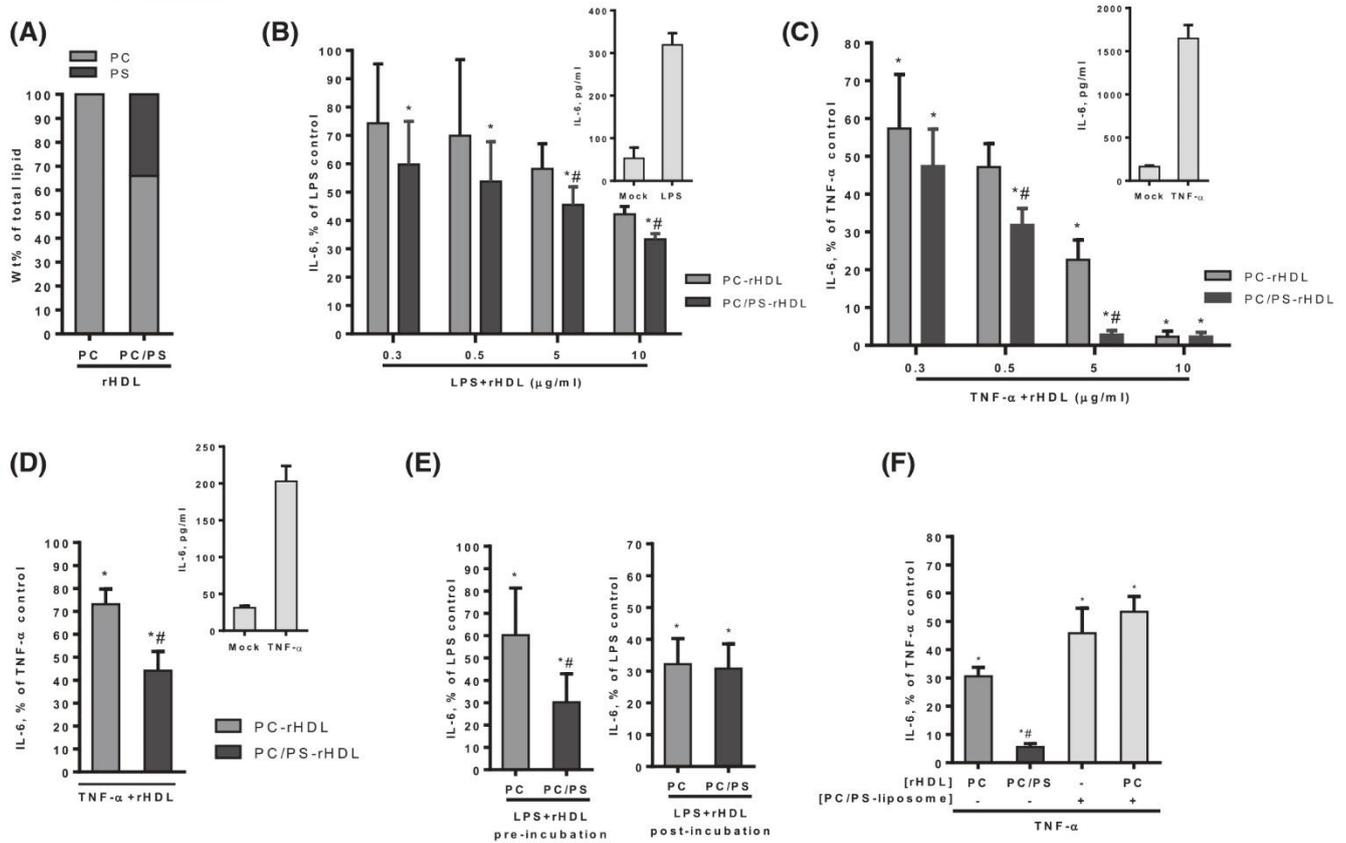


FIGURE 1 PS incorporation enhances rHDL-mediated inhibition of IL-6 secretion induced by LPS or TNF- α in macrophages.

Composition of the reconstituted HDLs (A). Discoidal rHDLs were prepared by cholate dialysis from a mixture of apoA-I isolated from human plasma and synthetic PC 16:0/18:1 with or without synthetic PS 16:0/18:1 at a molar ratio of 1:90 for PC-rHDL and 1:70:30 for PC/PS-rHDL. LC/MS/MS was used to characterize the phospholipidome of rHDLs. Wt: weight. THP-1 cells that had differentiated into macrophages were treated with either PC-rHDL or PC/PS-rHDL at varying concentrations ranging from 0.01–0.9 μ mol apoA-I/L (equivalent to 0.3–10 μ g protein/ml) during LPS (100 ng/ml)-induced stimulation (B) or TNF- α (8.5 pmol/L)-induced stimulation (C) for 8 h. HMDM cells were stimulated with TNF- α at 8.5 pmol/L and treated with PC- or PC/PS-rHDL at 5 μ g protein/ml for 8 h (D). In (B–D), insets show absolute levels of IL-6 in cell media. Concentrations of IL-6 measured at baseline and after LPS stimulation were 53 ± 61 and 318 ± 67 pg/ml, respectively (6-fold induction, $p < .001$); concentrations measured at baseline and after TNF- α stimulation were 163 ± 33 and 1648 ± 380 pg/ml (10-fold induction, $p < .001$) in THP-1 cells and 30 ± 5 and 203 ± 43 pg/ml in HMDM cells respectively (6-fold induction, $p < .001$). The cells were pre-incubated before stimulation with LPS (100 ng/ml) or post-incubated after stimulation with LPS (100 ng/ml) with either PC-rHDL or PC/PS-rHDL at 357 nmol apoA-I/L, equivalent to 10 μ g protein/ml (E). For liposome experiments (F), differentiated cells were treated with PC-rHDL or PC/PS-rHDL at 5 μ g protein/ml (178 nmol apoA-I/L) alone or 6.7 μ M phospholipid/L equivalent to 5.13 μ g phospholipid/ml of PC/PS-rHDL, in addition to 5.13 μ g phospholipid/ml of PC/PS-liposomes so that the total phospholipid concentration was equal to the final concentration of phospholipid present in 5 μ g protein/ml of PC/PS-rHDL for 8 h. Data are presented as mean \pm SEM of percentage change from three independent experiments performed in duplicate; * $p < .05$ vs. no rHDL; # $p < .05$ vs. PC-rHDL

concentration of 5 and 10 μ g protein/ml equivalent to 178 and 357 nmol apoA-I/L, respectively, Figure 1B). In TNF- α -stimulated macrophages, the presence of PC/PS-rHDL at 0.5 μ g protein/ml (17.8 nmol apoA-I/L) and 5 μ g protein/ml (178 nmol apoA-I/L) inhibited IL-6 secretion significantly more effectively as compared to PC-rHDL (–68% vs. –53% and –95% vs. –72%, respectively, Figure 1C). Remarkably, IC₅₀ values were 1.9- and 1.8-fold lower for PC/PS-rHDL relative to PC-rHDL treatment in LPS (3.1 vs. 6.0 μ g/ml) and TNF- α (0.25 vs. 0.46 μ g/ml)-stimulated THP-1 cells, respectively, consistent with enhanced anti-inflammatory activity of PC/PS-rHDL particles. In addition, these data

demonstrated enhanced potency of rHDLs toward TNF- α vs. LPS-induced macrophage activation.

The elevated anti-inflammatory activity of PC/PS-rHDL versus PC-rHDL observed in THP-1 macrophages was confirmed in primary HMDM cells. Indeed, PC/PS-rHDL attenuated TNF- α -induced IL-6 secretion by HMDM significantly stronger as compared to PC-rHDL (–56% vs. –27%, respectively, at 5 μ g protein/ml, $p < .05$; Figure 1D), a difference which was comparable to that observed in THP-1 macrophages (Figure 1C).

To evaluate whether direct interaction with LPS was required for the inhibitory effects of rHDL, similar

experiments were performed involving pre-incubation or post-incubation of cells with rHDL in the absence of LPS. As in the co-incubation experiments, a shift to lower IL-6 concentrations was observed when the cells were pre-incubated with PC/PS-rHDL as compared to pre-incubations with PC-rHDL (Figure 1E). However, this difference was not observed in post-incubation experiments when the cells were first activated with LPS in the absence of rHDL (Figure 1E).

To further determine whether the anti-inflammatory effects of PC/PS-rHDL were associated with PS, we used PC/PS-liposomes possessing the same molar phospholipid ratio as rHDLs (PC:PS at 70:30 mol/mol). As shown in Figure 1F, PC/PS liposomes significantly attenuated TNF- α -induced IL-6 secretion. However, this effect was markedly weaker than that of PC/PS-rHDL, suggesting that apoA-I conveyed enhanced anti-inflammatory properties to PS. To assess if a simple additive effect of apoA-I and PS took place, PC/PS-liposomes were added to PC-rHDL at a final concentration equal to that of the phospholipids present in the incubations with PC/PS-rHDL. As shown in Figure 1F, co-incubation with PC/PS-liposomes did not enhance the anti-inflammatory effect of PC-rHDL. One can speculate that the lack of the additive effect of PC/PS-liposomes and PC-rHDL on the suppression of IL-6 secretion by THP-1 cells could reflect both liposomal and lipoprotein remodeling in the mixture. Indeed, it is well established that co-incubation of rHDL and liposomes results in their extensive remodeling.³⁷

Cytokine analysis revealed that secretion of IL-1 β (–71% vs. –42%), TNF- α (–98% vs. –79%), VEGF (–40% vs. 14%), and MCP-1 (–21% vs. –76%) in the macrophage culture medium was significantly reduced upon treatment with PC/PS-rHDLs as compared to PC-rHDL, resulting in the enhanced anti-inflammatory activity of PC/PS-rHDL of 29%, 19%, 26%, and 55%, respectively (Figure 2A–D). In parallel, three weekly injections of PC/PS-rHDLs in a dyslipidemic *Ldlr*^{–/–} mouse model fed a high-cholesterol diet resulted in a significant reduction of plasma levels of IL-6 and IL-12 p40 (–36% and –24%, respectively) as compared to mice injected with PC-rHDL (Figure 2E,F). By contrast, no alteration of plasma levels of these cytokines was observed in the PBS-treated group relative to the baseline (data not shown).

The anti-inflammatory effects of rHDL were consistently stronger toward TNF- α relative to LPS-induced stimulation (Figure 1B vs. Figure 1C). Since elevated production of TNF- α is pivotal in triggering inflammatory cascades associated with vascular pathophysiology and atherosclerosis,³⁵ subsequent experiments predominantly focused on macrophages challenged by TNF- α . In an effort to better characterize the effects of rHDL on the inflammatory response of macrophages, we analyzed gene expression of selected cytokines. The mRNA levels of both IL-6 (–97% vs. –83%, Figure 3A) and TNF- α (–95% vs. –65%, Figure 3B) were more strongly decreased by

PC/PS- than by PC-rHDL upon TNF- α stimulation (by –14% and –30%, respectively). Similarly, a greater decrease in the expression of IL-6 (–63% vs. –23%, Figure 3D) and TNF- α (–79% vs. –58%, Figure 3E) was observed upon LPS stimulation by PC/PS- than by PC-rHDL (by –40% and –21%, respectively). Consistent with enhanced anti-inflammatory activity of rHDL containing PS, PC/PS-rHDL slightly but significantly enhanced expression of anti-inflammatory cytokine IL-10 (+9%, $p < .05$), while PC-rHDL reduced it (–57%, $p < .01$; Figure 3C) during stimulation with TNF- α . As a result, PC/PS-rHDL was a 1.5-fold more potent activator of IL-10 expression than PC-rHDL ($p < .01$) in this setting. Further along this line, PC/PS-rHDL less markedly inhibited IL-10 expression as compared to PC-rHDL (–62% vs. –75%, respectively) in LPS-stimulated macrophages (–13% vs. PC-rHDL, $p < .01$, Figure 3F). Finally, expression of anti-inflammatory transforming growth factor- β (TGF- β , Figure 3G) and activating transcription factor 3 (ATF3, Figure 3H) was not affected by PC/PS-rHDL but decreased slightly by PC-rHDL (–11% and –12%, respectively, $p < .05$) upon TNF- α stimulation, resulting in the enhanced expression of the both genes in the presence of PC/PS- vs PC-rHDL.

3.3 | Phosphatidylserine incorporation mildly induces ABCA1 and ABCG1 gene expression and weakly modifies the cholesterol efflux capacity of rHDL

To test whether the enhanced anti-inflammatory effects of PC/PS-rHDL were related to accelerated lipid efflux from cells, we performed a cholesterol efflux assay with [³H]-cholesterol. This assay showed only a trend (+19% as compared to PC-rHDL, $p < .16$, at 0.5 μ mol/L, equivalent to 15 μ g protein/ml) toward increased cholesterol efflux capacity upon the incorporation of PS into PC-rHDL (Figure 4A). In addition, stable knockdown of ABCA1 and downregulation of SR-BI reduced cellular cholesterol efflux to PC/PS-rHDL to the same extent as that to PC-rHDL (data not shown). Formation of plasma membrane lipid rafts was studied in the presence of rHDL due to their implication in the inflammatory response of activated macrophages.³¹ Exposure to PC/PS-rHDL decreased the formation of plasma membrane lipid rafts as compared to PC-rHDL treatment (–12%, $p = .04$; Figure 4B), consistent with stronger anti-inflammatory effects of PC/PS-rHDL toward macrophages. In addition, PC-rHDL significantly accelerated efferocytosis of apoptotic Jurkat cells by THP-1 macrophages, whereas PC/PS-rHDL significantly reduced it. (Figure 4C).

Finally, quantification of gene expression did not reveal any effect of rHDLs on SR-BI mRNA levels in TNF- α -activated cells (Figure 5A). By contrast, expression of both ABCG1 and ABCA1 was significantly reduced by both PC- and PC/PS-rHDL; elevated ABCG1 mRNA expression

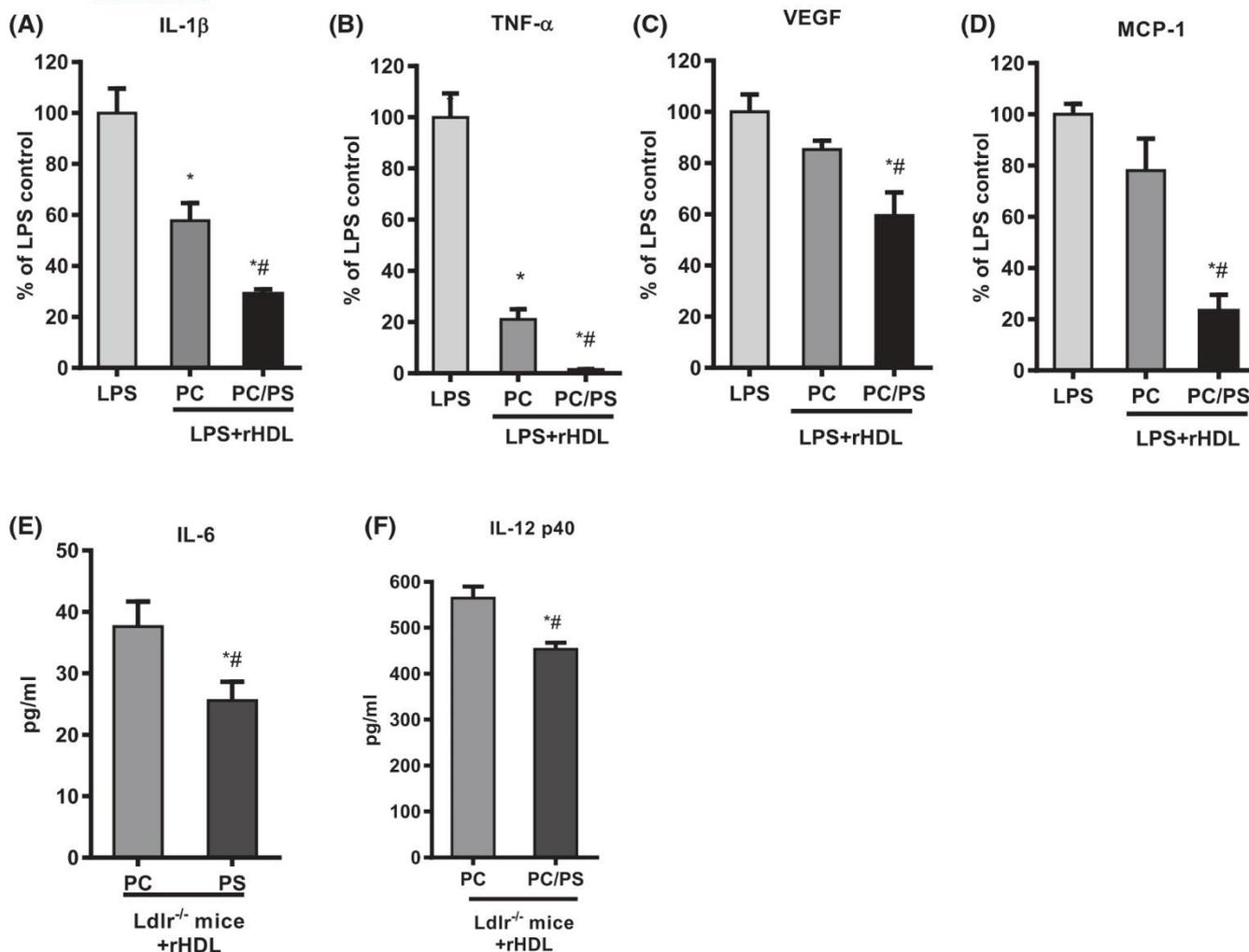


FIGURE 2 PS incorporation enhances rHDL-induced inhibition of cytokine secretion in macrophages and decreases cytokine levels in mouse plasma. THP-1 cells differentiated into macrophages were treated with either PC-rHDL or PC/PS-rHDL at a concentration of 5 μ g protein/ml (178 nmol apoA-I/L) during stimulation with LPS and cytokine levels of IL-1 β (A), TNF- α (B), VEGF (C) and MCP-1 (D) were measured. Baseline cytokine production and induction levels were as follows: IL-1 β , 12.6 \pm 0.8 and 44 \pm 7 pg/ml, respectively (3.5-fold induction, $p < .02$); TNF- α , 2 \pm 2 and 620 \pm 388 pg/ml, respectively (310-fold induction, $p < .001$); VEGF, 128 \pm 33 and 242 \pm 158 pg/ml, respectively (1.9-fold induction), and MCP-1, 41 \pm 4 and 171 \pm 9.8 pg/ml, respectively, (4-fold induction, $p < .003$). LDL receptor knock-out (*Ldlr*^{-/-}) mice were fed a high-cholesterol diet, PC- or PC/PS-rHDLs or PBS as a vehicle was administered as three weekly retro-orbital injections of 40 mg protein/kg, as described in the Methods section and plasma levels of IL-6 (E) and IL-12 p40 (F) were measured. Data are presented as mean \pm SEM of the percentage change from at least three independent experiments performed in duplicate; * $p < .05$ vs. no rHDL; # $p < .05$ vs. PC-rHDL

(1.30-fold, $p < .01$, Figure 5B) and a trend toward elevated ABCA1 mRNA expression (1.11-fold, $p = .10$, Figure 5C) were found in the presence of PC/PS- relative to PC-rHDL.

3.4 | Phosphatidylserine incorporation enhances rHDL-mediated anti-inflammatory effects through SR-BI receptor

The cell surface receptor SR-BI and ABCA1 and ABCG1 transporters play major roles in mediating HDL

interactions with cells. Therefore, we tested whether these membrane proteins were involved in the enhanced anti-inflammatory activity of PC/PS-rHDL in macrophages. As shown in Figure 5D, mRNA levels of SR-BI measured 48 h after transfection with siRNA targeting SR-BI were reduced by 70% as compared with cells transfected with control scrambled siRNA. Differences between inhibitory effects of PC/PS- and PC-rHDL on IL-6 secretion observed in control cells (Figure 1B,C) were negligible in both anti-SR-BI blocking antibody-treated cells (Figure 5E) and SR-BI knockdown cells (Figure 5F). The slightly diminished inhibitory effects of

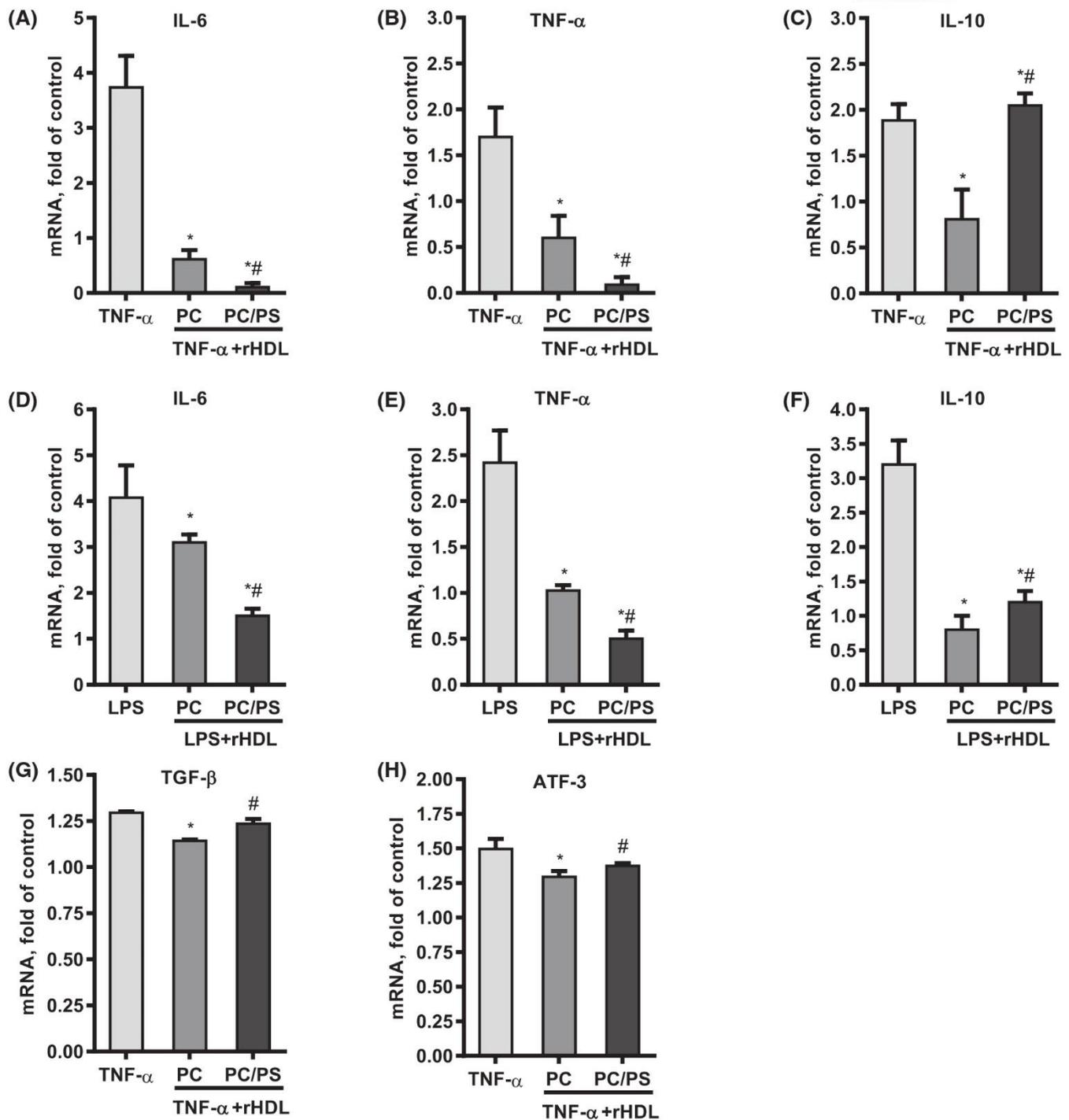


FIGURE 3 PS incorporation improves rHDL-mediated restoration of LPS- and TNF- α -induced alterations in the cytokine expression profile of macrophages. THP-1 cells that had differentiated into macrophages were treated with either PC-rHDL or PC/PS-rHDL at 178 nmol protein/L equivalent to 5 μ g protein/ml during stimulation with TNF- α (8.5 pmol/L) or LPS (100 ng/ml) for 8 h. Levels of IL-6, TNF- α , and IL-10 gene expression are shown in A–C for TNF- α -stimulated and D–F for LPS-stimulated cells, while those of TGF- β and ATF-3 are shown in G and H for TNF- α stimulation. Data are presented as the mRNA ratio relative to the mean of three housekeeping gene expression and are shown as mean \pm SEM from three independent experiments performed in duplicate; * p < .05 vs. no rHDL, # p < .05 vs. PC-rHDL

PC/PS-rHDL relative to PC-rHDL observed in this set of experiments may reflect off-target effects of the unspecific antibody and scrambled siRNA.

In addition, we used stable ABCA1 and ABCG1 knockdown cells to test whether ABCA1 and ABCG1 downregulation

altered anti-inflammatory activity of PC/PS-rHDL. Figure 5G,H shows that PC/PS-rHDL was still able to suppress TNF- α -induced IL-6 secretion more strongly as compared to PC-rHDL in the stable ABCA1 and ABCG1 knockdown cells.

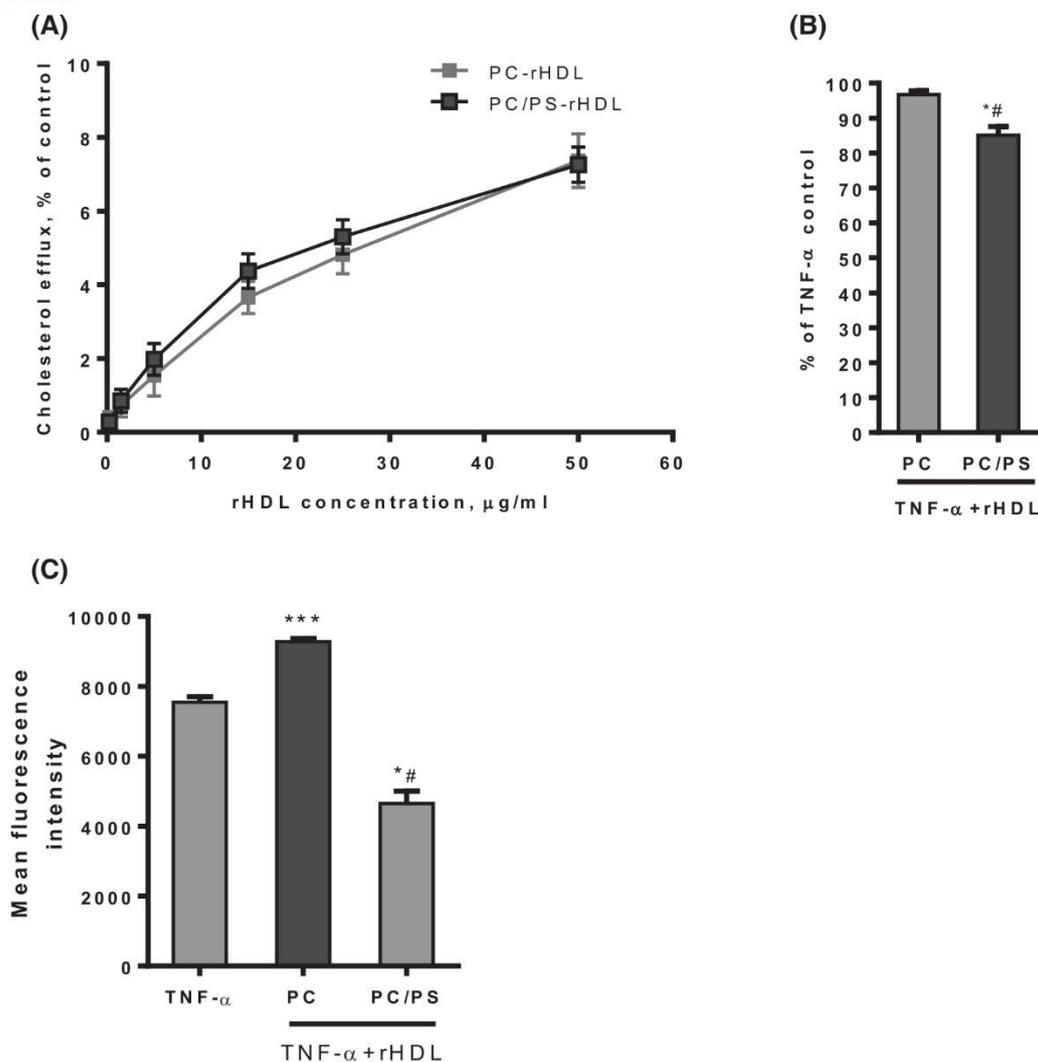


FIGURE 4 PS incorporation weakly modifies rHDL-mediated cholesterol efflux from macrophages and reduces abundance of plasma membrane lipid rafts as well as efferocytosis of apoptotic cells. Cellular [^3H]-cholesterol efflux to rHDLs (A), plasma membrane lipid raft formation (B), and macrophagic efferocytosis capacity in the presence of HDL (C) were measured in PC-rHDL- or PC/PS-rHDL-treated THP-1 cells. In the lipid raft and efferocytosis assays, cells were treated with 178 nmol apoA-I/L equivalent to 5 µg of protein/ml rHDLs during TNF- α -induced stimulation at 8.5 pmol/L for 8 h. Data are presented as mean \pm SEM of the percentage change relative to control incubations without rHDL obtained from three independent experiments performed in duplicate; * $p < .05$ vs. no rHDL, *** $p < .001$ vs. no rHDL, # $p < .05$ vs. PC-rHDL

3.5 | Phosphatidylserine incorporation modulates rHDL-mediated intracellular signaling but not lesion area

Our results, therefore, suggested that PS-containing rHDL induced a pattern of intracellular signaling that differed from that of PC-rHDL. Therefore, relative abundance of phosphorylated forms of Akt1/2/3, p38 MAPK, and NF- κ B was analyzed in cellular extracts. We also attempted to evaluate the role of ASK1 and JNK phosphorylation but did not detect phosphorylated forms of these proteins under our experimental conditions (data not shown). We found that PC/PS-rHDL induced a greater increase

in Akt1/2/3 phosphorylation (1.5-fold, $p = .02$) than PC-rHDL (Figure 6A,B). In addition, PC/PS-, but not PC-, rHDL significantly decreased p38 MAPK phosphorylation in THP-1 cells activated by TNF- α (-21% , $p = .01$; Figure 6C,D). However, both PC- and PC/PS-rHDL decreased NF- κ B phosphorylation to a similar extent (approximately -70% of the control, Figure 6E). In order to determine functional role of these signaling pathways in the rHDL-mediated suppression of inflammatory activation, Akt and p38 MAPK were blocked with specific inhibitors. As shown in Figure 6F, the PI3K/Akt pathway inhibitor MK2206 and the p38-MAPK inhibitor SB203580 did not appear to interfere with the rHDL-mediated

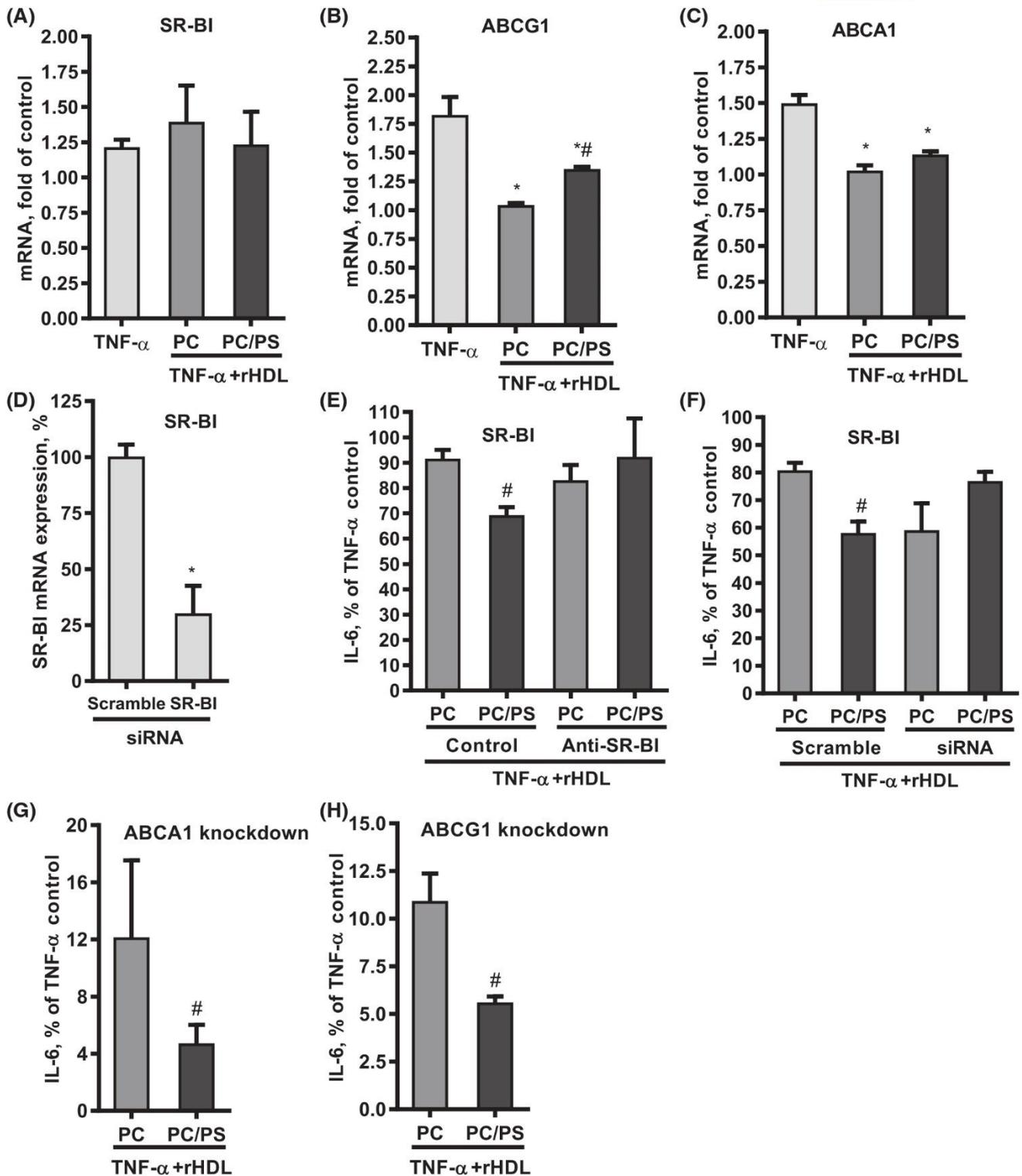


FIGURE 5 SR-BI but not ABCA1 and ABCG1 inhibition attenuates inhibitory effects toward IL-6 secretion induced by PS-containing rHDL in macrophages. Gene expression levels of SR-BI (A), ABCG1 (B) and ABCA1 (C) were measured in differentiated THP-1 cells. IL-6 levels were measured in culture media of THP-1 cells transfected with scrambled or SR-BI siRNA (D and F) or incubated with an anti-SR-BI antibody or an unspecific non-blocking antibody as a control (E) as well as of stable ABCG1- (G) and ABCA1-deficient knockout (KO) (H) cells treated with either PC-rHDL or PC/PS-rHDL at 178 nmol apoA-I/L, equivalent to 5 μ g protein/ml during TNF- α -induced stimulation at 8.5 pmol/L for 8 h. Gene expression levels of SR-BI were measured using quantitative real-time polymerase chain reaction and presented as the mRNA ratio relative to the mean of three housekeeping gene expression shown as a percentage of the expression obtained for the negative control scrambled siRNA (D). Data are presented as mean \pm SEM of the percentage change from three independent experiments performed in duplicate; * p < .05 vs. no rHDL (B, C) or no siRNA (D), # p < .05 vs. PC-rHDL

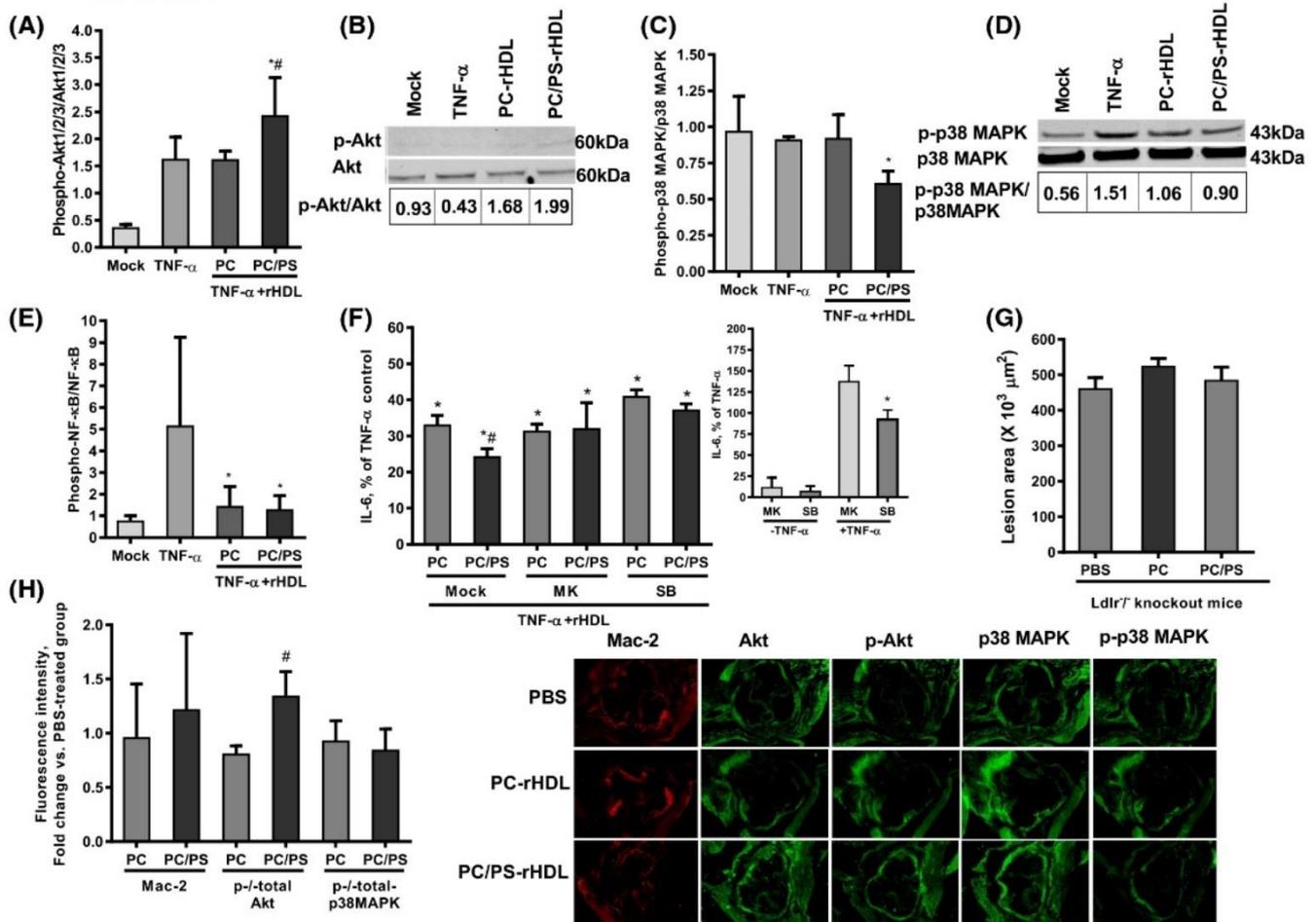


FIGURE 6 Incorporation of PS into rHDL induces intracellular signaling through Akt-dependent pathway while attenuating signalling through p38 MAPK-dependent pathway in macrophages. Differentiated THP-1 cells were treated with either PC-rHDL or PC/PS-rHDL at 178 nmol apoA-I/L equivalent to 5 μg protein/ml alone or in combination with chemical inhibitors during TNF- α stimulation at 8.5 pmol/L for 8 h. Levels of total and phosphorylated p-Akt1/2/3 were measured using enzyme-linked immunosorbent assay (A) and confirmed by Western blotting as shown in the representative images of total (t) and phosphorylated (p) proteins (B). Levels of total and phosphorylated p38 MAPK were measured using ELISA (C) and were confirmed by Western blotting as shown in the representative images of total (t) and phosphorylated (p) proteins (D). The levels of total and phosphorylated NF- κ B were measured using enzyme-linked immunosorbent assay (E). IL-6 concentrations in the culture media were evaluated after treatment with 0.1- μM PI3K/Akt pathway inhibitor MK2206 (MK) and 5- μM p38-MAPK inhibitor SB203580 (SB) (F). Atherosclerotic lesion area (G) and immunofluorescence images of mouse aortic sections (H) were quantified in *Ldlr*^{-/-} mouse model fed a high-cholesterol diet. Mac-2 (red) and phosphorylated/total Akt1/2/3 (Akt/p-Akt) and p38 MAPK (p38/p-p38) (green) staining are shown in sections from control (PBS-treated) and rHDL-treated mice at 20 \times magnification. Data are presented as mean \pm SEM of the percentage or fold change from at least three independent in vitro experiments performed in duplicate, or from 18 aortic sections produced using 6 mice in each group; * $p < .05$ vs. no HDL, # $p < .05$ vs. PC-rHDL

inhibition of IL-6 secretion. However, both inhibitors negated the enhanced IL-6-suppressive effect of PC/PS- vs. PC-rHDL (Figure 6F).

Ex vivo analysis of atherosclerotic lesions in *Ldlr*^{-/-} mice showed that PC-rHDL or PC/PS-rHDL infusions did not affect surface area of the lesions (Figure 6G). Immunofluorescence of mouse aortic tissue sections for Mac-2 showed that the administration of PC/PS-rHDL to *Ldlr*^{-/-} mice did not significantly change aortic macrophage infiltration at day 30 as compared to animals treated with PC-rHDL (Figure 6H). However, the PC/

PS-rHDL mouse group showed a significant increase in the ratio of phosphorylated to total Akt1/2/3 (1.66-fold, $p = .024$) compared with the PC-rHDL group; no significant change was observed in the ratio of phosphorylated to total p38 MAPK levels (Figure 6H).

4 | DISCUSSION

Novel rHDL particles enriched in PS were formulated to explore their anti-inflammatory activity and,

subsequently, their mechanisms of action. Our data revealed that the incorporation of PS into rHDLs enhanced their anti-inflammatory effects in TNF- α - or LPS-challenged macrophages in vitro by inhibiting pro-inflammatory cytokine secretion and expression. Similar results documenting superior anti-inflammatory properties of PC/PS-rHDL relative to PC-rHDL were obtained in *Ldlr*^{-/-} mice fed a high-cholesterol diet in vivo. Of note, macrophagic SR-BI was determined to be involved in the potent anti-inflammatory effects of PC/PS-rHDL coupled with the Akt1/2/3- and p38 MAPK-mediated intracellular signaling pathways.

Recent data demonstrate clinical benefits of anti-inflammatory agents, such as an antibody targeting interleukin-1 β , in reducing cardiovascular events beyond effects achieved by lipid lowering alone.³⁸ HDL may play a key role in the development of atherosclerosis through its capacity to inhibit inflammation in endothelial cells¹² and to mediate reverse cholesterol transport by promoting cholesterol efflux from macrophages. Both native HDL and apoA-I isolated from healthy volunteers display anti-inflammatory effects when incubated with macrophages.¹³⁻¹⁵ However, the effects of HDL on the inflammatory response of macrophages may vary as a function of differences in the basal and activated cellular signaling pathways. In addition, effects on a given inflammatory state can differ depending on experimental settings and macrophage types. Indeed, pro-inflammatory effects of HDL were observed in murine and human primary macrophages at relatively high concentrations (>100 μ g protein/ml), which were attributed to passive cholesterol depletion.³⁹ Thus, the anti-inflammatory function of HDL toward macrophages remains inconclusive.

Our results showed that both PC/PS- and PC-rHDL formulations displayed dose-dependent anti-inflammatory effects while suppressing production of inflammatory cytokines upon proinflammatory stimulation in human THP-1 macrophages. These findings rule out the possibility of non-specific neutralization of LPS by rHDLs since TNF- α is a proinflammatory protein that lacks the lipid moiety underlying LPS interaction with HDL. In addition, anti-inflammatory effects of rHDLs added before LPS stimulation further support the notion of specific signaling events rather than non-specific LPS capturing by rHDLs. The lack of such interaction can reflect the absence of LPS-binding protein from rHDLs, which is shown to be responsible for the neutralization of LPS by natural HDL.^{40,41}

Interestingly, the degree of the inhibitory effects of rHDLs was more pronounced in TNF- α - than in LPS-stimulated macrophages. A similar observation was reported for the inhibition of cytokine secretion by human aortic endothelial cells in the presence of HDL mimetic

CER-001.⁴² Since TNF- α -stimulated cells appear to represent a better model of chronic inflammatory response in the context of atherogenesis,⁴³ this in vitro setting may be more relevant to atherosclerosis-related inflammation. Overall, TNF- α was chosen by us as a non-lipid proinflammatory stimulator because of its biological relevance in the context of atherosclerosis and also the enhanced anti-inflammatory response observed in the presence of PC/PS relative to PC-rHDL.

In addition to the marked suppression of proinflammatory cytokines by PC/PS-rHDL compared to the typical PC-rHDL formulation and PC/PS liposomes, we observed a significant induction or preservation of anti-inflammatory cytokine expression with PC/PS-rHDL treatment. PS-mediated recognition and subsequent uptake of PC/PS-rHDL by macrophages can be envisaged as a possible mechanism that contributes to enhanced IL-10 secretion. According to the literature, PS (but not PC)-containing liposomes can mimic beneficial effects of apoptotic bodies in resolving inflammation.²⁰ Therefore, PS-mediated recognition and consequent activation of macrophagic apoptotic machinery can result in the reduction of inflammation as well as the induction of anti-inflammatory cytokine secretion. Consistent with this suggestion, we observed reduced efferocytosis of apoptotic cells in the presence of PC/PS-rHDL, which potentially reflected saturation of cellular PS-receptors and/or PS-triggered signaling pathways by the PS-containing particles. According to our results, combination of apoA-I and PS within a single HDL particle produced the strongest anti-inflammatory effects. Thus, it can be hypothesized that apoA-I and PS act synergistically to provide reconstituted particles possessing potent anti-inflammatory activities.

PS-expressing apoptotic cells were reported to upregulate ABCA1 expression in macrophages,^{44,45} a phenomenon required for macrophage cholesterol homeostasis. However, as such upregulation is accompanied by accelerated cholesterol efflux to apoA-I but not to HDL, it was suggested that PS-expressing apoptotic cells can only promote apoA-I-specific cholesterol efflux.⁴⁵ Our results indicated that cellular cholesterol efflux was not different in the presence of PC/PS- and PC-rHDL. In addition, earlier data indicated that ABCG1 was not essential for cholesterol efflux from human macrophagic foam cells.²⁸

Our results suggest that the enhanced anti-inflammatory effects of PS-containing rHDL did not depend on ABCA1 and ABCG1, as such effects were not affected by the silencing of these transporters. In contrast, PS failed to enhance the anti-inflammatory function of PC-rHDL in SR-BI-silenced cells or in cells incubated with an anti-SR-BI antibody. These findings are consistent with the notion that SR-BI functions as a PS receptor for the recognition

and clearance of apoptotic cells.⁴⁶ Accordingly, SR-BI-deficient macrophages display a proinflammatory phenotype.⁴⁶ As a result, PS-containing particles are able to compete with the SR-BI-mediated binding of apoptotic cells to phagocytic cells.^{47,48} As a negatively charged phospholipid constituent of these particles, PS can ensure such binding through electrostatic interactions with SR-BI.^{49,50}

Studies of HDL-induced signaling cascades typically focus on acute effects over the course of minutes. However, ultimate challenge of HDL-based therapy is to achieve a new equilibrium by creating persisting anti-atherosclerotic effects. A delayed response to HDL was evidenced by studies that investigated transcription of cytokines, nuclear receptors, and scavenger receptors,^{51–53} as well as using phosphorylation assays.⁵⁰ To identify molecular mechanisms underlying the enhanced anti-inflammatory effects of PS-containing rHDLs, we examined the delayed effect of rHDL on the phosphorylation of activation sites on Akt1/2/3, p38 MAPKs, and NF- κ B. Our results demonstrated that PS incorporation increased the Akt1/2/3 activation induced by rHDL and reduced p38 MAPKs phosphorylation accompanied by reduced lipid raft formation in stimulated monocytic cells. However, there was no further decrease in NF- κ B phosphorylation with PS-containing rHDL particles relative to PC-rHDL. PS-containing rHDLs also increased the Akt1/2/3 activation in vivo. These data indicate that the incorporation of PS into rHDLs bear a potential to promote their anti-inflammatory effects in aortic plaques. These effects may be particularly relevant in atherosclerosis associated with chronic inflammation when macrophage infiltration is prominent. Treatment with a PI3K/Akt inhibitor and a p38 MAPK inhibitor abolished differences in the anti-inflammatory effects of PC- and PC/PS-rHDL. These results suggest that the enhanced effects of PC/PS-rHDL are mediated by the Akt- and p38 MAPK-dependent pathways. Our findings are, therefore, consistent with those documenting that the activation of the PI3K/Akt signaling pathway leads to anti-inflammatory responses.⁵⁴ Indeed, Akt and ATF3 can function as upstream negative regulators of NF- κ B activation.^{55,56} Furthermore, ATF3, a transcriptional repressor of proinflammatory cytokines,⁵⁷ negatively regulates the transcriptional activity of NF- κ B.⁵⁸ According to our results, PS-containing rHDL may be particularly protective against inflammation because of its enhanced potential in preserving ATF3 and TGF- β activity in macrophages.

It remains to be shown whether the capacity of HDL to reduce inflammation can be translated into reduced atherogenesis in vivo. In our studies, we did not observe any inhibitory effect of PS-containing rHDL on atherosclerotic lesion area in Ldlr^{-/-} mice fed a high cholesterol diet. As PC-rHDLs were equally ineffective in this experimental setting, these data are consistent with an extreme character

of atherogenesis in this model, which can be too harsh for the clinical relevance of anti-inflammatory effects of rHDL. Further experiments performed under milder proatherosclerotic conditions are required to address this controversy.

In conclusion, incorporation of PS into rHDLs improves their anti-inflammatory activity by modulating Akt1/2/3- and p38 MAPK-mediated signaling through the SR-BI receptor in stimulated macrophages. These data identify PS as a potent anti-inflammatory component capable of enhancing therapeutic potential of rHDL-based therapy. Our results, therefore, provide a biochemical basis for the development of highly functional PS-based rHDL formulations to reduce excessive cardiovascular risk in patients presenting with a proinflammatory phenotype, such as acute myocardial infarction and stroke.

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DISCLOSURES

Dr Anatol Kontush has received a research grant from CSL (Australia) and is a co-author of a patent on the enhancement of HDL function by PS.

AUTHOR CONTRIBUTIONS

Anatol Kontush, Wilfried Le Goff, and Maryam Darabi designed the research. Maryam Darabi, Eric Frisdal, Marie Lhomme, Lucie Poupel, Emilie Tubeuf, Veronica D. Dahik, and Wilfried Le Goff performed the research. Maryam Darabi, Anatol Kontush and Wilfried Le Goff wrote the paper. Maryse Guerin, Kerry-Anne Rye, Philippe Lesnik, Mili Patel, Isabelle Guillas, Emmanuel L. Gautier and Thierry Huby contributed new reagents or analytic tools.

DATA AVAILABILITY STATEMENT

Data will be available upon reasonable request.

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ABSTRACT (FRENCH VERSION)

Les macrophages du tissu adipeux (MTA) sont des acteurs majeurs dans l'inflammation et l'insulinorésistance du tissu adipeux au cours de l'obésité induite par le régime. Le métabolisme intrinsèque des MTA est fortement lié à leur phénotype, qui est lui-même modulé par le flux constant des lipides caractéristique des états obèses. Les mécanismes gouvernant ces changements phénotypiques dans les MTA au cours de l'obésité sont cependant mal connus. Nos études montrent que le transporteur membranaire Atp-binding cassette g1 (Abcg1) est impliqué dans le contrôle de la réponse des MTA aux acides gras (FA). L'inactivation de ce transporteur dans la lignée myéloïde des souris soumises à un régime gras améliore leur état inflammatoire et diminue leur résistance à l'insuline. Les MTA issus de ces souris présentent un phénotype moins inflammatoire accompagné par l'acquisition d'un profil bioénergétique réduit et la modification de leur métabolisme lipidique. En effet, l'analyse du lipidome des MTA déficients pour Abcg1 montre une redirection des acides gras (AG) saturés au sein des phospholipides membranaires vers les gouttelettes lipidiques. Cela conduit à une diminution de la rigidité membranaire et à la neutralisation des AG pro-inflammatoires. Cette même relation entre le niveau d'expression d'Abcg1 et l'état inflammatoire des MTA est également observée dans des MTA humains issus de patients obèses. Le phénotype anti-inflammatoire associé à l'inhibition d'Abcg1 est aboli par l'inhibition de l'activité de la lipoprotéine lipase (Lpl) soulignant l'importance de l'axe Abcg1/Lpl dans le contrôle de l'inflammation métabolique dans les MTA. Ces travaux mettent en évidence le rôle majeur du transporteur Abcg1 dans la distribution des AG au sein du macrophage ainsi que les conséquences associées à ce remodelage au cours de l'obésité.

Mots clés : obésité, macrophages du tissu adipeux, Abcg1, métabolisme des lipides.

ABSTRACT

Adipose tissue macrophages (ATM) are central players in obesity-associated inflammation and its comorbidities. ATM metabolism is strongly linked to their functional phenotype and dysregulated or disproportionate ATM responses to environmental lipid fluxes during diet-induced obesity (DIO) influence the activation of specific inflammatory and metabolic programs that contribute to the onset of obesity-induced insulin resistance. The mechanisms governing this metabolic adaptation of ATM in DIO are however poorly understood. Our study demonstrates that the membrane Atp-binding cassette g1 (Abcg1) transporter controls ATM functional response to fatty acids (FA) carried by circulating triglyceride-rich lipoproteins (TRL), which are abundant in high energy diets. Mice genetically lacking Abcg1 in the myeloid lineage present an ameliorated inflammatory status in adipose tissue and a reduced insulin resistance. Abcg1-deficient ATM exhibit a less inflammatory phenotype accompanied by a low bioenergetic profile and a modification of FA metabolism pathways. A closer look into ATM lipidome reveals a shift in the handling of FA pools, including a redirection of saturated FA from membrane phospholipids to lipid droplets, and leading to a reduction in membrane rigidity and neutralization of pro-inflammatory FA. ATM from obese human subjects presented the same reciprocal relationship between Abcg1 expression and this inflammatory and metabolic status. Abolition of this protective, anti-inflammatory phenotype in Abcg1-deficient ATM was achieved through the inhibition of lipoprotein lipase (Lpl) activity, thus delineating the importance of the Abcg1/Lpl axis in controlling ATM metabolic inflammation. Overall, our work identifies the rewiring of fatty acid pools by Abcg1 as a new major pathway orchestrating ATM plasticity and insulin resistance in DIO.

Key words: obesity, adipose tissue macrophage, Abcg1, lipid metabolism.