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Modification of The Macrophage Efferocytosis Process in Obesity

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1. INTRODUCTION

Every day, billions of dead cells are eliminated from our bodies through a process called efferocytosis. Efferocytosis is a part of normal tissue homeostasis, as it contributes to the maintenance of tissue integrity, to the clearance of dead cells, and through molecules released by phagocytosing macrophages also to suppression of inflammation.

Obesity is characterized by the development of chronic low-grade inflammation in adipose tissue. In the increasing adipose tissue (primarily abdominal), fat cells die through apoptosis and necrosis due to excessive lipid overload. Dying fat cells release chemotactic and inflammatory signals to recruit bone marrow-derived macrophages from the bloodstream, which initiate inflammation. During the clearance of dead adipocytes, adipose tissue macrophages also take up lipids packaged in extracellular vesicles. If macrophages are exposed to high lipid levels, they increase and sustain inflammation by producing pro-inflammatory cytokines. Furthermore, they influence the adipokine production of adipocytes. These two effects together lead to decreased insulin sensitivity (Type 2 diabetes). When macrophages become saturated with lipid from disintegrating fat cells, the lipid is transferred to the liver tissue (fatty liver).

In obesity, the concentration of free saturated fatty acids also increases, which reduces the efferocytosis capacity of macrophages throughout the entire body. This increases the inflammatory predisposition throughout the body, which is why obesity is often associated with other chronic inflammatory diseases such as atherosclerosis, chronic obstructive pulmonary disease, osteoarthritis, periodontitis, or prolonged wound inflammation. In the first part of my dissertation, I investigated the inhibitory effect of palmitate on the clearance of apoptotic cells.

Mer tyrosine kinase is an efferocytosis receptor expressed by tissue and M2 macrophages. It may also be involved in suppressing inflammation and taking up lipid-containing extracellular vesicles, thereby reducing the lipid load on fat cells. We hypothesized that macrophage Mer plays a protective role in maintaining adipose tissue homeostasis. Therefore, in the second part of my dissertation, I investigated whether the absence of Mer affects the development of metabolic syndrome in mice fed a high-fat diet.

2. LITERATURE REVIEW

2.1. Obesity

Obesity is a chronic, complex disease defined by excessive fat deposits that can impair health. In clinical practice, the Body Mass Index (BMI) is used as a measure to determine the extent of obesity. BMI measures the ratio of your height to your weight. According to the World Health Organization (WHO) classification, a BMI ranging from 25 to 29.9 is considered overweight, 30 to 39.9 indicates obesity, and above 40 signifies severe obesity.

Although in most cases obesity is triggered by excessive calorie intake and a sedentary lifestyle, its development can also be influenced by genetic, epigenetic, and familial factors, as well as changes in the microbiome. Researches show that individuals with a family history of high blood pressure, obesity, diabetes, or stroke are at a greater risk of developing obesity or hyperlipidemia. In recent years, more than 100 genes have been discovered that are linked to an elevated BMI. Numerous publications confirm that genes predisposing individuals to obesity are influenced by environmental factors, lifestyle, and various pharmacological treatments.

2.2. Types of Adipocytes

There are three types of fat cells: white, beige, and brown adipocytes. The differences between them lie in their fat metabolism, thermogenic capacity, size, morphology, location, and various functions. White adipose tissue makes up the majority of our body's fat tissue. White adipose tissue stores energy in the form of triglycerides, and its most important function is to provide energy for the body's operation through the oxidation of stored fat during periods of low-calorie intake. Changes in the size and morphology of white adipose tissue vary widely, and a close relationship has been observed between the increase in white adipose tissue mass and the likelihood of developing insulin resistance, type 2 diabetes, and high blood pressure. Brown adipose tissue is more active metabolically, contains more mitochondria, and can produce energy in the form of heat under low-temperature conditions. The third type of adipocytes are beige adipocytes, which originate from white adipocytes under thermogenic stimulation. This process is called browning. This process is reversible, and the regulation of mitochondrial autophagy plays a key role in it. Beige adipocytes share some properties with brown adipocytes, such as the expression of uncoupling protein 1 (UCP1), the formation of multilocular lipid droplets, and a high mitochondrial count; however, they also show numerous differences in terms of location, markers, and thermogenic mechanisms.

2.3. Development of Hypertrophy in Adipose Tissue

Obesity is primarily characterized by an increase in adipose tissue size. Adipose tissue mass can grow through two mechanisms: hyperplasia and hypertrophy. During hyperplasia, the number of fat cells increases, while during hypertrophy, the size of fat cells increases. The hyperplasia leads to healthy fat storage, while hypertrophy is primarily linked to abdominal fat tissue, resulting in fat deposition that can lead to metabolic syndrome. The hypertrophic type of fat storage is partly attributed to the higher expression of 11β-hydroxysteroid dehydrogenase activity in abdominal fat tissue. Numerous signaling pathways are involved in the process of hypertrophy.

One such pathway is the insulin signaling pathway. Insulin promotes adipose tissue hypertrophy by inhibiting fat breakdown (lipolysis), promoting fatty acid synthesis, and increasing fat storage in adipose tissue. Insulin negatively regulates lipolysis and promotes the storage of fatty acids in the form of triacylglycerol. During the activation of lipolysis, the level of the secondary messenger molecule cyclic adenosine monophosphate (cAMP) increases, which promotes the phosphorylation and thus the activation of the hormone-sensitive lipase enzyme. Insulin's negative regulatory effect is exerted by activating the phosphodiesterase 3B enzyme, which reduces cAMP levels. When cAMP levels are low, hormone-sensitive lipase will not be active, and thus lipolysis will also be inhibited.

The perilipin 1 protein is highly expressed in adipocytes and plays a role in binding lipid droplets, and is also essential in lipolysis. Perilipin 1 is activated by phosphorylation via protein kinase A. However, this phosphorylation is inhibited when the insulin signaling pathway is activated, which in turn suppresses lipolysis. This ultimately contributes to the accumulation of lipids in fat cells.

Another crucial regulator of adipose tissue hypertrophy is peroxisome proliferator-activated receptor gamma (PPARγ). This ~~is a~~ nuclear transcription factor is essential for adipogenesis, lipid metabolism, inflammation regulation, and maintenance of metabolic homeostasis of adipocytes. PPARγ forms a heterodimer with the retinoid X receptor. By binding to specific DNA sequences, it enhances the expression of genes involved in adipocyte differentiation and maturation. It increases the expression of certain genes (e.g., lipoprotein lipase, CD36) leading to enhanced fatty acid uptake and storage by adipocytes.

Signal transducer and activator of transcription (STATs) proteins are important regulators of adipocyte development. In adipocytes, STAT1, 3, 5A, 5B, and 6 proteins are expressed. STAT5 enhances the expression of genes like acyl CoA oxidase, fatty acid synthase, and adiponectin, an adipokine that improves insulin sensitivity. STAT1 protein is important in cases of high-fat diets when INFγ levels are elevated. The increase in INFγ levels is caused by immune cells migrating into visceral fat tissue, which activates the STAT1 signaling pathway and inhibits adipocyte differentiation.

Increased STAT3 protein expression is also observed in the early stages of adipogenesis and enhances the extent of adipocyte differentiation.

Growth and differentiation factors (GDFs) belong to the transforming growth factor-beta (TGFβ) protein family, and play a crucial role in adipocyte functions and metabolism. GDF-15 is involved in maintaining energy homeostasis and body weight regulation. Its deficiency leads to the development of type 2 diabetes and an increase in adipose tissue mass. GDF-15 also promotes the M2 anti-inflammatory polarization of macrophages in adipose tissue and enhances insulin sensitivity. GDF-5 is a critical protein that protects against obesity. Its overexpression leads to a lean phenotype and protects against diet-induced obesity, partly by inducing the development of beige adipocytes. Similar to GDF-5, GDF-3 is responsible for maintaining adipose tissue homeostasis and regulating adipocyte energy balance. Under lipid overload, it causes an increase in fat mass by enhancing lipid storage; consequently, its deficiency results in reduced fat mass gain.

Growth hormone, a polypeptide produced by the pituitary gland in the brain, plays a multifaceted role in the regulation of adipose tissue. Growth hormone primarily promotes the proliferation of adipocytes and the differentiation of preadipocytes into mature fat cells. Furthermore, it influences lipolysis, the process of fat breakdown. Growth hormone achieves this by inhibiting the lipoprotein lipase enzyme, which in turn prevents the accumulation of triglycerides within adipocytes. Concurrently, growth hormone activates hormone-sensitive lipase, an enzyme crucial for the release of triglycerides from adipocytes through lipolysis. This dual action of inhibiting triglyceride storage and promoting their release helps fat accumulation within the body.

Some signaling pathways are triggered by signals that influence adipose tissue growth mediate their effects through the regulation of intracellular cAMP levels, while others affect mTOR function. mTOR (mammalian target of rapamycin) is a protein kinase that consists of two large complexes: mTORC1 and mTORC2. In adipocytes, mTOR plays a role in regulating adipogenesis, lipid metabolism, thermogenesis, and enhancing insulin sensitivity. The essential role of mTOR in adipogenesis is also demonstrated by the fact that mTOR deficiency leads to a reduction in adipose tissue mass and fat cell size. Furthermore, mTOR can enhance the browning process of white adipose tissue by regulating PPARγ expression. mTORC1 can also exert its effects via sterol regulatory element-binding protein (SREBP) transcription factors involved in regulating the expression of enzymes involved in fatty acid and triglyceride synthesis. Their inhibition ~~also~~ results in reduced adipocyte size.

2.4. Efferocytosis

Efferocytosis is the engulfment of particles larger than 0.5µm into plasma membrane-derived vesicles, which are called phagosomes. On the surface of most cells in our body, there are molecules that prevent the engulfment of cells by macrophages. These molecules are the so-called "don't eat me" signals. Examples of such molecules include CD24, CD31, CD47, major histocompatibility complex I (MHCI), programmed death-ligand 1 (PD-L1), and salicylic acid. The first step of phagocytosis is the migration of macrophages, which is controlled by "find me" signals. These signals can be found intracellularly under normal physiological conditions. Thus, adenosine triphosphate (ATP), uridine triphosphate (UTP), or other molecules like lysophosphatidylcholine, sphingosine 1-phosphate, fractalkine, and monocyte chemoattractant protein-1 (MCP-1) can act as a „find me" signal. As a result of cell death, these molecules are released from apoptotic cells as part of the apoptotic program, creating a gradient that promotes the migration of macrophages towards the apoptotic cells.

"Eat me" signals facilitate the distinction of apoptotic cells from healthy cells. The most studied "eat me" signal is phosphatidylserine (PS). PS is normally located on the inner surface of the plasma membrane due to flippase enzymes, and during apoptosis, it translocates to the outer surface of the plasma membrane. The recognition of PS by phagocytic receptors can occur directly or indirectly via bridging molecules. PS can be directly recognized by brain-specific angiogenesis inhibitor 1 (BAI1), T-cell immunoglobulin mucin protein 4 (TIM-4), and stabilin 2. It is recognized indirectly by three the TAM tyrosine kinases Mer, Axl, Tyro3, and the α(v)β(3) integrin receptor. Bridging molecules include Gas6, milk fat globule-EGF factor 8 (MFG-E8), protein S, and complement component 1q (C1q).

Another "eat me" signal is calreticulin, which is recognized by CD91 receptors on macrophages. During apoptosis, due to the action of caspase enzymes, calreticulin translocates from the cytosol to the outer surface of the plasma membrane, along with PS. Intracellular adhesion molecule 3 (ICAM3) and oxidized low-density lipoproteins, which contain oxidized lipids, can also be present on the surface of apoptotic cells. These act as ligands for receptors found on macrophages. Recent research has also confirmed the role of phosphatidyl inositols as "eat me" signals, and it is recognized by CD14-positive macrophages.

The recognition of apoptotic cells leads to the rearrangement of the actin cytoskeleton. This process is regulated by small GTPases. Rho family GTPases function as molecular switches. They are active in the guanosine triphosphate (GTP)-bound state, and inactive in the guanosine diphosphate (GDP)-bound state. Guanine exchange factors facilitate the formation of the active state, while their GTPase activity promotes the inactive state. The GTPase activity is regulated by GTPase-activating proteins. RhoA enhances acto-myosin contraction and F-actin stabilization in the early phase, but later

functions as a negative regulator of the process. Rac1 promotes F-actin branching, which is essential for lamellipodium formation. Finally, CDC42 is required for the formation of short actin filaments and filopodia. The main effectors of Rac1 and CDC42 are the Wiskott-Aldrich syndrome protein (WASP) and Wiskott-Aldrich syndrome protein family verprolin homologous (WAVE) complexes, which perform the activation of Arp2/3 complexes. Arp2/3 promotes actin polymerization. After the apoptotic cell is enclosed by pseudopodia, the fusion of the ends results in the formation of phagosomes. The actin structures that facilitated phagosome formation detach from the membrane, and the phagosome maturation process begins.

The main regulators of the phagosome maturation process are the Rab proteins, which are also GTPases. Rab5 is responsible for orchestrating the early stages of phagosome maturation, while Rab7 is responsible for the later stages of the maturation process. Rab5's function is essential for the fusion of the phagosome with early endosomes. One of its key effectors is early endosome-associated protein 1 (EEA1). EEA1 forms a complex with phosphatidylinositol 3-phosphate and Rab5, promoting the recruitment of even more EEA1 to endosomes. By interacting with vacuolar protein sorting (Vps) proteins, EEA1 participates in transport processes and also helps SNARE (Soluble N-ethylmaleimide-Sensitive-Factor Attachment Protein Receptor) proteins bind to the endosome, thereby facilitating endosome docking. As a result of these docking processes, endosomes come into proximity and fuse, leading to the formation of multivesicular endosomes. The pH of the early endosome is nearly neutral, around 6.3. Phosphatidylinositol 3-phosphate, together with Rab5 and EEA1, promotes the recruitment and subsequent activation of numerous proteins involved in phagosome maturation. These include hepatocyte growth factor-regulated tyrosine kinase substrate and other components of the endosomal sorting complexes required for transport (ESCRT), as well as Rab7, which is a marker for late endosomes. As the maturation process progresses, the amount of Rab5 decreases, and the amount of Rab7 protein increases.

Rab7's effector protein is Rab-interacting lysosomal protein (RILP), which interacts with the dynein-dynactin microtubule-associated motor complex. This complex, on one hand, directs the endosomes towards the cell's interior, and on the other hand, promotes the extension of phagosomal tubules towards endocytic compartments. As the process advances, the pH of the late phagosome continues to decrease to approximately 4.5-5.5, with the help of V-ATPases that pump protons from the cytoplasm into the phagosome lumen. Lysosomes contain about 70 hydrolytic enzymes responsible for the hydrolysis of macromolecules. Examples of these enzymes include various acid proteases, lipases, glycosidases, nucleases, phosphatases, and sulfatases.

2.5. Phagocytosis of apoptotic adipocytes

A prerequisite for efficient efferocytosis by macrophages is that the size of the dead cell is manageable for the macrophage. Small cells, such as thymocytes, meet this size requirement, and whether they die by apoptosis or necrosis, they are directly engulfed by surrounding macrophages. The size of adipocytes, however, is so large that during the biological process of apoptosis, they must fragment into apoptotic bodies so that the significantly smaller macrophages can engulf them. In our lab's previous studies, we visualized the apoptosis of adipocytes in vitro and, interestingly, found that the dying fat cells package their lipid content into extracellular vesicles, which are taken up first by macrophages. Then, once the majority of the lipid content has been engulfed, the classical apoptotic program is activated, and the remainder of the cell fragments into apoptotic bodies, which are then cleared. It is well-known that in tissue sections, macrophages form crown-like structures around dying adipocytes, meaning that multiple macrophages participate simultaneously in the processing of a single large fat cell. Other labs hypothesize that the uptake of lipids occurs via lysosomal exocytosis, where the macrophage's lysosomal membrane fuses with its plasma membrane, releasing the lysosome's contents into the space between the macrophage and the adipocyte, and the lysosomal enzymes then degrade the adipocyte. Based on our visual data, we instead propose that macrophages engulf these vesicles directly, perhaps into the lysosome. Verification of this hypothesis is currently underway in our laboratory.

2.6. Mer Tyrosine Kinase

The Tyro-3, Axl, and Mer proteins belong to the TAM (Tyro3, Axl, and Mer) receptor tyrosine kinase family. They are crucial for regulating homeostasis in various tissues and organs, and their proper functioning is essential for the immune, reproductive, hematopoietic, vascular, and nervous systems. All three are known efferocytosis receptors. TAM receptors can be categorized by their ligands (Protein S and Gas6). Gas6 can bind to all three TAM receptors, while Protein S only binds to Tyro3 and Mer receptors. A characteristic feature of TAM receptors is their two-domain structure. The extracellular domain consists of two immunoglobulin-like domains and two fibronectin type III domains, both involved in ligand binding. The intracellular domain is a tyrosine kinase domain, necessary for autophosphorylation, and can be activated with or without extracellular stimulation. Axl is expressed throughout the body, and is involved in various cellular processes, including cell proliferation, survival, angiogenesis, and immunosuppression. Tyro3 shows high expression during the development of the gastrointestinal, nervous, and reproductive systems. It also participates in cell proliferation, survival, tumorigenesis, and migration.

Mer is primarily expressed in tissue-resident and M2 phenotype macrophages. Its role in tissue homeostasis is evident from its anti-inflammatory signaling pathway. Mer expression is significantly increased during the M1-M2 polarization of macrophages. Elevated Mer expression leads to increased GAS6 production, which, in turn, increases the amount of anti-inflammatory IL-10. Mer's anti-inflammatory effect can also be achieved by inhibiting the production of pro-inflammatory TNFα (tumor necrosis factor alpha), thereby contributing to reduced inflammation.

Mer shows high expression in many tumors. Due to its anti-inflammatory effects, it can create an immunosuppressive tumor microenvironment, not only by increasing the production of anti-inflammatory cytokines but also by regulating immune cells. For this reason, Mer is currently a target for cancer treatment. Targeted inhibition of Mer, by reducing efferocytosis, both slows tumor growth and enhances the infiltration of anti-tumor immune cells into the tissue. Furthermore, Mer also contributes to promoting tissue regeneration following injury.

2.7. Steps and Consequences of the Development of Metabolic Syndrome

Besides storing lipids, adipose tissue also has an important endocrine function. It produces adipokines, such as adiponectin, leptin, and resistin, which regulate functions not only in the liver but also in muscles and in the brain. In obesity, the balance of these hormones is disrupted: adiponectin production is decreased, while leptin and resistin levels are increased. Due to the rapid growth of fat mass, the hypertrophic adipose tissue doesn't develop proper vascularization, leading to hypoxic conditions. As a result of hypoxia, adipocytes die. Adipocyte cell death can occur via both caspase-dependent and caspase-independent pathways. The extrinsic pathway is activated by death ligands binding to their receptors, such as Fas, TNF-R1, and TRAIL-R. These receptors then activate enzyme cascades, primarily containing proteolytic enzymes like caspases. The intrinsic pathway can be activated by stress, such as endoplasmic reticulum stress. This leads to the activation of pro-apoptotic proteins like Bid or Bax, which help the release of pro-apoptotic proteins from the mitochondria. These proteins participate in the formation of the apoptosome, which initiates apoptosis.

Under normal conditions, adipose tissue contains numerous immune cells, including eosinophils and neutrophil granulocytes. The cytokines secreted by these cells create an anti-inflammatory environment within the adipose tissue. However, during obesity, increased adipose tissue cell death leads to macrophages exiting the bloodstream and entering the adipose tissue. These macrophages differentiate into the M1 (pro-inflammatory) direction, intensifying the development of an inflammatory environment.

Adipocytes are capable of releasing fatty acids not only through lipases but also via exosome-sized lipid-filled vesicles, which can be taken up by macrophages. Obesity completely alters the

dynamics of adipose tissue, changing both adipose tissue and lipid turnover. As a result, fat storage extends beyond the waist and hips to also appear in the liver and muscles. All these factors contribute to the development of metabolic syndrome.

A growing body of research supports the hypothesis that adipose tissue macrophages play a crucial role in the development of pathological processes associated with obesity (metabolic syndrome). Consequently, if macrophages are either depleted, or the MCP-1 protein—which is a key regulator of macrophage migration—is genetically knocked out, then the development of both fatty liver and insulin resistance can be prevented.

Metabolic syndrome is a chronic, non-communicable disease of the modern world. When associated with obesity, metabolic syndrome refers to a cluster of metabolic disorders. These include insulin resistance, dyslipidemia (abnormal fat metabolism leading to increased lipid levels in the blood), abdominal obesity, and high blood pressure. Metabolic syndrome is strongly linked to the development of type 2 diabetes and cardiovascular diseases.

During obesity, energy intake is increased, which results in the activation of mechanisms that prevent the accumulation of energy in cells. One mechanism is the reduction of the insulin sensitivity of the cells, which leads to insulin resistance. Since adipose tissue can influence the insulin sensitivity of the entire body, insulin resistance is very common in individuals with obesity. This phenomenon is partly attributed to inflammatory cytokines released from adipose tissue, such as TNFα and IL-6. These cytokines can inhibit the insulin signaling pathway through several routes. One such route is the serine/threonine phosphorylation of insulin receptor substrate 1 (IRS-1), which negatively regulates the insulin signaling pathway. Another potential route involves the inhibition of PPARγ's function. Since PPARγ enhances the expression of genes involved in lipogenesis (fat production) and lipid storage, its inhibition contributes to the development of insulin resistance.Furthermore, increased level of FFAs in blood plasma, resulting from heightened lipolysis and triglyceride synthesis, also contributes significantly to the development of insulin resistance.

Insulin resistance can also develop due to decreased levels of adiponectin. Adiponectin is secreted by adipocytes and helps to enhance insulin sensitivity in cells, promote fatty acid oxidation, and reduce inflammation. Adiponectin levels show an inverse correlation with the degree of obesity, meaning lower levels contribute to the development of insulin resistance. Another important adipokine that regulates insulin sensitivity is resistin. Although initially it was discovered in adipocytes, resistin is also expressed in mononuclear leukocytes, macrophages, spleen, and bone marrow cells. High levels of resistin contribute to both insulin resistance and inflammation through its pro-inflammatory effects.

Non-alcoholic fatty liver disease (NAFLD) has a global prevalence of 25%. NAFLD is the leading cause of cirrhosis and hepatocellular carcinoma. NAFLD is characterized by the accumulation of triglycerides in the liver. The main metabolic substrates from which these triglycerides are formed

can be fats, fructose, or glucose. Excess dietary fat is typically stored in adipose tissue. However, as adipocyte fat stores increase, more fatty acids are released during fasting periods due to the action of hormone-sensitive lipase under the influence of glucagon. At the same time, the fatty acid oxidation capacity of adipose tissue macrophages becomes saturated, making them unable to take up fats released from fat cells in extracellular vesicles. Consequently, these released fats are transported from abdominal fat cells to the liver and deposited there due to circulatory conditions. If large amounts of glucose and fructose are consumed, they are converted into fatty acids through several enzymatic steps in a process called de novo lipogenesis. Some of these fatty acids can be oxidized by the liver, or they can be converted into triglycerides and released into the bloodstream as very low-density lipoproteins (VLDLs), leading to hyperlipidemia. However, under high substrate loads, these pathways don't function properly, which leads to the formation of lipotoxic lipids like diacylglycerol and ceramide, both contributing to processes that damage liver tissue.

The development of high blood pressure caused by obesity is a very complex process that includes excessive activity of the sympathetic nervous system, stimulation of the renin-angiotensin-aldosterone system, changes in the amount of cytokines produced by adipose tissue, insulin resistance, and changes in kidney function. Decreased adiponectin production by adipocytes leads to decreased insulin sensitivity, while increased pro-inflammatory cytokine production and free fatty acids lead to inflammation. These processes cause endothelial dysfunction, which increases arterial stiffness. Decreased leptin production by adipocytes increases sodium reabsorption through the nervous system, and free fatty acids increase the kidneys' burden; these all contribute to the development of high blood pressure.

2.8. The Relationship Between ER Stress and Autophagy

An elevation of free, primarily saturated fatty acid levels in the bloodstream contributes significantly to the dysfunction or damage of organs (primarily non-adipose cells) by causing lipid accumulation outside of fat cells. This process is called lipotoxicity. The accumulation of improperly folded proteins, which leads to ER stress, plays a decisive role in the activation of cell-damaging processes during lipotoxicity. The cell attempts to correct the error by not only refolding the improperly folded proteins but also trying to get rid of the defective proteins through the activation of autophagy.

2.9. The Relationship Between Inflammation and Deffective Efferocytosis

In obese individuals, high levels of saturated fatty acids affect macrophage efferocytosis in any part of the body. Reduced efferocytosis can lead to chronic inflammation through various mechanisms. A generally compromised efferocytosis program due to high fatty acid levels predisposes obese individuals to various inflammatory diseases, such as atherosclerosis, osteoarthritis, asthma, chronic obstructive pulmonary disease (COPD), and apical periodontitis. Genetic or external factors, such as smoking or high cholesterol levels determine which specific tissue develops inflammation.

Atherosclerosis begins with endothelial activation, followed by lipid accumulation and calcification. This not only causes narrowing of the arteries but also activates inflammatory pathways. The efferocytosis capacity of macrophages in atherosclerotic lesions is significantly reduced. As a result of impaired efferocytosis, uncleared apoptotic cells undergo necrosis, which, on one hand, causes inflammation, and on the other hand, creates a necrotic core. This necrotic core can lead to the detachment of the formed plaque, thereby inducing thrombosis.

Osteoarthritis is a common, chronic degenerative joint disease, where obesity is a significant risk factor for its development. One study showed that obese mice had increased joint cartilage damage and a higher number of synovial apoptotic cells. M1 macrophages infiltrating the tissues secreted less Gas6, which resulted in reduced efferocytosis. Additionally, these macrophages produced increased amounts of inflammatory cytokines. Injecting Gas6 into the joints successfully restored macrophage efferocytosis, thereby preventing the development of obesity-associated osteoarthritis.

Asthma is a condition caused by an immune response to inhaled allergens. Clinical studies have shown that a reduction in the efferocytosis capacity of airway macrophages predisposes individuals to airway inflammation. Indeed, a significant proportion of asthmatic patients show reduced efferocytosis in their airway macrophages.

COPD (chronic obstructive pulmonary disease) is caused by inhaling harmful particles, primarily tobacco smoke and pollens. COPD leads to chronic bronchitis and emphysema, both of which result in narrowed airways and shortness of breath. Basal epithelial cells play a crucial role in defense and post-injury remodeling. As a consequence of cell reprogramming by inhaled particles, the airways develop squamous metaplasia in epithelial cells, goblet cell hyperplasia, and ciliary dysfunction. Additionally, mucus production increases, creating an environment in the lungs prone to local inflammation and infection.

Apical periodontitis is an inflammation that develops around the tooth root, caused by bacterial infection. One study investigated the link between efferocytosis and apical periodontitis. ARA290 (an anti-inflammatory peptide) treatment enhanced macrophage efferocytosis and reduced inflammation through M2 macrophage polarization, thereby resolving apical periodontitis.

3. AIMS OF THE STUDY

I. Based on available scientific literature, increased fat cell death and their inadequate clearance by macrophages play a significant role in the development of chronic low-grade inflammation and other pathological processes associated with obesity. It's also known that obese individuals have an elevated concentration of circulating saturated fatty acids in their blood, which inhibits the efferocytosis capacity of macrophages. We aimed to determine the mechanisms through which palmitate affects the efferocytosis process in macrophages.

II. It's known that Mer tyrosine kinase is a crucial component of the efferocytosis process. In our experiments, we used Mer-deficient mice to model impaired clearance of dead cells. With this model, we investigated whether this impaired clearance, due to Mer deficiency, could exacerbate the development of metabolic syndrome in mice fed a high-fat diet.

4. MATERIALS AND METHODS

4.1. Reagents

All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except when indicated otherwise.

4.2. Experimental Animals

4-week-old and 2-4-month-old C57BL/6, Mer-deficient, and their wild-type littermates were used for the experiments. In the feeding experiments, 8-week-old Mer-deficient and wild-type littermates were kept in separate cages. Mer tyrosine kinase-deficient mice were characterized by a full-body Mer deficiency, which was created using the Cre-loxP method. Both Mer-deficient and wild-type mice were divided into two groups: HFD groups were maintained on a high-sucrose and high-fat diet (45% kcal% fat, 17% kcal% sucrose; Research Diets Inc., D12451), while control groups (ND) were maintained on a normal diet (13% kcal% fat; 4.6% kcal% sucrose; Special Diets Services, VRF1(P)). Animals were kept under a 12 h light: 12 h darkness, fed and watered ad libitum. The body weight and food intake of the animals were registered weekly. Study protocols were approved by the Animal Care and Use Committee of the University of Debrecen, (with permission number 7/2016/DEMÁB and 7/2021/DEMÁ).

4.3. Generation of BMDMs and Treatments

Bone marrow progenitors were obtained from the femur of 2- to 4-month-old mice by lavage with sterile physiological saline. Cells were differentiated and maintained at 37°C in 5% CO_2 for 5 days in DMEM (10% FBS, 20% supernatant from L929 fibroblast cell line [M-CSF source], 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin). The floating cells were removed by washing on day 3, and a fresh medium was added to the differentiating cells. On day 6, the cells were treated for 24 h with different concentrations of BSA, BSA-conjugated palmitate alone, or the AMP-activated protein kinase enzyme activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), the specific inhibitor of the mTORC1 complex rapamycin, the ROCK inhibitor Y-27632, or the pan-caspase inhibitor Z-VAD-FMK. For the preparation of sodium palmitate, a 100 mM stock solution was diluted in 150 mM NaCl and added to 10% fatty acid-free, low endotoxin BSA (adjusted to pH

7.4) in a 1:6 ratio to give a 4 mM palmitate-BSA stock solution, which was filtered through a 0.22 µm low protein-binding capacity filter.

4.4. *In Vitro* Generation of Apoptotic Thymocytes

Apoptotic thymocytes were obtained from thymi extracted from 4-week-old C57BL/6 mice. After removal of the thymus, the cells were pressed through a sterile metal filter, washed with PBS, and incubated in RPMI 1640 medium (2 mM L-glutamine, 100 U/ml penicillin/100 µg/ml streptomycin) for 24 hours (37°C, 5% CO_2).

4.5. Cell Viability Assay

On the sixth day, bone marrow-derived macrophages were exposed to different concentrations of BSA conjugated palmitate for 24 hours. Cells were then treated with 10 µl Cell Counting Kit-8 reagent for 1 hour at 37°C in the dark. Absorbance was measured at 450 nm using a BioTek microplate reader (Agilent Technologies, Santa Clara, CA, USA).

4.6. Efferocytosis Assays

Apoptotic thymocytes were stained with 2.5 µM Cell Tracker DeepRed fluorescent dye (Invitrogen, Carlsbad, CA, USA) for 24 hours. Apoptotic cells were then added to $2x10^5$ macrophages at a ratio of 1:5 and incubated for 40 min. After incubation, the non-phagocytosed labeled apoptotic cells were removed by washing, and the macrophages were then collected following trypsin treatment. The fluorescence of the cells was analyzed using a Becton Dickinson FACSCalibur flow cytometer. Macrophage populations were selected based on their size and density (SSC/FSC). Within this population, the percentage of macrophages that phagocytosed dead cells was determined by detecting the fluorescence signal in the FL2 and FL4 channels.

4.7. Fluorescent Microscopy

Macrophages were stained with 5µM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) for 24 h. Apoptotic thymocytes were stained with 2.5µM Cell Tracker DeepRed fluorescent dye for 24 hours. Macrophages and apoptotic cells were then co-incubated at a ratio of 1:5 for 40 min. Dead cells were removed by washing, and macrophages were fixed with 1% paraformaldehyde.

Pictures were taken on a fluorescence microscope (4471136, FLoidTM Cell Imaging station) (ThermoFisher, Waltham, MA, USA)

4.8. Western Blot Analysis

To obtain the total protein content of the cells, the cells were harvested and lysed in cold lysis buffer (50 mM Tris–HCl; 0.5% Triton X-100; 1 mM EDTA; 17 mM 2-mercaptoethanol (pH:8.3) and proteinase inhibitors). The protein concentration of the samples was determined with Bio-Rad Protein Assay dye (Bio-Rad, Budapest, Hungary). The samples were boiled in SDS sample buffer for 10 minutes at 100°C. The samples were run on a 12% polyacrylamide gel and electroblotted onto Immobilon-P polyvinylidene fluoride membranes (Bio-Rad, Budapest, Hungary) using a Bio-Rad semi-dry blotter. The membranes were blocked for 1 hour at room temperature with TBST buffer containing either 5% BSA or 5% milk powder. The membranes were then incubated overnight at 4°C with primary monoclonal anti- mouse p70 S6 Kinase (Cell Signaling Technology, Inc.), P-p70 S6 Kinase (T389) (Cell Signaling Technology, Inc.), Akt (pan) (Cell Signaling Technology, Inc.), P-Akt (Ser473) (Cell Signaling Technology, Inc.), myosin phosphatase-targeting subunit-1 (MYPT1) (ThermoFisher), and P-MYPT1 (ThermoFisher), Rock1 (MyBioSource, Inc., San Diego, CA, USA, AMPKα (Cell Signaling Technology, Inc., Beverly, MA, USA), P-AMPKα (Thr172) (Cell Signaling Technology) antibodies in 1:1000, and monoclonal anti-β-actin antibodies (A5441) in 1:5000 dilutions. Following three washes with TBST buffer, membranes were incubated for 1 hour at room temperature with HRP-conjugated mouse IgG or goat anti-rabbit IgG(H+L) secondary antibodies. The proteins were detected with Immobilon Western Chemiluminescent HRP Substrate (Advansta Inc., San Jose, CA, USA). The pixel density of the bands was determined using ImageJ software.

4.9. Determination of Rac1, Cdc42, and RhoA Activity in Macrophage

Macrophages from 2 wells cultured under the same conditions were collected as one sample, and 2.8×10^6 from them were used for the determinations. The activity of the three G proteins was then determined by the G-LISA Rac1, Cdc42, and RhoA activation assay kits (Cytoskeleton Inc., Denver, CO, USA) according to the manufacturer's instructions.

4.10. Staining of Engulfing BMDMs for Confocal Laser-Scanning Microscopy

BMDMs were cultured on µ-Slide 8 Well ibiTreat plates and stained with 50 nM LysoTracker™ Green DND-26 (Invitrogen) fluorescent dye for 1 h. Apoptotic thymocytes were stained with 2.5 µM Cell Tracker DeepRed fluorescent dye (Invitrogen) for 24 hours. Apoptotic cells were added to $6x10^4$ macrophages in a 1:5 macrophage: apoptotic cell ratio and incubated for 40 min. After incubation, non-phagocytosed labeled apoptotic cells were removed by washing. Confocal images were taken without fixing the cells.

4.11. Confocal Laser Scanning Microscopy

Fluorescence images were recorded on a Nikon A1 Eclipse Ti2 confocal laser-scanning microscope (Nikon, Tokyo, Japan) using a Plan Apo 60 water objective [NA = 1.27]. Laser lines of 488 nm and 647 nm were used for the excitation of LysoTracker® Green DND-26 and CellTracker™ Deep Red Dye. The fluorescence emissions were detected through bandpass filters of 500–550 nm and 660–740 nm, respectively. Images of approximately 1 µm-thick optical sections, each containing 512 × 512 pixels, with pixel sizes of 100 or 410 nm, were acquired with 2.2 µs pixel dwell time. Images were taken in sequential mode to minimize crosstalk between the channels, and an average of 4 lines was used for denoising the images.

4.12. Bone Marrow Transplantation

Recipient Mer-null and Mer+/+ male mice (7 weeks old, male) were irradiated with 11 Gy using a Theratron 780C cobalt unit. The mice were immobilized in a circular "mouse pie cage" designed to accommodate up to 11 animals during the irradiation procedure. Following irradiation, bone marrow cells were flushed out from the femurs, tibias, and humeri of donor Mer−/− or Mer+/+ mice (in RPMI-1640 medium). Mice were transplanted by a retro-orbital injection (20×10^6 bone marrow cells per mouse).

4.13. Collection of Tissue Samples

At the end of the feeding phase, the animals were sacrificed with isoflurane and body weight, body length, liver and gWAT weights were measured, and the body mass index was determined. Liver and gWAT samples were collected for further analysis. For histological analysis adipose tissue and

liver samples were fixed in 4% paraformaldehyde. Samples for gene expression analysis and liver triglyceride determination were frozen in liquid nitrogen and stored at −80°C prior use.

4.14. Adipocyte and Adipose Tissue Macrophage (ATM) Isolation

gWAT tissue was dissected, washed, and kept in transport buffer (DMEM + 1% penicillin-streptomycin) solution with 1% BSA. The tissue was finely minced and digested for 30-60 minutes at 37°C in a digestion solution containing 20 g/L BSA, 0.5 g/L collagenase Type I, and HEPES buffer (pH 7.4). Following the digestion, it was filtered through a 100 μm nylon filter. After filtration, samples were centrifuged, and adipocytes were collected and stored at −80∘C prior to RNA isolation.

During the isolation of adipose tissue macrophages, red blood cells were removed by hemolysis (ACK Lysing Buffer, Thermo Fisher Scientific), and the stromal vascular cell fraction was resuspended in staining buffer (PBS with 0.5% BSA and 2 mM EDTA). Adipose tissue macrophages were isolated from the stromal vascular cell fraction using MACS technology (F4/80-based) positive selection according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany).

4.15. Liver Triglyceride Levels

The triglyceride concentration was determined in saponified, neutralized liver extract (liver samples were digested overnight at 55°C in ethanolic KOH (P5958)) using an enzymatic glycerol assay according to the manufacturer's instructions Free Glycerol Reagent, Glycerol Standard Solution, F6428, G7793).

4.16. Histology

Liver and adipose tissue samples were fixed in 4% formaldehyde and embedded in paraffin. The paraffin sections were stained with hematoxylin-eosin. Sections were analyzed using the EVOSTM XL Core Imaging System (Thermo Fisher Scientific). ImageJ (National Institutes of Health, Bethesda, USA) was used for the evaluation of adipose tissue images. The cross-sectional areas of adipocytes are reported in μm^2.

4.17. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis of mRNA Expression

Total RNA isolation of samples was performed using TRI reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Budapest, Hungary) according to the manufacturer's instructions. Gene expression levels were determined by qRT-PCR in triplicate using FAM-MGB-labelled probes (Life Technologies) on a Roche LightCycler LC480 real-time PCR instrument. Relative levels of mRNA were calculated by the comparative CT method. The GAPDH housekeeping gene was used for normalization of expression values.

4.18. Insulin Resistance Test and Serum Insulin Determination

The insulin resistance test was performed in week 17. Following a 6-hour fasting period, 0.75 IU/kg body weight of insulin (ACTRAPID Penfill 100IU/ml) (Novo Nordisk, Copenhagen, Denmark) was injected intraperitoneally. After insulin administration, blood glucose concentrations were measured at specified time points using a DCont Trend blood glucose meter (DCont, Budapest, Hungary). Plasma insulin levels were determined using a mouse insulin ELISA 80-INSMS-E01 kit (ALPCO, Salem, NH, USA) according to the manufacturer's instructions.

4.19. Statistical Analysis

Data were presented as mean ± SD or individual values. All statistical analyses were performed using GraphPad Prism 6.01 software. A p value < 0.05 was considered statistically significant. In the experiments with BMDMs for differences between 2 groups, two-tailed unpaired Student's t-test was used, and for comparisons $n > 2$ groups one-way ANOVA (with Tukey's multiple comparisons test) was used. In the experiments investigating Mer deficiency for differences between four or six groups one-way ANOVA (Sidak's multiple comparison test) was used. For datasets split on two independent factors, two-way ANOVA (multiple comparison procedures with Tukey post hoc test) was used.

5. RESULTS

5.1. Mechanism of the Inhibitory Effect of Palmitate on BMDM Efferocytosis

5.1.1. Palmitate Exposure Inhibits Phagocytosis of Apoptotic Cells by BMDMs without Significantly Affecting the mRNA Expression of Various Phagocytosis-Related Molecules

We investigated whether palmitate directly inhibits BMDM efferocytosis. We exposed BMDMs to various concentrations of palmitate for 24 hours, then added apoptotic thymocytes for 40 minutes to measure the BMDMs' phagocytic capacity. We found that palmitate inhibited BMDM efferocytosis in a dose-dependent manner.

Previous studies showed that palmitate can increase macrophage cell death by inducing endoplasmic reticulum stress. To determine if the reduced efferocytosis in our case was due to palmitate-induced macrophage apoptosis, we treated BMDMs with different palmitate concentrations and assessed their viability. We observed that the palmitate concentrations we used did not affect BMDM viability. To further confirm this, we treated BMDMs with a pan-caspase apoptosis inhibitor. Inhibiting apoptosis in BMDMs did not enhance palmitate-inhibited phagocytosis. These results demonstrate that the decrease in BMDM phagocytic capacity is not related to palmitate-induced macrophage cell death. We determined that 0.4 mM palmitate was the most effective concentration as this concentration significantly reduced phagocytosis, but did not affect the viability of BMDMs, so we used this concentration in our further experiments.

Since the recognition of apoptotic cells by phagocytic receptors is a key step in phagocytosis, our next experiment aimed to determine if palmitate exerts its efferocytosis-reducing effect by influencing the mRNA expression of key molecules involved in phagocytosis. Palmitate did not significantly affect the mRNA expression of receptors involved in apoptotic cell uptake, with the exception of CD36, which is involved in fatty acid uptake and whose expression significantly increased.

5.1.2. Exposure to Palmitate Affects Efferocytosis Very Likely via the Energy-Sensing Mechanisms in BMDMs

In the form of palmitate, BMDMs take up excess energy. AMP-activated protein kinase (AMPK) plays a key role in cellular energy sensing; its activity depends on the cell's energy status. When cells have enough nutrients, AMPK activity is inhibited. However, if cells don't have enough nutrients,

AMPK activity increases. For maximum AMPK activity, phosphorylation of threonine 172 in the kinase domain is required.

Since palmitate doesn't exert its inhibitory effect through phagocytosis receptors, we examined how palmitate treatment affects the activity of energy-sensing pathways. Western blot data showed that palmitate treatment reduced AMPK enzyme activity in BMDMs.

To investigate whether the energy overload originating from palmitate affects efferocytosis, we created conditions in BMDMs that mimicked low energy levels. We did this by adding AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), an AMPK activator, during the last 1.5 hours of palmitate treatment. Our results demonstrated that AICAR-induced activation of AMPK was able to counteract the inhibitory effect of palmitate on phagocytosis.

5.1.3. Palmitate Exposure Triggers mTORC1 Activity in BMDMs

It's known that AMPK can inhibit the activity of the mammalian target of rapamycin (mTOR) protein. Since palmitate treatment decreased AMPK activity, we wanted to determine if palmitate treatment could alter mTOR activity in BMDMs. With the Western blot technique, we determined the levels of threonine 389 phosphorylated protein S6 kinase. Since protein S6 kinase is a substrate of the mTORC1, its increased phosphorylation indicates increased mTORC1 activity. Increased phosphorylation of Akt protein at serine 473, however, indicates increased mTORC2 activity.

Our experiments demonstrated that palmitate increased mTORC1 activity but had no significant effect on mTORC2 activity. To find out whether mTORC1 activation is involved in palmitate-related efferocytosis inhibition, we treated macrophages with rapamycin, a selective mTORC1 inhibitor. Rapamycin, at a concentration of 100 nM, completely inhibited the phosphorylation of protein S6 kinase but did not affect Akt phosphorylation, confirming its selective effect on mTORC1. Rapamycin treatment at this concentration significantly counteracted the inhibitory effect of palmitate.

In summary, our results indicate that palmitate enhances mTORC1 activity in mBMDMs, and inhibiting mTORC1 with rapamycin reverses the negative effect of palmitate on efferocytosis.

5.1.4. Palmitate Exposure Triggers an mTORC1-Dependent Rho Kinase Activation to Inhibit Efferocytosis

G proteins are crucial for cytoskeletal rearrangement during phagocytosis. Since palmitate did not affect the mRNA expression of phagocytosis receptors, we hypothesized it might impact G protein activity. After 24 hours of palmitate treatment, we examined the activity of Rac1, Cdc42, and RhoA

in macrophages. We found no significant change in Rac1 and Cdc42 activity; however, RhoA activity increased, and we observed an mTORC1-dependent regulation of RhoA activity.

Given that RhoA is known to negatively regulate efferocytosis through the regulation of RhoA-associated protein kinase 1 (ROCK1), we investigated whether palmitate, rapamycin, or a combination of both affected ROCK1 protein expression. With Western blot technique, we found no significant changes in ROCK1 protein expression with any of the treatments.

During cytoskeletal rearrangement, MYPT-1 protein is one of ROCK1's substrates, activated by phosphorylation at threonine 850. With Western blot, we determined the levels of phosphorylated MYPT1 protein. Palmitate significantly increased the amount of phosphorylated MYPT1 protein in BMDMs. Conversely, rapamycin treatment, even in the presence of palmitate, resulted in a significant decrease. From these results, we can conclude that mTORC1 can activate RhoA, and through RhoA, it also activates ROCK1.

We hypothesized that if ROCK1 activation contributes to palmitate's inhibitory effect on efferocytosis, then inhibiting ROCK1 would reverse palmitate's negative impact, thereby enhancing BMDM efferocytosis. Our results indeed showed that inhibiting ROCK1 with the inhibitor Y-27632 eliminated the inhibitory effect of palmitate on efferocytosis.

5.1.5. Palmitate Exposure Induces Autophagy but Inhibits the Fusion of Autophagic Vacuoles and Lysosomes

It's known that palmitate can induce ER stress in cells. One cellular response to ER stress, among others like the unfolded protein response or changes in mitochondrial function, is autophagy. During autophagy, LC3-II protein levels increase, which results from the post-translational modification of the protein by ATG proteins during autophagy. p62 protein also plays an important role in marking molecules for degradation during autophagy. p62, along with its associated proteins, is degraded in the lysosome, so a decrease in its amount is a significant indicator of the extent of autophagy.

As mTORC1 can inhibit autophagy, we would expect autophagic processes to be inhibited following its inhibition by palmitate. However, ER stress can enhance autophagy through an mTORC1-independent pathway. Therefore, we were curious to see how palmitate affects autophagic pathways in macrophages. Our experiments showed that treatments with rapamycin, AICAR, and Y-27632—which inhibit the mTORC1 pathway—each individually increased the LC3-II/LC3-I ratio, indicating increased autophagy in BMDMs. The amount of p62 protein synchronously decreased with these treatments.

However, after adding palmitate, we detected a significant increase in p62 levels. This suggested either a delayed fusion of autophagosomes with lysosomes or an inhibition of this fusion, resulting in a consequential accumulation of LC3 in autophagosomes that couldn't fuse with lysosomes.

When rapamycin, AICAR, and Y-27632 treatments were applied in the presence of palmitate, they significantly reduced p62 levels. From this, we can conclude that inhibitors of the mTORC1 pathway are capable of resolving the autophagosome-lysosome fusion block caused by palmitate. This allows the ER stress-induced autophagic process, triggered by palmitate, to proceed without obstruction.

5.1.6. Palmitate Also Inhibits the Uptake of Apoptotic Thymocytes Into the Lysosome

We hypothesized that if palmitate treatment activates the mTORC1 pathway, and this activation inhibits the fusion of autophagic vacuoles with lysosomes, it might also inhibit the fusion of phagosomes with lysosomes. This is because essentially the same proteins are involved in the fusion of these organelles. To support our assumptions with microscopic examination, we labeled BMDM lysosomes with LysoTracker and apoptotic cells with DeepRed fluorescent dye. Then, we tracked the appearance of apoptotic cells within the lysosomes. In the case of palmitate treatment, we detected a very low number of apoptotic cells in the lysosomes compared to other treatments. Our observations suggest that the activation of the mTORC1-RhoA-ROCK signaling pathway interferes with the phagosome-lysosome fusion pathway. However, it's important to note that we cannot rule out the possibility that the delayed uptake of apoptotic cells alone explains their late appearance in the lysosomes.

5.2. Effect of Mer Deficiency on High-fat Diet-induced Pathological Changes in Mice

5.2.1. Mer-Null Mice Respond to High-Fat Diet with Attenuated Body Weight Gain

In our study, first, we investigated the impact of high-fat diet (HFD) compared to a standard control diet (ND) on the body weight gain of mice. As expected, wild-type mice exposed to HFD developed significant obesity, with body weights of 38.6±2 g versus 29.6±1.5 g compared to ND-fed mice.

Interestingly, Mer deficiency did not significantly affect body weight at the start of the feeding period (19.0±0.6 g for Mer+/+ vs. 18.1±2.4 g for Mer-/- mice). However, over the longer term, Mer-null mice on a normal diet showed slower weight gain compared to their wild-type counterparts

(27.0±0.9 g vs. 29.6±1.5 g). This reduced weight gain in Mer-null mice was even more pronounced when they were exposed to HFD. This difference was also reflected in their BMI.

Furthermore, the mass of gonadal white adipose tissue (gWAT) was significantly lower in Mer-/- mice compared to Mer+/+ mice exposed to HFD. The increase in gWAT mass in wild-type mice was accompanied by an increase in average adipocyte size, as detected by histological sections. Previous research has shown that adipocyte hypertrophy is associated with increased inflammatory cytokines and altered adipokine production. Therefore, we measured the mRNA expression levels of several pro-inflammatory cytokines in gWAT adipocytes. Consistent with existing literature, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNF), interleukin 6 (IL6), resistin, and leptin showed increased expression in wild-type mice exposed to HFD, while adiponectin levels decreased. However, in Mer-null mice exposed to HFD, we did not detect significantly altered expression of these molecules.

In summary, our findings indicate that Mer deficiency in mice exposed to HFD protects against weight gain and the development of inflammation.

5.2.2. Loss of Mer in Mice Protects Against High-Fat Diet-Induced Hepatosteatosis and Insulin Resistance

We observed high mRNA expression of several pro-inflammatory cytokines and resistin in the adipose tissue macrophages of wild-type mice exposed to HFD compared with the macrophages isolated from Mer-null mice. We also detected high Mer expression in macrophages from wild-type mice exposed to ND, but Mer expression was low in pro-inflammatory macrophages isolated from HFD-fed mice. These findings suggest that in Mer-/- mice exposed to HFD, Mer-null adipocytes do not become hypertrophic, and inflammation does not develop in Mer-null adipose tissues.

It is known that HFD-induced inflammation and altered adipokine production are linked to the development of insulin resistance and fatty liver. Therefore, we also investigated these processes. We found no difference in liver weight between ND and HFD-exposed mice. However, in the livers of HFD-exposed wild-type mice, we detected significantly higher triacylglycerol (TAG) concentrations compared to mice exposed to ND. In contrast, the TAG concentration measured in the livers of Mer-/- mice did not increase even when mice were exposed to HFD. Our results align with the images from hematoxylin-eosin-stained liver sections.

Subsequently, we determined the extent of insulin resistance. Wild-type mice exposed to HFD showed a greater degree of insulin resistance compared to Mer-/- mice. These results were further supported by the increased compensatory serum insulin levels measured in Mer+/+ mice exposed to HFD. In contrast, there was no significant change in the serum insulin levels of Mer-null mice.

Based on our findings, Mer deficiency in mice protects against the development of inflammation, fatty liver, and insulin resistance.

5.2.3. Loss of Mer Expressed by Nonhematopoietic Cells Protects Against HFD-Induced Obesity

Our initial findings with Mer-null mice, which showed an unexpected protection against HFD-induced obesity, led us to question whether this protective effect originates from the absence of Mer in hematopoietic cells or other cell types. To answer this question, we performed bone marrow transplantations on wild-type and Mer-null mice after irradiating them with a cobalt unit to eliminate their bone marrow. As controls, wild-type bone marrow was transplanted into wild-type mice. To address our core question, we transplanted Mer-deficient bone marrow into wild-type mice and wild-type bone marrow into Mer-deficient mice. We then repeated the experiments that we performed previously with these transplanted mice. Consistent with existing literature, irradiated mice exhibited significantly less weight gain at the end of the feeding period compared to their non-irradiated counterparts. Despite this, the observed body weight changes demonstrated that while Mer deficiency in hematopoietic cells did not favorably impact HFD-induced weight gain, Mer deficiency in non-hematopoietic cells proved to be protective against the obesity-inducing effects of the high-fat diet, at least in the early stages of the feeding period.

As we initially anticipated, Mer deficiency in hematopoietic cells led to a larger gWAT mass in mice exposed to HFD compared to wild-type mice. This difference was even more pronounced when Mer was missing in non-hematopoietic cells. Hematoxylin-eosin-stained tissue sections also revealed that mice lacking Mer in hematopoietic cells already showed larger adipocyte sizes on ND compared to other groups.

Consistent with the body weight data, an increase in triacylglycerol content was detectable in the livers of all three types of mice exposed to HFD, and this increase was most pronounced in mice lacking Mer in hematopoietic cells. Regarding insulin sensitivity, as a result of low weight gain, no significant difference had developed between the groups at the time of measurement.

In summary, our results lead us to conclude that Mer deficiency in non-hematopoietic cells plays a crucial role in protecting mice against HFD-induced obesity, and this effect masks the myeloid Mer-deficient phenotype.

6. DISCUSSION

Obesity is a widespread global health issue, affecting over 650 million adults and 340 million children today. It's characterized by an abnormal accumulation of fat tissue in the body, posing significant health risks and often accompanied by chronic low-grade inflammation.

The development of chronic low-grade inflammation is closely linked to macrophage efferocytosis, the process by which macrophages remove apoptotic cells. Efferocytosis is crucial for maintaining normal tissue homeostasis, preserving tissue integrity, and preventing tissue-damaging inflammation. However, the engulfment of large, lipid-laden apoptotic adipocytes by macrophages can itself promote inflammation. Our research group previously showed that macrophages can take up and oxidize the lipid content of apoptotic adipocytes in the form of lipid-containing extracellular vesicles, thereby reducing ectopic fat overload in peripheral tissues. In the absence of TG2, this process is even more efficient, likely due to the compensatory higher integrin β expression in adipocytes, which is known to be involved in the uptake of PS-expressing extracellular vesicles. We hypothesize that Mer tyrosine kinase may play a similar role, as its involvement in the uptake of PS-positive extracellular vesicles has also been demonstrated.

If the removal of apoptotic cells is impaired, the uncleared cells undergo secondary necrosis. During necrosis, intracellular molecules are released from the cells, leading to inflammation. Their continuous formation, combined with a disruption in macrophage polarization due to impaired clearance, can collectively lead to chronic inflammation. This occurs in adipose tissue during obesity, but the accumulation of saturated fatty acids in circulation can also reduce the efferocytosis capacity of macrophages in other tissues. The development of chronic inflammation forms the basis for numerous obesity-associated diseases, including atherosclerosis, COPD, rheumatic diseases, and oral cavity inflammations.

Chronic low-grade inflammation causes macrophage accumulation in adipose tissue, leading to adipose tissue dysfunction. This causes increased lipogenesis, but over time, the fat tissue's capacity for further lipid storage decreases. As a consequence, changes are observable in adipokine production, and fat cell necrosis within the adipose tissue increases. This is partly due to pathological interactions between macrophages and adipocytes. Research indicates that adipose tissue macrophages are promising targets for treating obesity. Potential targets include macrophage polarization, inflammatory pathways, metabolic pathways, insulin signaling pathways, and energy-sensing pathways within macrophages. By influencing these signaling pathways, we could impact adipocyte function, potentially preventing obesity and related metabolic diseases.

In the first part of my dissertation, I investigated how palmitate affects the uptake of apoptotic cells by macrophages, aiming to identify new drug targets. In the second part of my dissertation, I examined the role of Mer tyrosine kinase, which is highly expressed by tissue macrophages and involved in clearing apoptotic cells, in maintaining adipose tissue homeostasis.

Our investigation into palmitate's effect on efferocytosis aimed to determine if palmitate truly reduces macrophage uptake of apoptotic cells, and if so, we wanted to know what mechanisms are involved in this process. Our initial studies confirmed that palmitate decreases dose-dependently the uptake of apoptotic cells by macrophages. Next, we examined the mechanism behind palmitate's efferocytosis-reducing effect. Palmitate has previously been shown to induce ER stress and thereby initiate apoptosis in macrophages. To determine whether decreased efferocytosis is caused by increased macrophage death caused by palmitate exposure, we examined how palmitate treatment affects macrophage viability. Our results showed that palmitate treatment did not significantly reduce macrophage viability in concentrations at which it already strongly interfered with the efferocytosis process.

Since the first step of efferocytosis in vitro involves recognition via "eat me" signals, we hypothesized that palmitate might influence the expression of phagocyte receptors. To test this, we treated macrophages with palmitate and examined the expression of several genes involved in phagocytosis. Our results indicated that palmitate did not affect the expression of these genes. We did, however, find a significant increase in the expression of CD36, a gene also involved in fatty acid uptake.

The uptake of large quantities of fatty acids causes energy excess in macrophages. Therefore, we investigated how palmitate affects energy-sensing pathways and whether these pathways could influence phagocytosis. Palmitate treatment reduced the activity of AMPK in macrophages, which is the cell's primary energy-sensing protein and shows decreased activity during nutritional excess. AICAR is a known activator of AMPK, so we examined whether activating AMPK, thereby indicating a low energy state, could enhance phagocytosis. Indeed, by activating AMPK, we were able to counteract the negative effect of palmitate on efferocytosis and increase the uptake of apoptotic cells.

Next, we investigated how palmitate affects mTOR activity in macrophages, as AMPK is known to exert some of its effects by regulating mTOR. Palmitate treatment significantly increased mTORC1 activity but did not affect mTORC2 activity. Similar to AMPK, we wanted to know if inhibiting mTORC1 with rapamycin, a selective mTORC1 inhibitor, can enhance efferocytosis. By inhibiting mTORC1 in palmitate-treated macrophages, we were able to significantly increase efferocytosis.

Since palmitate did not exert its effect by influencing the expression levels of genes involved in phagocytosis, we examined its impact on efferocytosis signaling pathways. Palmitate significantly increased RhoA activity in macrophages. RhoA is a negative regulator of efferocytosis. Conversely,

the mTORC1 inhibitor rapamycin suppressed RhoA activity. Further supporting the role of the RhoA pathway in palmitate-induced efferocytosis inhibition, we detected increased ROCK1 activity after palmitate treatment, which was then reduced by rapamycin treatment. Following this, we investigated whether efferocytosis could be enhanced by inhibiting RhoA. By treating macrophages with Y27632, a specific RhoA inhibitor, we successfully reversed the inhibitory effect of palmitate. Based on these results, we conclude that mTORC1 is involved in regulating the RhoA/ROCK1 signaling pathway.

The AMPK and mTOR signaling pathways are important regulators of autophagy and overlap at several points with the efferocytosis signaling pathway. However, the mTORC1 pathway does not overlap with LC3-mediated phagocytosis, so we investigated the effect of palmitate on autophagy. Palmitate can inhibit autophagy at multiple points by activating the AMPK/mTORC1 pathway, which could inhibit autophagosome-lysosome fusion. This inhibition can occur via both AMPK and mTOR. However, palmitate also causes ER stress, and through ER stress, the activation of an autophagy process, which is not regulated by mTORC1. ER-activated autophagy and the mTORC1 pathway-inhibited autophagosome/lysosome fusion lead to the accumulation of autophagosomes in palmitate-exposed macrophages, which we detected through the accumulation of LC3-II and p62 levels. Inhibition of the mTORC1 pathway with AICAR, rapamycin, and Y-27632 in macrophages was able to release the palmitate-induced autophagosome/lysosome fusion blockage leading to lysosomal degradation, which we detected through decreased p62 levels.

The membrane surrounding the engulfed cell must be replaced during phagocytosis; it is recycled from endosomes and lysosomes. Based on our results, we hypothesize that the inhibitory effect of palmitate on efferocytosis is due to insufficient membrane availability for uninterrupted phagocytosis because of defective autophagy caused by palmitate treatment. It is also conceivable that the activated mTORC1 pathway inhibits the fusion of phagosomes with lysosomes, as many identical proteins are involved in both processes. AMPK activators are currently used in the treatment of obesity. Our results suggest that activating AMPK can contribute to the more efficient clearance of apoptotic cells in obesity. Our results also indicate that the use of ROCK inhibitors may promote the improvement of obesity-related reduced efferocytosis. The studies were conducted on macrophages, but the findings may also apply to other cell types. For example, in hepatocytes, palmitate also leads to mTORC1 and ROCK1 activation. Summarizing the results of the first part of my dissertation, AMPK activators or ROCK inhibitors may have a beneficial effect in the treatment of lipotoxicity by improving defective autophagy, which is caused by the accumulation of free saturated fatty acids in cells.

In the second part of my dissertation, I investigated the role of Mer tyrosine kinase in adipose tissue homeostasis. Mer is highly expressed in tissue-resident and M2 phenotype macrophages. In these cells, it's a crucial molecule for initiating both efferocytosis and anti-inflammatory processes

during engulfment. In adipose tissue macrophages, Mer may also play a role in the uptake of PS-positive, lipid-containing extracellular vesicles released from adipocytes.

Initially, we hypothesized that Mer will be involved in the protection of HFD-induced obesity and inflammation, and its absence would lead to increased obesity, inflammation, and insulin resistance. Surprisingly, our very first experiment, in which we exposed wild-type and Mer-/- mice to ND and HFD, showed the opposite of our hypothesis. Mer-deficient mice exposed to HFD exhibited less obesity compared to their wild-type counterparts. This was observable in the mass of gWAT, in the size of white fat cells, and in the BMI. In Mer-/- mice, neither gWAT mass nor fat cell size increased significantly in response to HFD. Consistent with these results, we observed neither increased pro-inflammatory cytokine expression nor altered adipokine production in the gonadal adipose tissue of Mer-/- mice exposed to HFD. In contrast, in wild-type mice, as reported in the literature, there was a significant increase in MCP-1, TNF-α, and IL-6 expression, indicating increased inflammation as a consequence of obesity. Furthermore, resistin and leptin mRNA levels increased, while adiponectin levels significantly decreased. All these changes are consequences of an imbalanced adipokine production. These findings suggest that in the absence of Mer, neither white adipose tissue hypertrophy nor the associated inflammation and adipokine production disorders develop in the adipose tissue.

Obesity is frequently associated with NAFLD, metabolic syndrome, and insulin resistance. Therefore, we investigated how Mer deficiency affects the development of these diseases. In wild-type mice exposed to HFD, we observed hepatic steatosis, indicated by increased liver mass, elevated liver triglyceride content, and confirmed by histological examination. Insulin resistance studies showed that wild-type mice exposed to HFD developed insulin resistance, which was accompanied by a compensatory increase in serum insulin levels. In contrast, neither fatty liver nor insulin resistance developed in Mer-/- mice, demonstrating that Mer deficiency also protects against the development of fatty liver and insulin resistance resulting from obesity.

As our initial findings were contrary to our expectations, we next investigated whether the protective effect against obesity observed in Mer-deficient mice was due to the absence of Mer in bone marrow-derived cells or other cell types. To examine this, we irradiated wild-type and Mer-null mice to ablate their bone marrow and performed bone marrow transplantation from either wild-type or Mer-null mice. Although irradiated mice showed less weight gain compared to non-irradiated mice, as a consequence of irradiation, it was still evident that Mer deficiency in hematopoietic cells did not protect against HFD-induced weight gain. When Mer was absent from hematopoietic cells, we detected larger gonadal adipose tissue and fat cells in both normal diet and high-fat diet groups compared to wild-type mice. These results supported our original hypothesis that Mer, when expressed in macrophages, positively impacts the maintenance of adipose tissue homeostasis.

In contrast, if Mer was absent from non-hematopoietic cells, mice were protected from HFD-induced weight gain, at least during the initial feeding period. This was confirmed by gWAT mass, fat cell size, and liver triacylglycerol content. Mice lacking Mer in non-hematopoietic cells showed significantly smaller gWAT mass, reduced fat cell size, and lower liver triacylglycerol content compared to mice where Mer was absent from hematopoietic cells. However, insulin resistance had not yet developed in either mouse group at this level of weight gain. These results suggest that the absence of Mer in non-hematopoietic cells leads to altered adipose tissue hypertrophy on both ND and HFD.

In further experiments, which I did not personally conduct, my colleagues demonstrated that Mer is also expressed on adipocytes. They found that in white adipocytes, Mer promotes hypertrophy, while in brown adipocytes, it has an inhibitory effect on thermogenesis. Mer's absence in mice shifts the ingested energy towards burning, so hypertrophy won't occur even on a high-fat diet. Therefore, the adipocyte Mer-deficient phenotype masks the characteristics of the macrophage Mer-deficient phenotype in mice where Mer is absent throughout the entire body.

In addition to the Mer tyrosine kinase, our group also detected the expression of the other two TAM kinases in adipocytes. The role of Tyro3 has not yet been studied in adipocytes, but the role of Axl has. Interestingly, these studies began by searching for kinases that regulate the differentiation of brown fat cells. They found that, similar to Mer tyrosine kinase, Axl inhibits heat production in brown adipose tissue. Consistent with our findings, the absence of Axl also protected against HFD-induced obesity. These studies did not test the potential role of Axl in mouse gonadal adipocytes. However, Axl expression has been demonstrated in human white adipocytes, and even more notably, increased Axl expression was found in the subcutaneous fat cells of obese individuals.

These studies did not test the expression of Mer in adipose tissue, but genetic studies suggest that the loss-of-function rs4374383 G > A variant of the Mer tyrosine kinase results in more pronounced fat oxidation and increased insulin sensitivity. Furthermore, in a nine-year follow-up of originally healthy, non-overweight, non-diabetic, and insulin-sensitive individuals, the MERTK G > A variant provided protection against the development and severity of non-alcoholic fatty liver disease (NAFLD) and the development of type 2 diabetes. These findings indicate that, similar to what was observed in mice, a reduction in Mer function in humans also provides protection against obesity and related pathological conditions.

My results indicate that Mer tyrosine kinase, expressed in two different cell types, regulates fat homeostasis. In adipose tissue macrophages, Mer contributes to maintaining fat homeostasis and protects against obesity. In macrophages, it contributes to the clearance of apoptotic adipocytes during normal tissue turnover. Its activation results in an anti-inflammatory effect and promotes the uptake of excess fat from adipocytes, thereby reducing their lipid content. Mer activation can be promoted

not only by interaction with apoptotic cells but also by the binding of its ligands, Protein S and Gas6. Interestingly, both macrophages and adipocytes are capable of producing these ligands. Thus, it would be intriguing to investigate which cell type regulates Mer activity under resting conditions. Are these ligands already present on vesicles released by adipocytes? Or are they located on macrophages?

However, in the case of a high-fat diet, tissue-resident macrophages are replaced by infiltrating inflammatory bone marrow-derived macrophages. These macrophages rather express Axl tyrosine kinase, which also belongs to the TAM kinase family. Therefore, macrophage Mer likely does not participate in regulating high-fat diet-induced adipose tissue inflammation. The fact that its complete absence protects against the development of obesity designates Mer tyrosine kinase as a potential target for the treatment of obesity.

In summary, in my dissertation, I investigated two aspects of macrophage efferocytosis behavior related to obesity. Both studies identified potential molecules whose regulation could influence obesity and its associated complications.

7. SUMMARY

Obesity is frequently associated with chronic low-grade inflammation. This is due to the continuous expansion of adipose tissue, and the consequently increased cell death of hypertrophic adipocytes. This induces transmigration of bone marrow-derived macrophages into the adipose tissue, which develop into inflammatory direction in the lipid rich environment. Previous reports have shown that the clearance of apoptotic cells is decreased in obesity due to elevated levels of circulating free fatty acids, such as palmitate, in the blood. In my dissertation, the inhibitory effect of palmitate on macrophage efferocytosis, and the metabolic consequences of the absence of Mer tyrosine kinase (an efferocytosis receptor expressed by macrophages) in obesity, were investigated.

My results demonstrate that palmitate inhibits the efferocytosis of bone marrow-derived macrophages (BMDMs) in a dose-dependent manner. Palmitate, on one hand, enhances autophagy, on the other hand, activates an mTORC1/ROCK1 signaling pathway which inhibits autophagosome-lysosome fusion. As a result, in palmitate-exposed macrophages cell membranes accumulate in the autophagosomes. We hypothesize that the inhibition of efferocytosis in BMDMs exposed to palmitate is related to the lack of sufficient plasma membrane availability for the undisturbed functioning of the efferocytosis process. Our studies suggest that AMP-activated protein kinase activators leading to mTORC1 inhibition or ROCK1 inhibitors are able to reverse the palmitate-induced inhibition of efferocytosis. From our results, we conclude that AMP-activated protein kinase activators may be useful in the treatment of obesity, not only due to their known beneficial effects on metabolism.

In the case of Mer tyrosine kinase, we hypothesized that it provides protection against obesity. In striking contrast we found that Mer-deficient mice are protected against high fat diet (HFD)-induced obesity and the associated inflammation. In the absence of Mer, HFD feeding did not lead to the development of fatty liver or insulin resistance either. Bone marrow transplantation experiments revealed that Mer expressed by non-hematopoietic cells (adipocytes) is responsible for this phenotype. If Mer was missing only from the hematopoietic cells, however, indeed resulted in increased fat storage in the mice kept on both normal diet (ND) and HFD. This phenotype, however, was suppressed by the loss of Mer in adipocytes. Our data altogether indicate that targeting Mer could be an effective strategy for treating obesity.

8. LIST OF PUBLICATIONS

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Registry number: DEENK/371/2025.PL
Subject: PhD Publication List

Candidate: László Sós
Doctoral School: Doctoral School of Dental Sciences
MTMT ID: 10076380

List of publications related to the dissertation

1. Köröskényi, K., **Sós, L.**, Rostás, M., Papp, A. B., Kókai, E., Garabuczi, É., Deák, D., Beke, L., Méhes, G., Szondy, Z.: Loss of MER Tyrosine Kinase Attenuates Adipocyte Hypertrophy and Leads to Enhanced Thermogenesis in Mice Exposed to High-Fat Diet.
   Cells. 13 (22), 1-20, 2024.
   DOI: http://dx.doi.org/10.3390/cells13221902
   IF: 5.1 (2023)
2. **Sós, L.**, Garabuczi, É., Sághy, T., Mocsár, G., Szondy, Z.: Palmitate Inhibits Mouse Macrophage Efferocytosis by Activating an mTORC1-Regulated Rho Kinase 1 Pathway: Therapeutic Implications for the Treatment of Obesity.
   Cells. 11 (21), 1-18, 2022.
   DOI: http://dx.doi.org/10.3390/cells11213502
   IF: 6

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List of other publications

3. Vincze-Fige, É., Sarang, Z., **Sós, L.**, Szondy, Z.: Retinoids Promote Mouse Bone Marrow-Derived Macrophage Differentiation and Efferocytosis via Upregulating Bone Morphogenetic Protein-2 and Smad3.
   Cells. 11 (18), 1-22, 2022.
   DOI: http://dx.doi.org/10.3390/cells11182928
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4. Vincze-Fige, É., Szendrei, J., **Sós, L.**, Kraszewska, I., Potor, L., Balla, J., Szondy, Z.: Heme Oxygenase-1 Contributes to Both the Engulfment and the Anti-Inflammatory Program of Macrophages during Efferocytosis.
   Cells. 10 (3), 652-669, 2021.
   DOI: http://dx.doi.org/10.3390/cells10030652
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Total IF of journals (all publications): 24,766
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The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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