Understanding macrophage activation in the adipose tissue

At the crossroads of immunology and metabolism

Lily Boutens

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PROPOSITIONS

1. Macrophages residing in different tissues should be considered as unique cell types.
   (this thesis)

2. Obese adipose tissue would benefit from having more rather than fewer macrophages.
   (this thesis)

3. Access to scientific publications, experimental setups and data is not sufficient to achieve true open science.

4. The gap between what is expected from science on the one hand and feasible for scientists to provide on the other fuels public distrust in science.

5. Whereas scientific research orders the universe, it increases entropy in personal life.

6. Just as in fashion, trends dominate the scientific agenda.

7. The reliability of scientific data is determined by the methods and materials used yet is too often appreciated as being directly linked to individuals or journals.

8. Dealing with serendipity makes up an essential part of a PhD-track.

Propositions belonging to the thesis, entitled

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Lily Boutens

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Thesis

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CONTENT

1

General introduction

Circulating in the bloodstream or nested in tissues, monocytes and macrophages protect against invaders and support tissue development and function. Central to these acts is their capacity to recognize and ingest foreign particles or microbes; a process called phagocytosis, and damaged or dying cells; a process called efferocytosis (1,2). Monocytes and macrophages are, however, not only involved in the clearance of stressors, but also modulate immune responses via antigen presentation and the release of cytokines, chemokines and growth factors (3). Their great phenotypic plasticity allows for such functional polarization and underlies their capacity to maintain or regain homeostasis while being challenged by a variety of environmental stressors (4).

Activation through Toll-like receptors

Pattern recognition receptors (PRRs) play a crucial role in recognition of various stressors. They enable immune cells including monocytes and macrophages to respond to structural components of microbes, called pathogen-associated molecular patterns (PAMPs), or structures formed upon tissue injury, so-called damage-associated molecular patterns (DAMPs). Despite their relatively recent discovery, the family of Toll-like receptors (TLRs) are the most extensively studied class of PRRs (5). To date, thirteen members have been identified in mammals (6). Located either on the cell surface or intracellular membranes of endosomes or lysosomes, TLRs recognize specific entities of pathogens (7). Whereas bacterial lipids and proteins are recognized by TLRs residing on the plasma membrane (TLR1, -2, -4, -5, -6, and -10), nucleic acids form the most prominent ligand of intracellular TLRs (TLR3, -7, -8, and -9) (7, 8). Being composed of at least two of the three main ligand entities (lipids, proteins, or nucleic acids), a pathogen generally activates more than one TLR (8). Such simultaneous activation of multiple TLRs or TLRs in combination with other PRRs enables the initiation of pathogen-tailored immune responses (8).

Upon ligand binding TLRs dimerize and activate adaptor molecules ultimately cumulating into effector functions including pathogen uptake, chemokine and cytokine release, and antigen presentation (7, 9). After the initial pro-inflammatory responses upon TLR activation that facilitate immune cell infiltration and pathogen killing, anti-inflammatory signalling is needed for resolution of inflammation and restoration of tissue homeostasis (10). This involves efferocytosis of infiltrated neutrophils and monocytes that have undergone apoptosis, and the release of anti-inflammatory cytokines including IL-10 and TGF-β (11, 12).

Classical versus alternative activation

The sharp contrast between phenotypes directly after activation versus those at later stages has led to the classification of macrophages into pro-inflammatory or classically activated on the one hand (13) versus anti-inflammatory or alternatively activated on the other hand (14). In vitro, stimulation with IFN-γ, often in combination with the TLR4 ligand lipopolysaccharide (LPS), generates a macrophage phenotype resembling classical activation (15, 16), whereas

IL-4, sometimes in combination with IL-13, induces an alternative macrophage phenotype (14, 17). Nowadays, these two divergent phenotypes are named 'M1' for classically activated versus 'M2' for alternatively activated macrophages (18).

Diversification of macrophage phenotypes into M1 versus M2 has enabled the in vitro investigation of functional polarization of macrophages as well as the identification of pathways or transcriptional patterns underlying macrophage polarization. Accordingly, several transcription factors including signal transducer and activator of transcription (STAT) proteins have been identified to initiate transcriptional patterns specifically required for a functional M1 or M2 response (19). On the one hand STAT1 is critically involved in activating transcription of opsonic receptors and pro-inflammatory cytokines and chemokines characterizing a M1 response (e.g. IL-12 and CCL5), whereas STAT6 predominantly drives the expression of mannose receptors and anti-inflammatory cytokines and chemokines like IL-10 and CCL24 (20).

Through recent years intracellular metabolism has regained attention as fundamental driver of immune responses (21). Although initially the M1 versus M2 classification was based on arginine metabolism either driving the production of nitric oxide (NO) or ornithine respectively (18), the last decade many other metabolites and metabolic pathways have been identified to underlie macrophage activation. Currently intracellular metabolism is generally acknowledged to promote macrophage function through the generation of energy, production of building blocks, and synthesis of signalling molecules (21, 22).

Metabolism as determinant of function

Upon activation immune cells strongly enhance glucose uptake and secrete high amounts of lactate (23). As such, they display great similarity with Warburg's observation in tumour cells opting for the fermentation of glucose despite sufficient oxygen and functional mitochondria (24). Importantly, enhanced glycolysis in classically activated macrophages is accompanied by impaired oxidative phosphorylation (OXPHOS). Although the yield of ATP is much lower per molecule of glucose, a shift from OXPHOS toward glycolysis allows for a more rapid increase in ATP production required for cytokine and chemokine secretion as well as phagocytic capacity. In addition, it enables immune cells to feed the pentose phosphate pathway (PPP) and use tricarboxylic acid (TCA) cycle intermediates and mitochondria for purposes other than ATP production. Indeed, the PPP supplies building blocks for nucleic acids and amino acids, and NADPH needed for fatty acid (FA) synthesis and reactive oxygen species (ROS) formation through NADPH oxidase (22, 25). Mechanistically, the TCA cycle is disrupted at two sites, allowing for the accumulation of the TCA intermediates citrate and succinate that respectively fuel FA synthesis required for proliferation and HIF-1α stabilisation that promotes IL-1β transcription (22, 26). Moreover, rather than being used for ATP production, mitochondria have been shown to fuel ROS formation through reverse electron transport (27). Hence, by activation of glycolysis in expense of OXPHOS M1 macrophages facilitate

bacterial killing, cytokine release, cell growth and proliferation. At the other side of the spectrum, M2 macrophages display an intact TCA cycle and enhanced mitochondrial activity, and mainly rely on OXPHOS in mitochondria for ATP production (25, 28).

Recent insights, however, point to metabolic choices of M1 versus M2 macrophages being less straight-forward than initially thought. For example, instead of FAs (29), M2 macrophages likely predominantly use glycolysis to fuel the TCA cycle for mitochondrial ATP production (30, 31). On the other hand, M1 macrophages that predominantly rely on glycolysis for pro-inflammatory functions, have been shown to require FA oxidation for inflammasome activation and IL-1β secretion as well (32).

Metabolic diversity in vivo

Whether metabolic profiles similar to those observed in M1 or M2 macrophages exist in vivo remains ambiguous. Given the great versatility and plasticity of macrophages, and complex combination of stressors – either pathogenic or non-pathogenic of origin – present in vivo, metabolic profiles of M1 versus M2 macrophages are not expected to generally reflect monocyte or macrophage phenotypes.

Indeed, growing evidence suggests that activation of different PRRs or stimulation with distinct cytokines drives unique intracellular signalling routes that translate into diverse macrophage phenotypes (33). Nutrient availability further increases the complexity of responses in vivo by driving metabolic choices made by monocytes or macrophages. For instance, lactate has been shown to inhibit glycolysis and pro-inflammatory M1 activity (34), whereas glutamine deprivation interferes with M2 polarization (35). Recently, it was shown that M1 macrophages switch toward using OXPHOS for ATP production in glucose-deprived medium (36). Interestingly, this did not affect their pro-inflammatory trait, suggestive of a complex interplay between intracellular metabolism and macrophage function that may critically depend on both the presence of stressors and nutrients.

Tissues are characterized by a distinctive combination of stressors and nutrients, and have indeed been shown to accommodate unique populations of macrophages that adopt tissue-specific ‘default settings’ to prevent continuous inflammation (37). This opens an interesting field of exploration to understand how macrophages in various tissue environments adopt intracellular metabolic routes that can be used to fuel tissue-tailored functional requirements.

Tissue-resident macrophages

Conventionally, monocytes and macrophages were seen as intermediate and end-point within one maturation flow in which pro-monocytes from the bone marrow enter the bloodstream to become monocytes that randomly infiltrate tissues at steady-state ultimately differentiating into macrophages (38). Nowadays, however, we know that infiltration of monocytes solely occurs in tissues depending on external replenishment (39-

41), or during injury or infection (42, 43). It has become apparent that virtually all tissues are characterized by a stable population of macrophages that mostly establishes before birth and maintains itself throughout adulthood (44-46). Although immune surveillance is part of their task, most tissue macrophages are predominantly occupied by clearing cellular debris and apoptotic cells as part of homeostatic turnover (2, 37, 47). In addition, they adopt various tailor-made phenotypes and functions driven by micro-environmental cues and tissue-specific challenges (3, 48). For example, consequential to their direct exposure to external stressors, macrophages populating the lungs display a high phagocytic capacity needed for clearing pathogens (49). In contrast, macrophages in the sterile environment of the testis have been shown actively involved in steroidogenesis by producing 25-hydroxycholesterol (50). Accordingly, each tissue harbours a unique population of macrophages executing highly specific functions and clearly distinguishable on transcriptional level (51, 52).

White adipose tissue as immunological organ

White adipose tissue plays a crucial role in cold insulation and protection of vital organs. Yet rather than being a passive storage entity for excess energy, research done in the last twenty-five years has revealed that the adipose tissue also is a highly dynamic organ that ensures metabolic flexibility. Via the uptake of glucose and FAs for storage into triglycerides (TGs) when energy is abundant, and breakdown of these TGs when energy is needed, the adipose tissue rapidly responds to metabolic needs (53). Moreover, it affects whole body metabolism by releasing a variety of signalling molecules called adipokines that may impact on food intake and key metabolic enzymes in peripheral organs including the liver (54).

The observation of increased levels of TNF-α (55) and identification of macrophages as source of TNF-α (56, 57) in obese adipose tissue instigated a plethora of studies that have identified the adipose tissue as an important immunological organ that accommodates various immune cells from the innate and the adaptive immune system both in lean and obese conditions (58). Accumulating evidence emphasizes tight interactions between immune cells and adipocytes and a pivotal role for immune cells in adipose tissue functioning (58, 59).

Ever since the discovery of enhanced numbers in obese adipose tissue, macrophages have gained particular attention and have been extensively studied. These studies revealed adipose tissue macrophages (ATMs) to be critically involved in adipose tissue functioning and whole body homeostasis. For example, in conditions of high adipocyte lipolysis as apparent during weight loss or induced by β-adrenergic stimulation, macrophages rapidly infiltrate the adipose tissue, locate around adipocytes, and adopt a lipid-laden appearance (60, 61). By buffering lipids released by adipocyte lipolysis, ATMs ensure gradual release of free fatty acids (FFAs) into the circulation and shield other tissues from potentially toxic levels of circulating FFAs (61). Moreover, they effectively remove adipocytes that have died as a consequence of continuous lipolysis (60, 61) or upon targeted activation of caspase-8 (62), and further facilitate tissue turnover by promoting extracellular matrix remodelling,

adipogenesis, and angiogenesis (59, 60, 63, 64). Although these ATM functions have been identified upon experimental manipulation, adipocyte turnover has been shown to exist at steady-state as well. Indeed, calculations of adipocyte replacement range from 10-100% cellular replacement a year (65, 66), suggestive of a continuous cycle of cell death and replenishment. Considering the above, ATMs presumably play a key role in this process.

The development of obesity is characterized by adipocyte hypertrophy and high numbers of cell death (67). As with the targeted adipocyte cell death in lean adipose tissue, adipocyte cell death in obese adipose tissue is accompanied by a sharp increase in the number of macrophages (56, 57) that surround dying or dead adipocytes in so-called crown-like structures (CLSs) and display a lipid-laden appearance (68-71). In contrast to the lean state, however, lipid-laden macrophages in CLSs of obese adipose tissue adopt a pro-inflammatory phenotype (67, 72-74), displaying profound similarity with foam cells present in atherosclerotic plaques (72). Importantly, the presence of CLSs in obese adipose tissue correlates with the development of insulin resistance in both mice and humans (68, 75, 76).

The exact cause of inflammatory activation of ATMs in obese adipose tissue is still under debate. Similarly to what has been reported for foam cells that display lipid-induced ER stress in atherosclerotic plaques (77, 78), ongoing lipid accumulation may cause lipotoxicity and thereby pro-inflammatory activation of ATMs part of obese adipose tissue (79). Indeed, ER stress has recently been shown to be enhanced in ATMs during obesity, and ablation of IRE-1α – a key sensor of ER stress – curtailed the inflammatory trait of ATMs (80). Otherwise, FAs but also other factors coming from stressed or dead hypertrophic adipocytes, such as HMGB1 (81, 82) or DNA (83), may act as DAMPs activating macrophages in obese adipose tissue via PRRs including TLRs (84).

Role for Toll-like receptors in obese adipose tissue

The family of TLRs may provide a link between nutrient abundance and adipose tissue inflammation (85). Especially TLR2 and TLR4 have gained attention because of their activation by various metabolic products, among which FAs (86). Although direct binding to TLRs is questionable (87), FAs may activate TLRs via the circulating plasma protein Fetuin-A (88). Alternatively, saturated FAs may modulate the propensity of TLR dimerization hence TLR activation via promoting the formation of lipid rafts and subsequent TLR translocation into lipid rafts, or through binding to co-receptors including the FA-transporter CD36 (89). Interestingly, the expression of both TLR2 and TLR4 is increased during atherosclerosis and obesity and associates with diabetes outcome (90). In murine models of diet-induced obesity, deletion or truncation of TLR2 or TLR4 has been shown to improve disease outcome by abating weight gain, attenuating adipose tissue inflammation, or improving insulin sensitivity (86, 88, 91-94).

Of the thirteen TLR family members, ten are expressed in humans and twelve in mice. Interestingly, all twelve TLRs expressed in mice were shown upregulated in obese adipose

tissue (95), some of them much more than could be explained solely by macrophage influx. Importantly, TLRs are expressed both in immune cells and adipocytes (96) and may affect adipose tissue inflammation via adipocyte-macrophage cross-talk as has been shown for TLR4 (97). However, the exact role of TLRs in interactions between macrophages and adipocytes in lean versus obese adipose tissue, or in the development of adipose tissue inflammation and Type 2 Diabetes in obese individuals, is not yet fully understood.

Metabolic activation of macrophages in obese adipose tissue

Because most of the studies on ATM activation have been done in mice or in vitro (most often using mouse cells), the relevance for humans remains ambiguous. Importantly, however, both human and mouse ATMs display a unique inflammatory phenotype, characterized by the expression of M1 as well as M2 markers (98-102). Recently, it has been shown that instead of pathogens or cytokines, a combination of insulin, glucose and palmitic acid drives macrophage activation toward a phenotype resembling ATMs part of obese adipose tissue (103). These 'metabolically activated' ATMs are characterized by enhanced expression of genes involved in lipid uptake (*Cd36*), storage (*Plin2*) and export (*Abcg1*) (103). Intriguingly, metabolic reprogramming in ATMs is in line with a previous study that revealed increased lysosomal biogenesis and activity in ATMs part of obese adipose tissue (104). Because macrophages in human adipose tissue display similar metabolic activation (103, 104), one could speculate activation of metabolism to be a general characteristic of ATMs that may importantly underlie their inflammatory trait in obese adipose tissue. How intracellular metabolism relates to inflammatory activation of ATMs in lean or obese conditions is, however, an intriguing aspect that is not yet understood.

Outline of this thesis

In this thesis we focus on the interface of metabolism and immunity, generally referred to as 'immunometabolism'. By examining the role of intracellular energy metabolism in activation and function of monocytes or macrophage exposed to various environmental cues, and by studying ATM activation and function in lean and obese conditions, we aim to enhance our understanding of metabolic choices and functions of monocytes and macrophages either challenged by pathogenic stimuli or residing in the complex environment of the adipose tissue.

Changes in intracellular metabolism upon stimulation of TLR4 with LPS are relatively well understood and have often been extrapolated to other forms of inflammatory activation of mononuclear phagocytes, presuming general characteristics of metabolic rewiring upon activation. We evaluated whether LPS-induced metabolic rewiring holds true upon stimulation of other TLRs in human monocytes. As such, in **Chapter 2** we describe metabolic reprogramming in freshly isolated human monocytes stimulated with various microbial

pathogens, and dig further into distinct metabolic rewiring as well as its consequences for functional outcome by using the synthetic TLR-ligands LPS and Pam_3CysSK_4 (P3C). This provides the basis for further elaboration on the role of intracellular metabolism for macrophage functioning in the adipose tissue; a more complex environment that accommodates a variety of stimuli. First, in **Chapter 3**, a contemporary overview of the origin and function of ATMs in both lean and obese adipose tissue is provided, as well as of stressors that may contribute to their inflammatory activation during obesity. Building upon studies that have plead for metabolic rather than inflammatory activation of ATMs (103, 104), we examine real-time energy metabolism in ATMs isolated from obese or lean adipose tissue in **Chapter 4**, and, by using various inhibitors of different metabolic routes, demonstrate the contribution of ATM metabolism to cytokine production. Moreover, we evaluate the role of HIF-1α, a key regulator linking energy metabolism to cytokine release (105), in metabolic rewiring and inflammatory activation of ATMs during obesity by feeding myeloid-specific *Hif-1α* knock-out versus wild-type mice a high-fat diet (HFD). Then, we zoom into the role of lipids as potential drivers of the ATMs phenotype. During obesity ATMs have been shown to enhance the expression of several genes involved in lipid uptake, including lipoprotein lipase (*Lpl*), that is crucial for breakdown of extracellular TGs, and cluster of differentiation 36 (*Cd36*), facilitating FA uptake (79, 103, 106). By exposing wild-type macrophages as well as macrophages lacking angiopoietin-like 4 (ANGPTL4), a well-known inhibitor of LPL, to adipose tissue-conditioned medium or a TG emulsion, we evaluate the role of TGs versus FAs in driving the inflammatory phenotype observed in macrophages in an adipose tissue environment in **Chapter 5**. In **Chapter 6**, we explore clearance of dead adipocytes by macrophages in the context of obese adipose tissue. Clearance of dead adipocytes by macrophages has only recently been studied in detail and so far its relevance during obesity and potential differences between the lean and obese state are far from completely understood. We therefore study regulation of the efferocytic machinery in ATMs, interfere with the phagocytic machinery in obese mice, and measure the uptake of lipids from dead adipocytes and subsequent inflammatory activation of macrophages primed with either obese or lean adipose tissue in vitro. In **Chapter 7** we investigate whether TLR10 is of relevance during the development of obesity. This TLR family member has been detected in human adipose tissue (96) and is the only TLR attributed with anti-inflammatory properties (107). In our study, we examine plasma adipokines and cell counts in the adipose tissue of obese and lean individuals carrying single nucleotide polymorphisms in *TLR10* or bearing wild-type alleles of *TLR10*, and include an animal experiment in which mice carrying a human *TLR10* transgene and wild-type controls are fed a HFD for 16 weeks. Lastly, in **Chapter 8** the insights obtained in this thesis are evaluated and integrated in a more broader exploration and discussion on the therapeutic potential of targeting intracellular metabolism for restricting inflammatory activation of macrophages, with particular emphasis on ATMs in obese individuals.

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2

Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes

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ABSTRACT

Microbial stimuli such as lipopolysaccharide (LPS) induce robust metabolic rewiring in immune cells known as the Warburg effect. It is unknown whether this increase in glycolysis and decrease in oxidative phosphorylation (OXPHOS) is a general characteristic of monocytes that have encountered a pathogen. Using $CD14^+$ monocytes from healthy donors, we demonstrated that most microbial stimuli increased glycolysis, but that only stimulation of Toll-like receptor (TLR) 4 with LPS led to a decrease in OXPHOS. Instead, activation of other TLRs, such as TLR2 activation by Pam_3CysSK_4 (P3C), increased oxygen consumption and mitochondrial enzyme activity. Transcriptome and metabolome analysis of monocytes stimulated with P3C versus LPS confirmed the divergent metabolic responses between both stimuli, and revealed significant differences in the tricarboxylic acid cycle, OXPHOS and lipid metabolism pathways following stimulation of monocytes with P3C versus LPS. At a functional level, pharmacological inhibition of complex I of the mitochondrial electron transport chain diminished cytokine production and phagocytosis in P3C- but not LPS-stimulated monocytes. Thus, unlike LPS, complex microbial stimuli and the TLR2 ligand P3C induce a specific pattern of metabolic rewiring that involves upregulation of both glycolysis and OXPHOS, which enables activation of host defence mechanisms such as cytokine production and phagocytosis.

INTRODUCTION

Otto Warburg first described the concept of metabolic rewiring in tumour cells in the 1930s and showed that differentiated cells rely on mitochondrial oxidative phosphorylation (OXPHOS) to generate ATP, whereas tumour cells switch to aerobic glycolysis under normoxic conditions[1]. In recent years, immunologists have discovered that this metabolic shift also underpins the activation and function of immune cells.

T-helper cells, such as activated T_H1 and T_H17 cells, are characterized by active glycolysis[2], whereas quiescent T cells, such as regulatory and memory T cells, rely on OXPHOS and fatty acid oxidation for their ATP production[2,3]. Similar parallels can be drawn between polarized macrophages, where pro-inflammatory M1 macrophages are highly glycolytic, but anti-inflammatory M2 macrophages rely mainly on OXPHOS and fatty acid oxidation[4,5]. These metabolic adaptations are thought to accommodate various functional outputs. For instance, intracellular metabolites such as succinate[6] and itaconate[7] serve not only as tricarboxylic acid (TCA) cycle intermediates but are also closely linked to cytokine production, macrophage polarization and microbial killing.

As such, the shift from OXPHOS to glycolysis following activation, known as the Warburg effect, has been adopted as a core paradigm in the field of immunology[8]. Currently, the field is dominated by studies in the murine system, which have primarily investigated the influence of Toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), on cellular metabolism[4,7,9,10]. Few studies have investigated this switch in human immune cells[11], and it is unclear whether all microbial stimuli induce the Warburg effect. To build on this, we examined whether different microbial stimuli could induce the Warburg effect in human monocytes. Whole-pathogen lysates were used to represent both Grampositive and Gram-negative bacteria, whereas TLR4 (LPS) and TLR2 (Pam_3CysSK_4 (P3C)) ligands were used at different concentrations for mechanistic studies. Additionally, we investigated the consequences of metabolic rewiring on two functional outputs: cytokine production and phagocytosis.

Surprisingly, we discovered that a shift from OXPHOS to glycolysis was observed only in monocytes stimulated with concentrations of 1–100 ng ml^{-1} LPS, and not in monocytes stimulated with P3C or other bacterial lysates. In monocytes stimulated with P3C, increased OXPHOS was needed for retention of their phagocytic capacity and cytokine production. These findings therefore challenge the notion that a shift from OXPHOS to glycolysis underlies activation of all immune cells upon microbial stimulation. Instead, we propose that each individual stimulus induces a complex set of metabolic programmes that govern the function of a given immune cell.

MATERIAL AND METHODS

Healthy volunteers

PBMCs were isolated from blood donated by healthy male volunteers after informed consent. Ethical approval was obtained from the CMO Arnhem-Nijmegen (NL32357.091.10).

Isolation of PBMCs and CD14+ monocytes

Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque PLUS (GE Healthcare Biosciences). Monocytes were separated from the PBMCs by hyperosmotic density gradient centrifugation over Percoll (Sigma-Aldrich) and washed once with PBS. Cells were counted in a Coulter counter (Coulter Electronics). Alternatively, CD14+ monocytes were purified from freshly isolated PBMCs using MACS microbeads for positive selection, according to the manufacturer's instructions (Miltenyi Biotec).

Stimulation experiments

Monocytes (2×10^5 per well) were used for Seahorse measurements, while 1×10^6 monocytes per well were used for cytokine, RNA and western blot analysis. Monocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (no glucose; Gibco) supplemented with 10 μg ml^{-1} gentamicin (Gibco), 10 mM pyruvate (Gibco), 10 mM HEPES (Sigma-Aldrich), 5.5 mM glucose (Sigma-Aldrich) and 10% human pooled serum, and stimulated with RPMI 1640, 0.1–10 μg ml^{-1} P3C (EMC Microcollections), 0.1–100 ng ml^{-1} LPS from E. coli (Sigma-Aldrich), 10 μg ml^{-1} poly(I:C) (Invivogen), 5 μg ml^{-1} Mycobacterium tuberculosis (strain H37Rv) lysate, 1×10^6 organisms ml^{-1} E. coli (ATCC 35218) or 1×10^6 organisms ml^{-1} S. aureus (clinical isolate). Unless stated otherwise, monocytes were stimulated with 10 ng ml^{-1} LPS or 10 μg ml^{-1} P3C for 24 h.

Monocytes were treated with 2DG (Sigma-Aldrich) to block glycolysis, with rotenone (Sigma-Aldrich) in combination with antimycin A to block complex I of the ETC, with TRIF inhibitory peptide (Pepinh-TRIF; InvivoGen) or with control peptide (Pepinh-Control; InvivoGen) to inhibit TRIF signalling, with GW9962 (Sigma-Aldrich) to inhibit PPAR-γ, with rosiglitazone (Sigma-Aldrich) to activate PPAR-γ, or with dimethyl itaconate (Sigma-Aldrich). For experiments related to hypoxia, monocytes were stimulated with LPS or P3C for 24 h and then incubated in hypoxic (1% oxygen) or normoxic (20% oxygen) conditions for 2 h before measuring the phagocytic capacity of the monocytes.

Cell culture supernatants were collected and stored at −20 °C. Cells were lysed in TRIzol reagent (Invitrogen) and stored at −80 °C until RNA isolation was performed, or stored at −80 °C until processed for western blot analysis as described below.

Cytokine measurements

The production of IL-1β and TNF-α was measured using commercial enzyme-linked

immunosorbent assay (ELISA) kits (R&D Systems), and IL-6 and IL-10 concentrations were also measured by ELISA (Sanquin).

Lactate measurements

Lactate was measured from cell culture supernatants after perchloric acid precipitation. The resulting supernatants were neutralized with NaOH. An enzymatic assay, in which lactate was oxidized and the resulting H_2O_2 was coupled to the conversion of Amplex Red reagent to fluorescent resorufin by horseradish peroxidase, was used to determine lactate levels. Either 30 µl lactate standard or 200-fold diluted sample was added to a black, 96-well, flat-bottomed plate, followed by 30 µl reaction mix, which consisted of 0.6 µl 10 U ml^{-1} horseradish peroxidase (Sigma-Aldrich), 0.6 µl 100 U ml^{-1} lactate oxidase (SigmaAldrich), 0.3 µl 10 mM Amplex Red reagent (Life Technologies) and 28.5 µl PBS. The assay was incubated for 20 min at room temperature, and the fluorescence of resorufin (excitation/emission maxima of 570/585 nm) was measured on a 96-well plate reader (Biotek).

Extracellular flux analysis

Real-time OCR and ECARs of monocytes were analysed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). In short, basal metabolic rates of monocytes seeded in quintuplicate were determined during four consecutive measurements in unbuffered Seahorse medium (8.3 g DMEM powder, 0.016 g phenol red and 1.85 g NaCl in 1 l Milli-Q water, pH set at 7.4 at 37 °C; sterile filtered) containing 5.5 mM glucose and 2 mM L-glutamine. After three basal measurements, three consecutive measurements were taken following the addition of 1.5 µM oligomycin, 1 mM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and 2 µM antimycin together with 1 µM rotenone. Glucose (20 mM) and pyruvate (1 mM) were added together with FCCP to fuel maximal respiration. All compounds used during the Seahorse runs were acquired from Sigma-Aldrich. SRC was determined as the absolute increase in OCR after FCCP injection compared with basal OCR. Equal cell numbers in the wells were assessed by measuring total DNA content using a Quant-iT dsDNA Assay kit (Thermofisher Scientific). The 96-well plates for Seahorse measurements were pretreated with Cell-Tak Cell and Tissue Adhesive (Corning).

Mitochondrial respiratory chain enzyme measurements

Assays for the measurements of the activity of the mitochondrial respiratory chain enzymes and citrate synthase were based on previously described methods[32–34]. The assays were performed on a KoneLab autoanalyser as described previously[35].

Western blotting

Monocytes were lysed in 100 µl lysis buffer (1 M Tris buffer, pH 7.4, 0.5 M EDTA, 5 M NaCl, 10% ND40, 0.5 M NaF, 2.5% sodium deoxycholate, PhosSTOP (Roche) and cOmplete (Roche)).

The cell homogenate was frozen, thawed and processed for western blot analysis according to the manufacturer's instructions. Western blotting was carried out using Mini-PROTEAN TGX precast gels (Bio-Rad). Proteins were transferred using the Trans-Blot Turbo system (Bio-Rad) according to the manufacturer's instructions. Primary antibodies against the following proteins were used: actin (Sigma-Aldrich), phospho-AKT (S473) (Cell Signalling), total AKT (Cell Signalling), hydroxylated HIF-1α (Cell Signalling), PARP (Cell Signalling) and caspase-3 (Cell Signalling).

Phagocytosis assay

For the phagocytosis assay, 1.5×10^5 CD14+ monocytes were seeded into a 96-well plate and stimulated for 24 h with RPMI 1640, LPS (10 ng ml^{-1}) or P3C (10 µg ml^{-1}) (four replicates each). Inhibitors (1 µM rotenone or 1 mM 2DG) were added to the cells 30 min before the phagocytosis assay. Phagocytosis was assessed using a Vybrant Phagocytosis Assay kit (Molecular Probes).

Cell viability assessment by annexin V staining

The percentage of cells that underwent early or late apoptosis was determined by labelling with annexin V–fluorescein isothiocyanate (Biovision) and staining with propidium iodide (Sigma-Aldrich), according to the manufacturer's instructions. Treated CD14+ cells were gently scraped after incubation with Versene solution (Gibco) for 15 min on ice, washed with PBS and resuspended in 200 µl RPMI 1640. The cells were incubated on ice in the dark with 1 µl annexin V–fluorescein isothiocyanate for 15 min, followed by a 5 min incubation with 1.5 µl propidium iodide. The relative level of apoptotic cells was detected by flow cytometry within 1 h, using a FC500 flow cytometer (Beckman Coulter), and the data were analysed using Kaluza 1.3 software (Beckman Coulter).

RNA isolation and real-time quantitative PCR (qPCR)

RNA was isolated from CD14+ cells using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. RNA was transcribed into complementary DNA by reverse transcription using an iScript cDNA Synthesis kit (Bio-Rad). Primer sequences used for the qPCR are listed in Supplementary Table 1. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for qPCR in an AB Step One Plus Real-time PCR System (Applied Biosystems) or a CFX384 Real-Time PCR Detection System (Bio-Rad). qPCR data were normalized to the housekeeping gene human β2-microglobulin.

Metabolite measurements

CD14+ monocytes (3×10^6) were seeded in each well of a six-well plate and stimulated for 24 h with RPMI 1640, LPS (10 ng ml^{-1}) or P3C (10 µg ml^{-1}) (two replicates each). Dry cell pellets were flash frozen and stored at −80 °C. Of note, the same samples were used

for both transcriptomics and metabolomics. Samples were shipped to Metabolon for processing and measurement. A total of 512 compounds of known structural identity were detected. Samples were prepared using the automated MicroLab STAR system from Hamilton Company. Raw data were extracted, peak identified and quality-control processed using Metabolon's hardware and software. Peaks were quantified by measurement of the area under the curve. Metabolite data normalized to protein concentration (measured by a Bradford assay; Metabolon) was used for all analyses. MetaboAnalyst 3.0 was used to explore and characterize the data set. Data were autoscaled, and an unsupervised analysis using PCA was performed to determine clustering and separation[36]. A heatmap representing the top 25 metabolites ranked by analysis of variance was generated.

Microarray analysis

RNA was purified from human CD14+ monocytes using TRIzol reagent (Invitrogen), followed by an additional round of purification with RNeasy Minikit columns (Qiagen). RNA quality was assessed using RNA 6000 nanochips on an Agilent 2100 Bioanalyzer (Agilent). Purified RNA (100 ng) was labelled with a WT PLUS Reagent kit (Affymetrix) and hybridized to a Human Gene 1.1 ST array plate (Affymetrix). Hybridization, washing and scanning were carried out on a GeneTitan platform (Affymetrix) according to the manufacturer's instructions. Arrays were normalized using the robust multiarray average method[37,38]. Probe sets were defined according to the method of Dai et al[39]. In this method, probes are assigned to Entrez IDs as a unique gene identifier. The P values were calculated using an intensity-based moderated t-statistic[40]. The microarray data have been submitted to the Gene Expression Omnibus (accession no. GSE78699). Expression changes in whole blood ex vivo (GSE55375)[41] and in vivo (GSE6269)[42] were extracted from publically available microarray data sets using the analysis pipeline described above.

Electron microscopy

For electron microscopy analysis of mitochondria, cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed for 1 h in 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M cacodylate buffer. After being washed in buffer, the cells were dehydrated in an ascending series of aqueous ethanol solutions and subsequently transferred via a mixture of propylene oxide and Epon to pure Epon 812 as embedding medium. Ultrathin grey sections (60–80 nm) were cut, contrasted with aqueous 2% uranyl acetate, rinsed and counterstained with lead citrate, air dried and examined in a JEM1010 electron microscope (JEOL) operating at 80 kV.

Statistics

For normally distributed parametric data, Student's t-test was used. For non-parametric data, the Wilcoxon signed-rank test was used.

Data availability

The data that support the findings of this study are available from the corresponding author on request. Microarray data is publically available in the Gene Expression Omnibus under accession no. GSE78699.

RESULTS

LPS uniquely downregulates OXPHOS compared with other bacterial stimuli

$CD14^+$ human monocytes were stimulated with a panel of commonly used concentrations of TLR ligands (LPS or P3C) or whole-pathogen lysates (Escherichia coli, Staphylococcus aureus or Mycobacterium tuberculosis) for 24 h to investigate changes in metabolism and cytokine production. An increased extracellular acidification rate (ECAR) (**Fig. 1a**) and lactate production (**Fig. 1b**) in response to all stimuli suggested a universal increase in glycolysis following microbial stimulation. This was accompanied by changes in gene expression of enzymes in the glycolysis pathway. Specifically, expression of the first enzyme of glycolysis, hexokinase 2 (HK2), and the rate limiting enzyme fructose 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) was increased by all stimuli (**Fig. 1c**). Cytokine production (tumour-necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β and IL-10), a function that has been linked to changes in cellular metabolism[6], was also increased by all stimuli (**Fig. 1d**).

Next, we assessed the oxidative capacity of monocytes by measuring oxygen consumption rate (OCR) and spare respiratory capacity (SRC). SRC is an indicator of the total mitochondrial capacity of a cell, which represents the amount of spare energy available during periods of stress[12]. As reported previously[13], LPS lowered the basal OCR and SRC of human monocytes. In contrast, P3C and bacteria lysates increased basal OCR and SRC (**Fig. 2a**,**b**). Supporting these results, LPS decreased the activity of several enzymes in the mitochondrial electron transport chain (ETC), whereas P3C increased their activity (**Fig. 2c**). Similarly, microarray analysis revealed lower expression levels of genes that comprise the five ETC complexes in monocytes stimulated with LPS compared with P3C (**Fig. 2d**).

Protein levels of classical regulators of energy metabolism, AKT and HIF-1α, were equally activated after LPS or P3C stimulation (**Fig. 2e**), suggesting that these regulators are unlikely to control OXPHOS. Mitochondrial function has recently been suggested to be linked to mitochondrial morphology[14]. Using electron microscopy, the mitochondria appeared to be larger in LPS- compared with P3C-stimulated monocytes (**Fig. 2f**).

The transcriptome of LPS- versus P3C-treated monocytes reveals specific patterns of metabolic rewiring

To validate the expression profiles of LPS- and P3C-stimulated monocytes, we used a publically available data set of ex vivo LPS- or P3C-stimulated whole blood. Principle component analysis (PCA) using genes of selected metabolic pathways derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed a clear separation of untreated versus LPS- versus P3C-treated cells in both data sets (**Fig. 3a**,**b**). We then compared the expression levels of individual genes in each metabolic pathway and, as expected, observed robust changes in gene expression induced by LPS- or P3C-stimulated monocytes (**Supplementary Fig. 1a**).

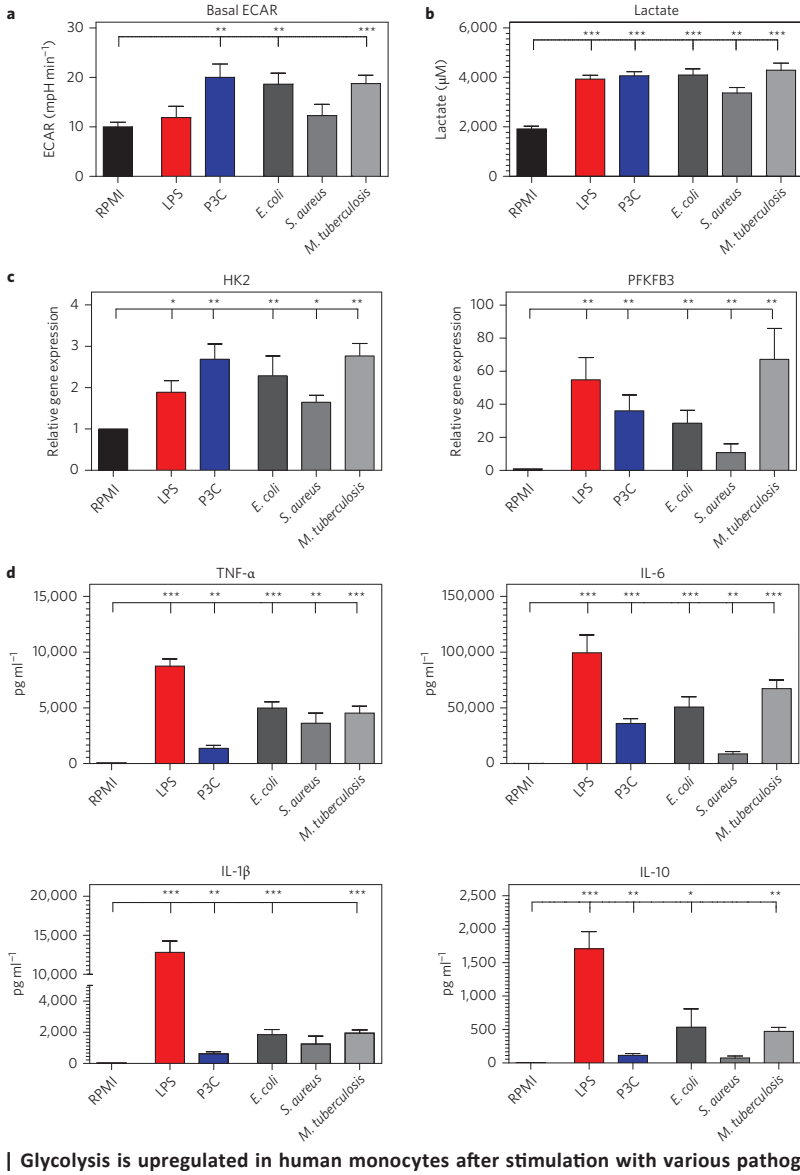

Figure 1 | Glycolysis is upregulated in human monocytes after stimulation with various pathogenic stimuli for 24 h.

a–d, ECAR (**a**), lactate production (**b**), expression of glycolytic enzymes (**c**) and cytokine production (**d**) of human monocytes treated with TLR ligands or whole-pathogen lysates were measured. All data (means ± s.e.m.) are from two experiments with a total of six to eight donors. *P < 0.05, **P < 0.01, ***P < 0.001 (paired two-tailed Student's t-test).

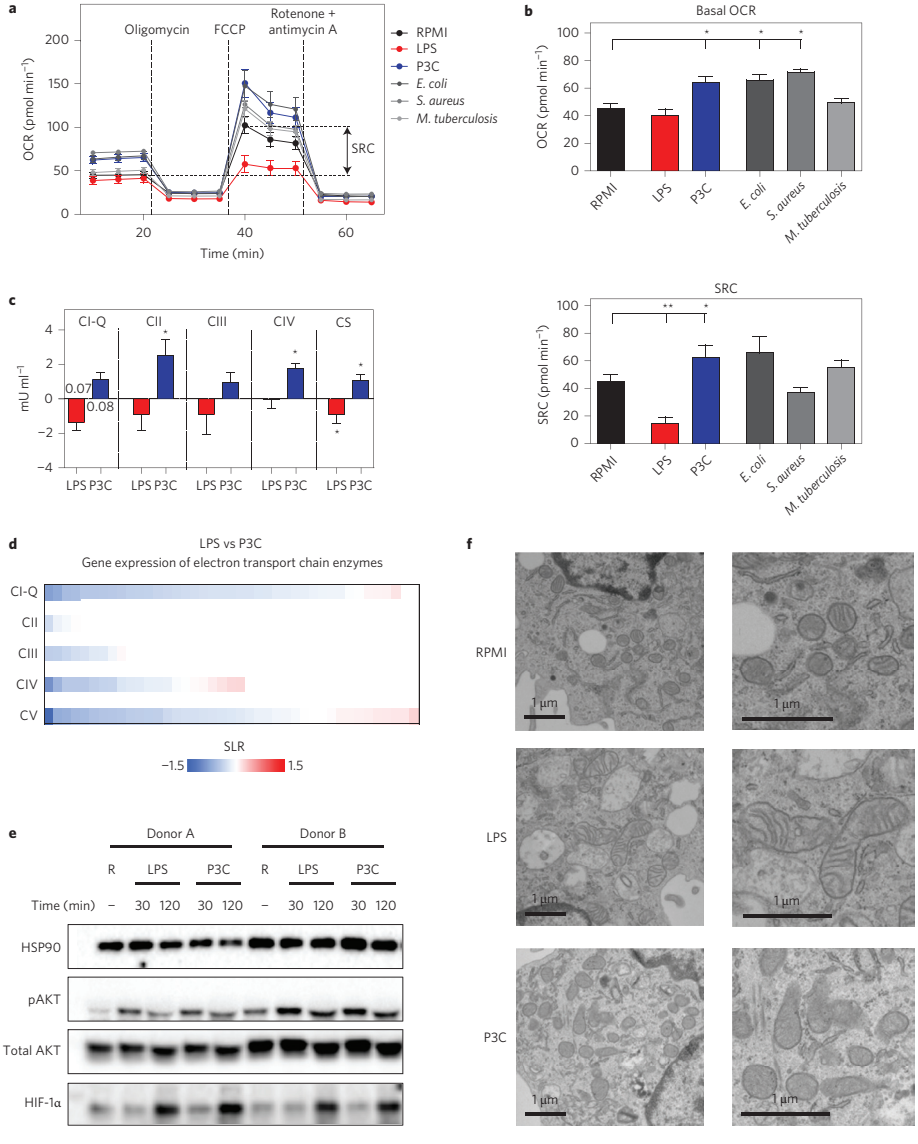

Figure 2 | LPS is unique in downregulating OXPHOS in human monocytes.
a, Basal OCR and real-time changes in the OCR of human monocytes stimulated with TLR ligands or whole-pathogen lysates, assessed during sequential treatment with oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and antimycin A + rotenone. **b**, Basal OCR and SRC of monocytes treated with TLR ligands or whole-pathogen lysates. **c**, Enzyme activity of mitochondrial complexes (C) I–IV (Q, ubiquinone) and citrate synthase (CS) in monocytes stimulated with LPS or P3C, normalized to untreated monocytes. **d**, Gene expression data of mitochondrial complexes I–V of monocytes stimulated with LPS or P3C. Expression levels are presented as signal log ratios (SLR), with red representing higher and blue lower gene expression in LPS- versus P3C-stimulated monocytes. **e**, Immunoblot of phosphorylated AKT (pAKT), total AKT and hydroxylated HIF-1α in human monocytes stimulated with LPS or P3C for 30 or 120 min. R denotes untreated cells. HSP90 served as a loading control. **f**, Mitochondrial morphology analysed by electron microscopy. Data (means ± s.e.m.) are from two experiments with a total of six to eight donors (**a**,**b**), two experiments with a total of seven donors (**c**), one experiment with five donors (d), a representative experiment with three donors (**e**) or one experiment with one donor (**f**). *P < 0.05, **P < 0.01 (paired two-tailed Student's t-test).

Overall, LPS led to a decrease in expression of metabolic genes compared with P3C, most noticeably those in the TCA cycle, OXPHOS and peroxisome proliferator-activated receptor (PPAR) signalling pathways (**Fig. 3c** and **Supplementary Fig. 1a**). These observations were validated in the ex vivo whole-blood data set (**Fig. 3d**).

Of note, a similar PCA performed with publically available expression data of peripheral blood mononuclear cells (PBMCs) from individuals with acute E. coli versus S. aureus infections showed clustering that was not as distinct as LPS- versus P3C-stimulated monocytes (**Supplementary Fig. 1b**). Similarly, the metabolic gene expression profiles of LPS- versus P3C-stimulated monocytes showed little overlap with the expression profiles of PBMCs from individuals with acute E. coli versus S. aureus infection (**Supplementary Fig. 1c**). Unlike a pure population of monocytes, PBMCs are a heterogeneous mix of circulating immune cells with distinct bioenergetic profiles and distribution of respiratory chain proteins[15].

LPS-treated monocytes displayed a pronounced decrease in the expression of genes involved in lipid handling and usage (**Fig. 3d** and **Supplementary Fig. 1a**). Many genes in this pathway, including PPAR-γ and its targets (LPL, FABP4 and PDK4), had lower expression in LPS- versus P3C-stimulated monocytes, suggesting that PPAR-γ may regulate the difference in OXPHOS between the two stimuli (**Fig. 3e**). To test this hypothesis, we inhibited PPAR-γ using the antagonist GW9662 and found that basal OCR was mostly unaffected (**Supplementary Fig. 2a**). GW9662 decreased IL-1β and increased IL-10 levels (**Supplementary Fig. 2b**). Treatment with rosiglitazone, a PPAR-γ agonist, had little effect on OCR and cytokine production in LPS- and P3C-stimulated monocytes (**Supplementary Fig. 2a,b**). Together, these results do not implicate PPAR-γ as the primary regulator of the oxidative capacity of P3C-stimulated monocytes. Instead, PPAR-γ may be upregulated as a consequence of the higher levels of polyunsaturated fatty acids found in monocytes stimulated with P3C compared with LPS (**Fig. 3f**). These fatty acids would function as natural ligands of PPAR-γ.

The TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway is specifically activated downstream of TLR4 but not TLR2. Therefore, we tested whether poly(I:C), a TLR3 ligand that signals exclusively via TRIF, would induce a similar pattern of metabolic rewiring to LPS. As with P3C, stimulation with poly (I:C) increased ECAR and OCR (**Supplementary Fig. 2c**), suggesting that TRIF is not involved in the metabolic rewiring of LPS-treated cells. Additionally, inhibition of TRIF using an inhibitory peptide did not affect cytokine secretion or metabolic gene expression of LPS-treated cells (**Supplementary Fig. 2d,e**).

LPS has dose-dependent effects on OXPHOS in human monocytes

The concentrations of 10 ng ml^{-1} LPS and 10 μg ml^{-1} P3C used in our experiments are those used most commonly in the literature. To test whether the differences in regulation of OXPHOS were dose dependent, we stimulated monocytes with a range of LPS (from 0.1 to 100 ng ml^{-1}) or P3C (from 0.1 to 10 μg ml^{-1}) doses and measured metabolic and functional outcomes. Various doses of LPS (1–100 ng ml^{-1}) induced greater levels of IL-1β (**Fig. 4a**) and

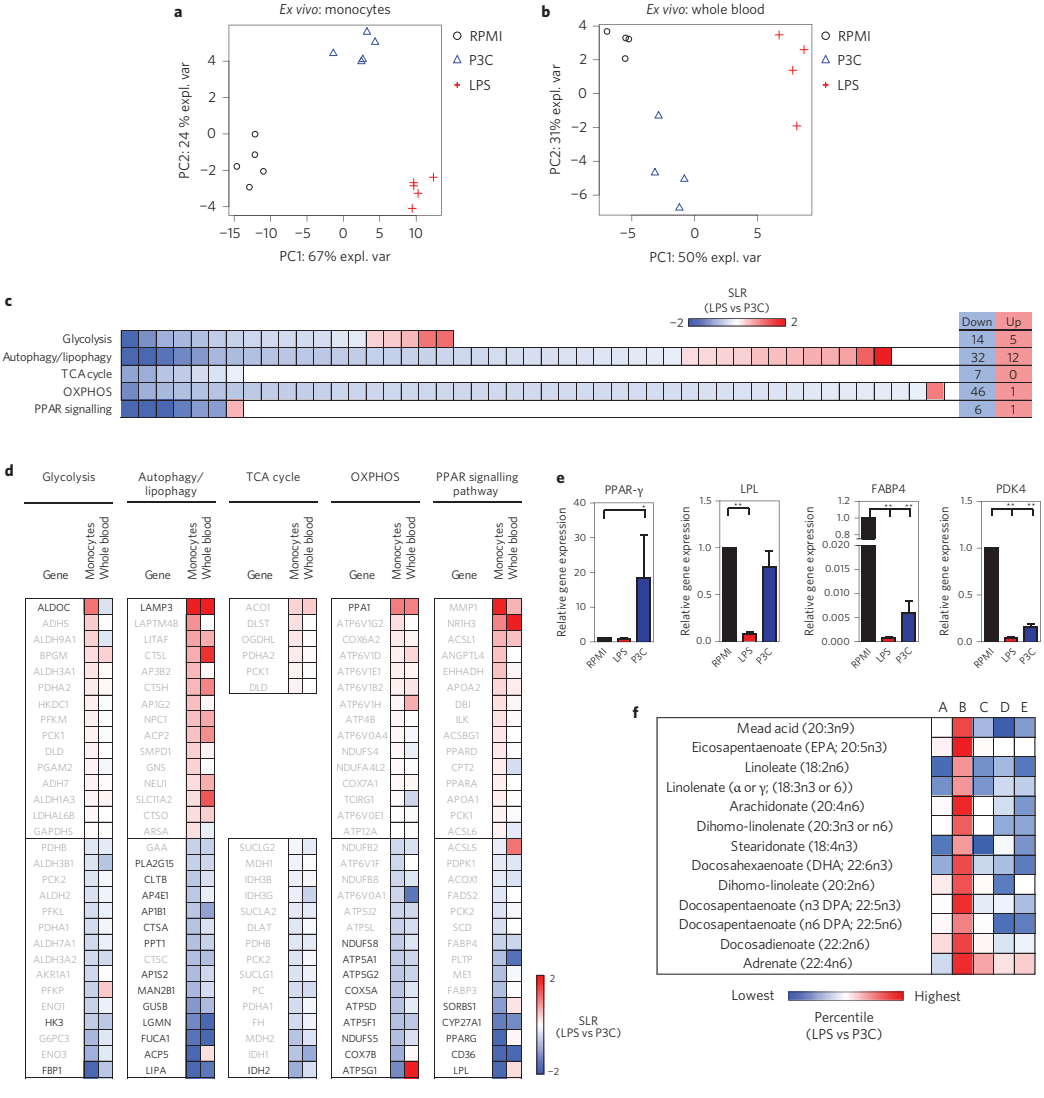

Figure 3 | Human monocytes stimulated with LPS versus P3C show differential expression of metabolic genes. **a**–**d**, Microarray analysis of human monocytes stimulated with LPS (10 ng ml^{-1}) or P3C (10 μg ml^{-1}) for 24 h (monocytes), and whole blood stimulated with LPS (1 ng ml^{-1}) or P3C (200 ng ml^{-1}) for 24 h (whole blood; data retrieved from Gene Expression Omnibus accession no. GSE55375). Shown are PCA plots based on genes from selected KEGGderived metabolic pathways, showing the percentage of explained variance (expl. var.) (**a**,**b**); heat map of genes from selected KEGG-derived metabolic pathways in human monocytes. Expression data are presented as the SLR of LPS versus P3C. For each pathway, significantly different upregulated (red) or downregulated (blue) genes ($q < 0.01$) are shown (**c**); and heat map of genes from selected KEGG-derived metabolic pathways in human monocytes and whole blood. Expression data are presented as SLR of LPS versus P3C. For each pathway, the top 15 most differentially higher (red) or lower (blue) expressed genes in LPS- versus P3C-stimulated monocytes are shown. Significantly different regulated genes ($q < 0.01$) are shown in bold (**d**). **e**, Relative gene expression of LPS- and P3C-stimulated monocytes measured by real-time quantitative PCR. Heat map showing the relative abundance of polyunsaturated fatty acids in LPS- versus P3C-stimulated monocytes per donor (A–E). Data (means ± s.e.m.) are from one experiment with five donors (**a**,**c**,**d**,**f**) or from two experiments with a total of eight donors (**e**). *$P < 0.05$, **$P < 0.01$; (paired two-tailed Student's t-test).

other cytokines (**Supplementary Fig. 3a**) compared with P3C, whereas the lowest dose of LPS (0.1 ng ml^{-1}) was similar to P3C. Basal ECAR was similar for all conditions (**Fig. 4b**); however, OCR and SRC varied across the different doses of LPS but not P3C (**Fig. 4c**). The higher doses of LPS (1–100 ng ml^{-1}) decreased the oxidative capacity of monocytes, whereas the lowest dose (0.1 ng ml^{-1}) increased OXPHOS similarly to P3C.

To test whether these changes were reflected at a transcriptional level, we measured the expression of genes involved in glycolysis (PFKFB3 and GAPDH) and fatty acid metabolism (PPAR-γ, LPL, LIPA and CPT-1α) (**Fig. 4d,e**). In line with our findings, expression of these genes in monocytes stimulated with the low dose of LPS (0.1 ng ml^{-1}) was comparable with monocytes stimulated with P3C (10 μg ml^{-1}), but not with 10 ng ml^{-1} LPS.

Lastly, time-dependent differences in OXPHOS were observed between the standard doses of 10 ng ml^{-1} LPS and 10 μg ml^{-1} P3C. At 4 h post-treatment with LPS, the basal OCR and SRC of stimulated monocytes were increased. With the exception of E. coli, the stimuli had no effect on OCR and SRC at 4 h post-stimulation. (**Fig. 4f**). Glycolysis, as measured by ECAR and lactate production, was similar among all stimuli (**Supplementary Fig. 3b,c**). Differences in expression of genes involved in glycolysis and fatty acid metabolism were less pronounced after 4 h compared with 24 h between the two conditions (**Fig. 4g,h**). These transcriptional differences in kinetics were validated in the whole-blood data set stimulated with LPS versus P3C (**Supplementary Fig. 3d,e**).

Although metabolic rewiring can be dependent on the maturation and activation state of cells, measurement of human leukocyte antigen (HLA)-DR revealed no differential effect on activation by 0.1 or 10 ng ml^{-1} LPS or by 10 μg ml^{-1} P3C (**Supplementary Fig. 4a,b**). P3C-stimulated monocytes expressed slightly more CD11c and CD83 compared with LPS. Nevertheless, stimulation with 0.1 ng ml^{-1} LPS and 10 ng ml^{-1} LPS led to a similar maturation pattern, despite differences in OXPHOS, suggesting that the extent of monocyte maturation probably cannot explain differences in metabolic rewiring following LPS and P3C stimulation.

Functional consequences: OXPHOS is needed for phagocytosis, while cytokine production relies on both OXPHOS and glycolysis in P3C-stimulated monocytes

We next investigated how cytokine production and phagocytosis are influenced by the different metabolic programmes induced by LPS or P3C. As described elsewhere[16], inhibition of glycolysis with 2-deoxy-D-glucose (2DG) decreased IL-1β (**Fig. 5a**) and other cytokines (**Supplementary Fig. 5a**) in both conditions. Inhibition of OXPHOS using the complex I inhibitor rotenone lowered cytokine production only in P3C-stimulated cells (**Fig. 5b**). This points to a P3C-specific reliance on OXPHOS for cytokine production. In contrast to the results of Kelly et al.[17], IL-10 levels were decreased in rotenone-treated cells after LPS stimulation (**Supplementary Fig. 5b**). Rotenone did not affect cell death, as assessed by annexin V/propidium iodide staining measurement of cleaved caspase-3 and poly(ADP–ribose) polymerase (PARP) (**Supplementary Fig. 5c,d**).

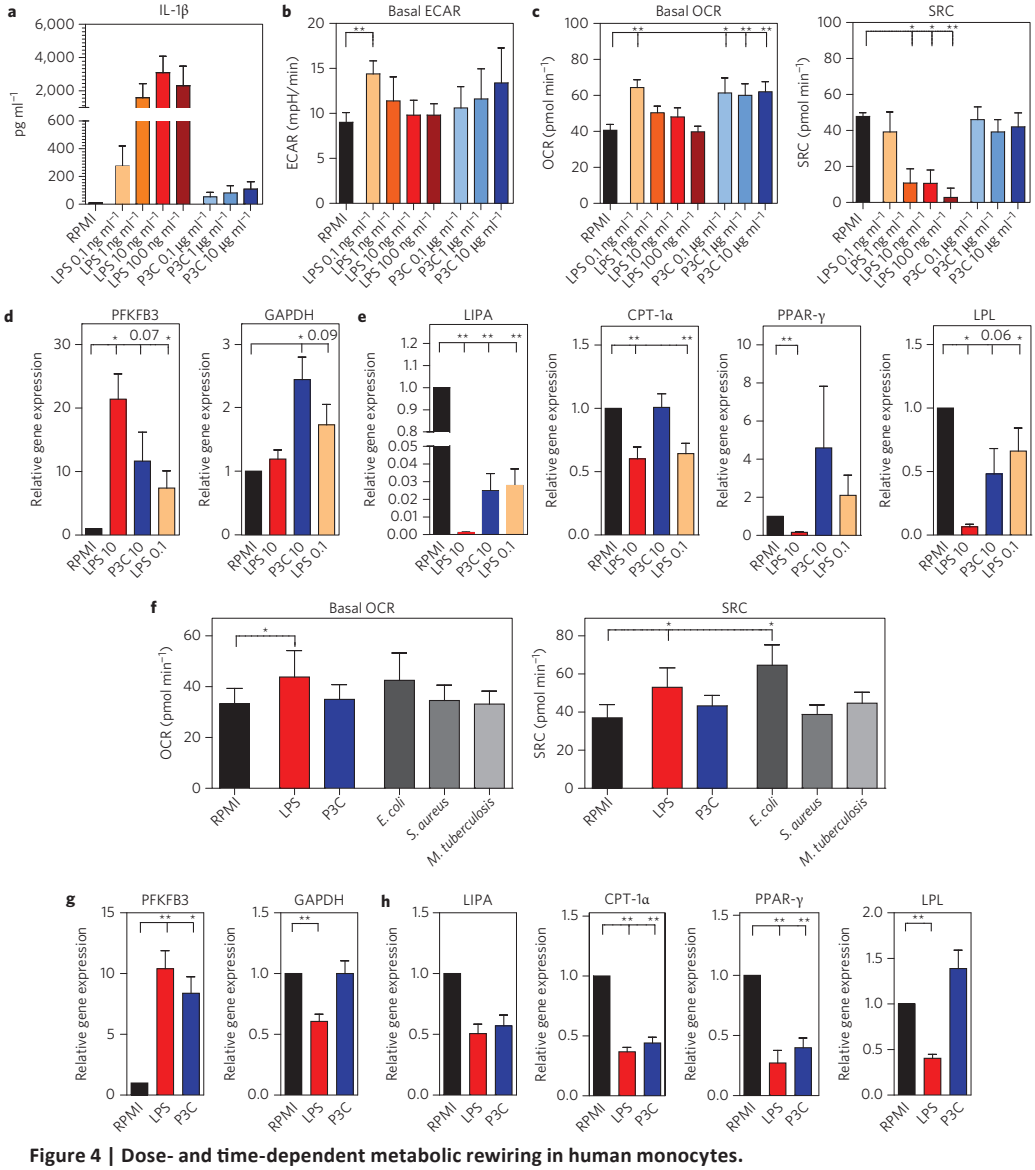

Figure 4 | Dose- and time-dependent metabolic rewiring in human monocytes.
a–**e**, IL-1β production (**a**), basal ECAR (**b**), basal OCR and SRC (**c**), and relative gene expression (**d**,**e**) of human monocytes treated with various doses of LPS and P3C for 24 h. **f**–**h**, Basal OCR and SRC (**f**) and relative gene expression (**g**,**h**) of human monocytes treated with TLR ligands or whole-pathogen lysates for 4 h. Data (means ± s.e.m.) are from one experiment with five donors (**a**–**e**), from two experiments with a total of five to six donors (**f**) or from two experiments with a total of eight donors (**g**,**h**). *P < 0.05, **P < 0.01; exact p values are also indicated in the graphs (paired two-tailed Student's t-test).

Next, we determined the phagocytic capacity of stimulated monoand cytes and found that LPS lowered phagocytic rates compared with P3C (**Fig. 5c**). To determine whether OXPHOS facilitated the higher phagocytic capacity of P3C- versus LPS-treated cells, we inhibited complex I of the ETC with rotenone. Treatment with rotenone significantly decreased the phagocytic capacity of P3C-stimulated monocytes, whereas untreated or LPS-treated monocytes were unaffected. However, lowering of oxygen consumption using hypoxic culture conditions (1 versus 5% O_2) had no effect (**Supplementary Fig. 5e**). 2DG also did not affect the phagocytic capacity of stimulated monocytes, suggesting that OXPHOS, but not glycolysis, is an important determinant of the phagocytic capacity of human monocytes (**Fig. 5c**).

Metabolite analysis reveals pronounced differences in TCA cycle metabolites between LPS- and P3C-stimulated monocytes

To increase our understanding of differences in metabolic rewiring between LPS- and P3C-treated monocytes, we measured intracellular levels of over 500 metabolites. PCA confirmed a robust separation in metabolic responses following LPS and P3C stimulation (**Fig. 5d**). A heat map of the top 25 differentially regulated metabolites showed increased levels of succinate, citrate and itaconate specifically in LPS-treated cells, whereas gulonate levels were highly increased upon P3C stimulation (**Fig. 5e**). These findings appear to match the two previously described breaks in the TCA cycle at citrate and succinate following LPS stimulation[18], but indicate that these breaks might be absent in P3C-stimulated monocytes (**Fig. 5f**). An increase in expression of IRG1, which converts citrate to itaconate, in LPS- but not in P3C-stimulated cells further supported these findings (**Fig. 5g**). Moreover, LPS stimulation decreased the expression of two TCA cycle enzymes, IDH1 and SDH, present after each break in the TCA cycle (**Fig. 5g**).

As shown above (**Fig. 5e**), itaconate has been reported to accumulate intracellularly in LPS-treated mouse macrophages[19]. To test whether itaconate plays a functional role in cytokine production, membrane-permeable dimethyl itaconate was added before LPS or P3C stimulation. Levels of IL-1β, IL-6 and IL-10 were all decreased, whereas levels of TNF-α were mainly unaffected following LPS or P3C stimulation (**Supplementary Fig. 6a**). Moreover, dimethyl itaconate did not change glycolytic metabolism, as assessed by lactate production, in either LPS- or P3C-stimulated monocytes (**Supplementary Fig. 6b**). These results highlight the importance of intracellular metabolites in the regulation of immune cell function and demonstrate their immunomodulatory potential independent of the pathogenic stimulus.

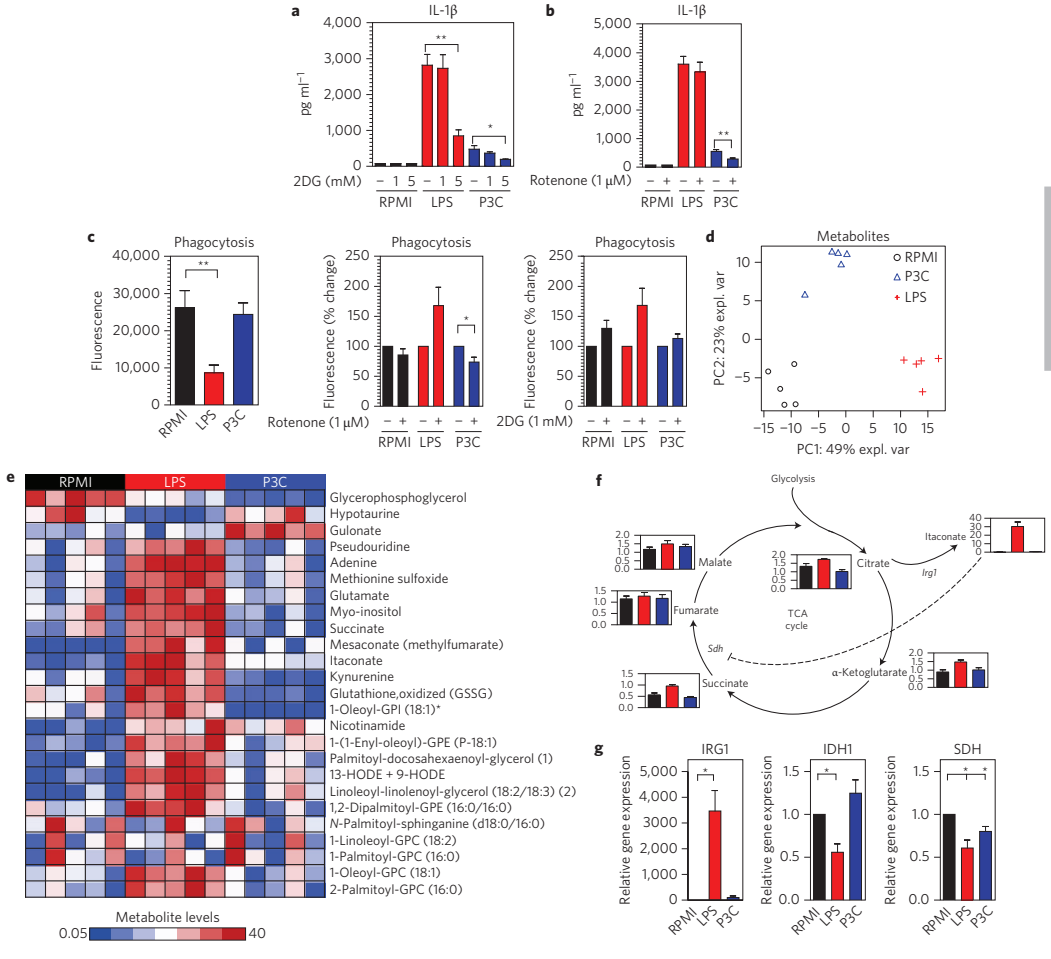

Figure 5 | Differential metabolic rewiring of human monocytes stimulated with LPS or P3C leads to functional differences in cytokine production and phagocytosis.

a,**b**, IL-1β production of monocytes pre-treated with 2DG (**a**) or rotenone (**b**) for 1 h before addition of LPS or P3C for 24 h. **c**, Phagocytosis of monocytes stimulated with LPS or P3C for 24 h and treated with 2DG or rotenone for 30 min before the start of the assay. **d**, PCA plot based on intracellular metabolites. **e**, Heatmap of the top 25 metabolites most differentially regulated in monocytes stimulated with LPS or P3C for 24 h, with red representing higher and blue representing lower abundance. **f**, Scheme showing the relative abundance of TCA cycle intermediates in monocytes stimulated with LPS or P3C for 24 h. Units on the y axis are given as relative metabolite levels normalized to cellular protein content. **g**, Relative expression of genes involved in regulating levels of TCA cycle intermediates. Data (means ± s.e.m.) are from two experiments with a total of five donors (**a**–**c**) or from one experiment with five donors (**d**–**g**). *P < 0.05, **P < 0.01 (paired two-tailed Student's t-test).

DISCUSSION

Evidence acquired mostly from in vitro stimulation of myeloid cells with LPS has pinpointed an increase in glycolysis and a decrease in OXPHOS as characteristic of activated pro-inflammatory innate immune cells[20]. Our study recapitulates these findings in human monocytes exposed to LPS, but also demonstrated that a decrease in OXPHOS following activation of myeloid cells is not a universal response to pathogenic stimuli. Furthermore, we have linked different metabolic routes adopted by human monocytes following microbial stimulation to host defence functions, such as cytokine release and phagocytosis.

Increased glucose utilization following activation with TLR ligands or bacterial stimuli has been demonstrated previously[9] and has led to the identification of various upstream regulators of glycolysis, including AKT and HIF-1α[6]. Our studies confirmed these findings in human monocytes[6], as inhibition of glycolysis with 2DG reduced IL-1β production after stimulation with LPS or P3C. Although some conflicting results in murine dendritic cells[9] versus macrophages[6] have been reported, in human monocytes 2DG also decreased TNF-α, IL-6 and IL-10 production. Together with higher glycolytic rates, LPS-stimulated monocytes displayed decreased OXPHOS and metabolic flexibility. In contrast, stimulation with P3C or lysates from E. coli, S. aureus or M. tuberculosis resulted in increased or unaltered OXPHOS and metabolic flexibility. Thus, the classical Warburg effect induced by LPS is not a universal prerequisite for pro-inflammatory cytokine responses.

Pathogen-specific metabolic rewiring may fuel different functional outputs. In this study, we showed that the phagocytic capacity of human monocytes is related to their oxidative capacity. LPS stimulation lowered OXPHOS as well as the phagocytic capacity of monocytes in comparison with P3C stimulation. Similarly, inhibition of complex I of the ETC with rotenone decreased the phagocytic capacity of P3C-stimulated monocytes. Phagocytosis is an energy-demanding process[21], and OXPHOS, unlike glycolysis, is characterized by a high ATP yield. Thus, OXPHOS seems to be more capable of supporting the bioenergetic demands of phagocytosis. Interestingly, the phagocytic capacity of monocytes was unaffected by short-term hypoxia, which, while activating HIF-1α, may not result in complete inhibition of mitochondrial respiration.

Previous studies have shown that macrophages are dependent on several metabolic pathways for phagocytosis[22,23], making it likely that other pathways, such as fatty acid oxidation, also contribute to phagocytosis. Similarly, as seen for phagocytosis, inhibition of OXPHOS decreased cytokine production from P3C- but not LPS-stimulated monocytes, further emphasizing the fact that different stimuli may use convergent or redundant metabolic pathways to execute their inflammatory functions.

We attempted to delineate the differences in OXPHOS between LPS- and P3C-stimulated monocytes in several ways. First, we examined the contribution of TRIF signalling, as both stimuli induce MyD88 signalling, whereas only LPS has been shown to activate TRIF. Poly(I:C),

a TLR3 ligand exclusively recruiting TRIF[24], increased the oxidative capacity of monocytes, thus making it unlikely that signalling via TRIF contributes to the LPS-induced decrease in OXPHOS. Second, we looked at PPAR-γ, which has been shown to promote OXPHOS and stimulate mitochondrial biogenesis in mouse macrophages[25] and has been linked to the regulation of phagocytosis in human macrophages[26]. Although expression of PPAR-γ was greater in P3C- than in LPS-stimulated cells, stimulation or inhibition of PPAR-γ did not affect OXPHOS or the cytokine production of LPS- or P3C-stimulated monocytes. It is more likely that the activation of PPAR-γ by P3C is a consequence of stimulation rather than responsible for P3C-induced metabolic rewiring. Third, mitochondrial morphology was visualized. A recent study linked changes in mitochondrial morphology to the metabolic differences observed between glycolytic T-effector cells and OXPHOS-dependent T-memory cells. Mitochondrial fusion in T-memory cells favoured OXPHOS and fatty acid oxidation, whereas fission in T-effector cells led to loosening of the cristae, which resulted in less efficient transport of electrons through the ETC and in turn promoted glycolysis as an alternative fuel source[27]. Electron microscopy revealed larger mitochondria after LPS stimulation, suggesting that changes in mitochondrial morphology might contribute to differences in OXPHOS and metabolic rewiring after LPS versus P3C stimulation. Last, metabolome analysis was executed and revealed an increased presence of metabolites that have been shown to accumulate in a broken TCA cycle in LPS-stimulated murine macrophages[6,7] in LPS- but not in P3Cstimulated human monocytes. These metabolites, such as citrate, succinate and itaconate, have been identified as important regulators of intracellular metabolism and inflammatory activation. For example, excess citrate is transported into the cytosol, where it is converted into acetyl-CoA for fatty acid synthesis, and into oxaloacetate, which promotes nitric oxide production, which can inhibit components of the mitochondrial ETC via nitrosylation[28]. These metabolites may thus serve to maintain the reduced respiratory capacity of LPS-stimulated monocytes.

Itaconate treatment of LPS-stimulated mouse macrophages has been shown to dampen the inflammatory response by modulating mitochondrial respiration[29]. Likewise, pretreatment of monocytes with itaconate lowered cytokine release not only in LPS- but also in P3C-stimulated cells, demonstrating that itaconate can modulate the inflammatory response of human monocytes, irrespective of the stimulus. The modulatory effects of itaconate may be relevant in septic patients, in whom IRG1, the enzyme responsible for itaconate production, was found to be highly upregulated in their PBMCs[30]. Itaconate also plays a key role in LPS-induced tolerance in mouse macrophages[30].

Additionally, we identified gulonate to be specifically induced in P3C-stimulated monocytes. Gulonate is part of the glucuronic acid pathway, an alternative pathway for the oxidation of glucose that produces activated UDP-glucuronate. Gulonate is ultimately converted to D-xylulose 5-phosphate, which enters the pentose phosphate pathway. Therefore, gulonate and gulonate-derived metabolites may represent an alternative pathway to oxidize glucose in P3C-treated cells. Specific metabolites that accumulate in P3C-treated

cells, including gulonate, may preserve OXPHOS through as yet unidentified mechanisms.

Although LPS and P3C are often used as a model for Gram-negative bacteria (such as E. coli) and Gram-positive bacteria (such as S. aureus) respectively, ex vivo stimulations of human monocytes with LPS, E. coli, P3C or S. aureus led to varied metabolic profiles. In the case of LPS, a low dose (0.1 ng ml^{-1}) induced similar metabolic changes to P3C, whereas higher doses led to the characteristic decrease in OXPHOS. This suggests that differences in signalling strength, rather than qualitative signalling differences between LPS and P3C, may explain the divergent metabolic phenotype. Of much interest, however, are the parallels of low-dose LPS (0.1 ng ml^{-1}) with acute in vivo infections of E. coli, and of high- dose LPS (1 –100 ng ml^{-1}) with sepsis, which has recently been characterized by defects in OXPHOS and energy metabolism in leukocytes[31]. Concentrations of LPS above 0.1 ng ml^{-1} seem to cross a certain signalling threshold at which not only is glycolysis increased but OXPHOS is also reduced to maintain ATP production. These dose-related effects therefore emphasize the complexity of intracellular metabolic adaptations and the importance of selecting representative in vitro models for disease.

This study highlights the complexity of metabolic rewiring in activated human immune cells and demonstrates the importance of understanding pathogen-specific metabolic reprogramming. We propose that each pathogen induces a highly specific metabolic programme in human innate immune cells, thereby determining the functional output of the cells. While some functional outputs, such as cytokine release, may be relatively similar, the underlying metabolic routes may vary significantly. Targeting metabolic routes to alleviate inflammatory conditions or improve antimicrobial host defence thus holds therapeutic promise, but tailor-made approaches are warranted.

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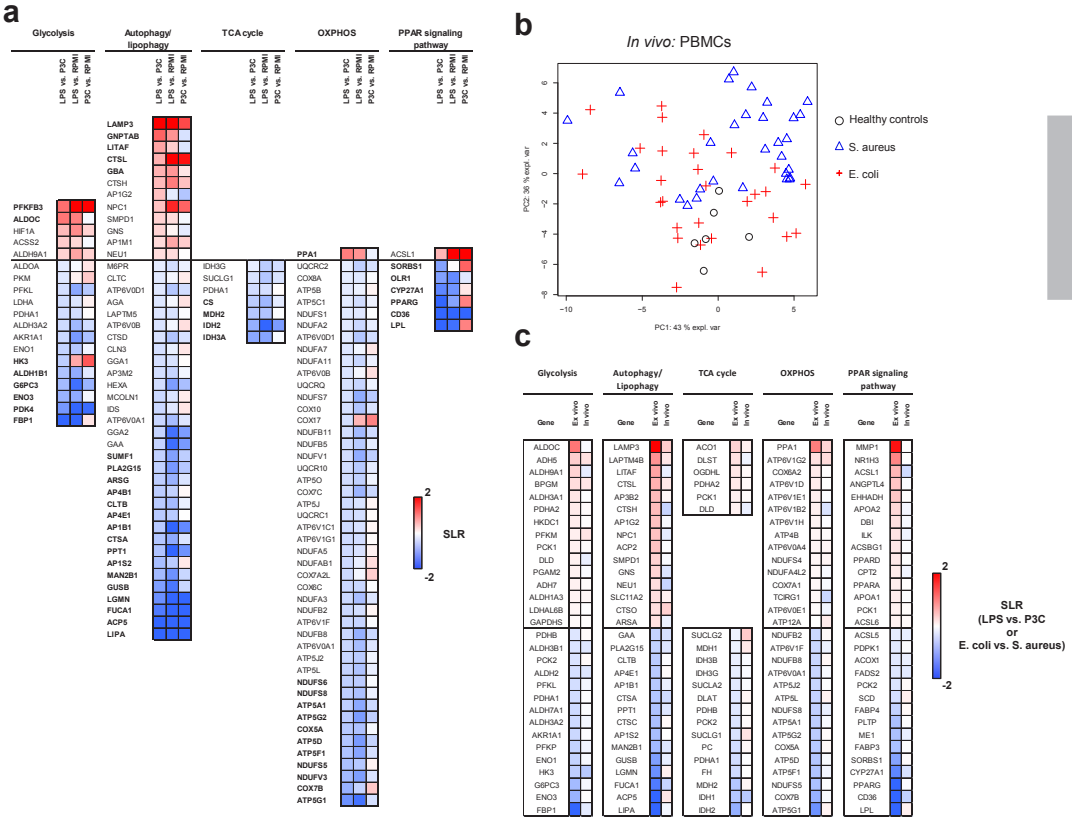
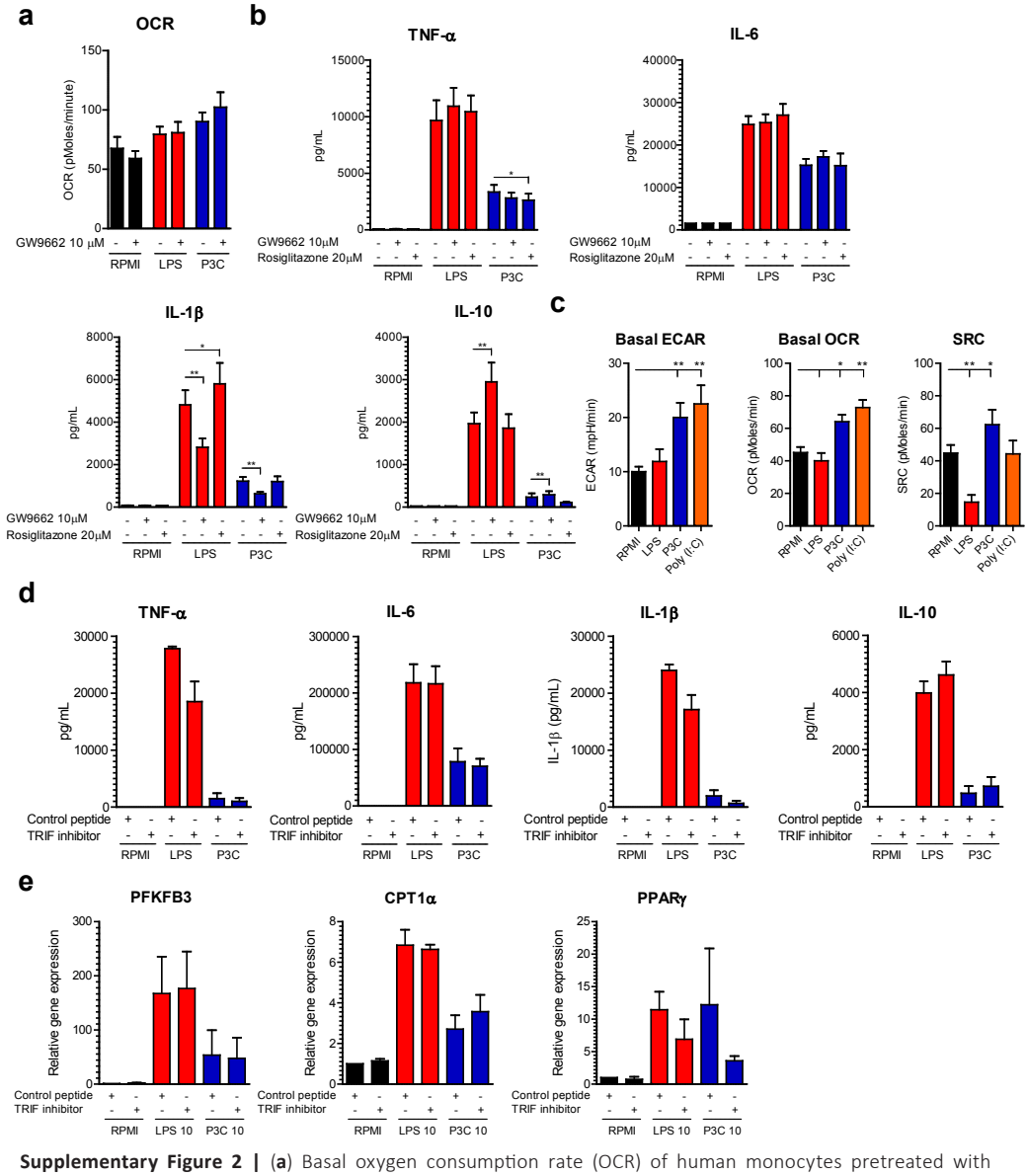
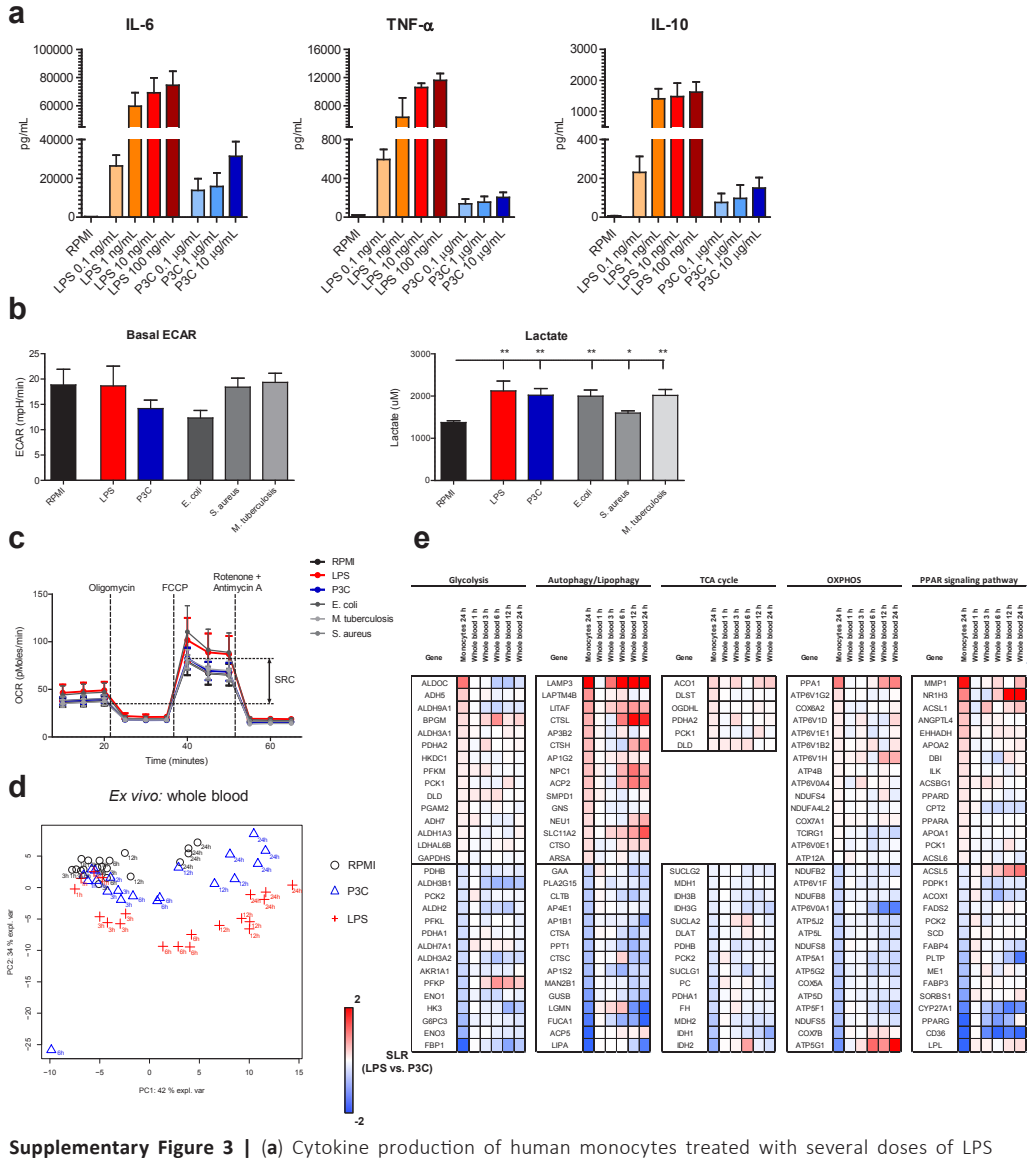
SUPPLEMENTARY FIGURES AND TABLE



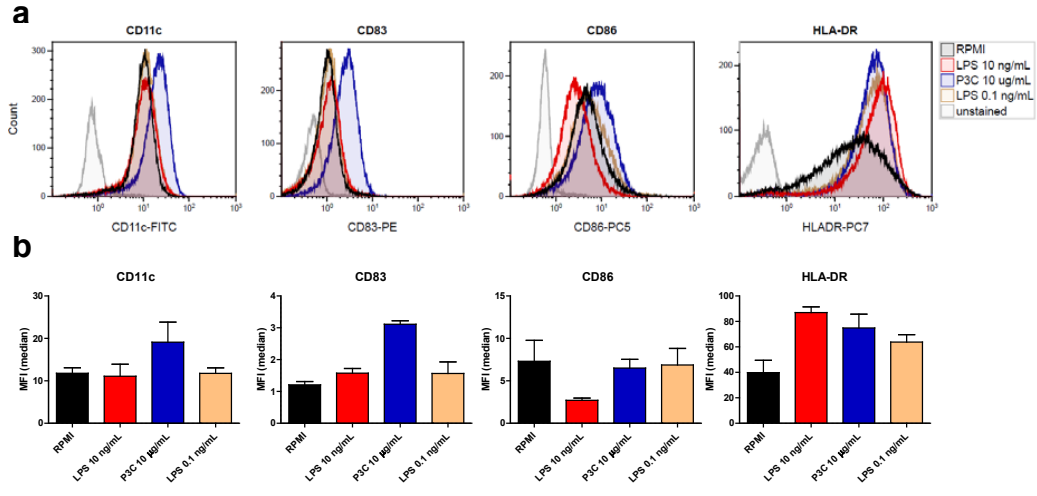
Supplementary Figure 1 | Microarray analysis of the following datasets: human monocytes stimulated with LPS (10 ng/mL) or P3C (10 µg/mL) for 24 h (*ex vivo: monocytes*), or PBMCs isolated from patients with an *E. coli* or *S. aureus* infection (*in vivo: PBMCs;* data retrieved from GSE6269). (**a**) Heat map of genes from selected KEGG-derived metabolic pathways in the *ex vivo* monocyte dataset. Expression data are presented as signal log ratio (SLR) of LPS vs. P3C, LPS vs. RPMI or P3C vs. RPMI. For each pathway significantly different regulated genes (q<0.01) are shown. Genes with a SLR>1.5 or SLR<-1.5 are highlighted in bold. (**b**) PCA plot of the *in vivo* PBMC dataset based on genes part of selected KEGG-derived metabolic pathways. (**c**) Heat map of genes of selected KEGG-derived metabolic pathways. Expression data are presented as signal log ratio (SLR) of LPS vs. P3C or *E. coli* vs. *S. aureus*. For each pathway the top fifteen most differentially higher (red) and lower (blue) expressed genes in LPS- vs. P3C-stimulated monocytes are shown.



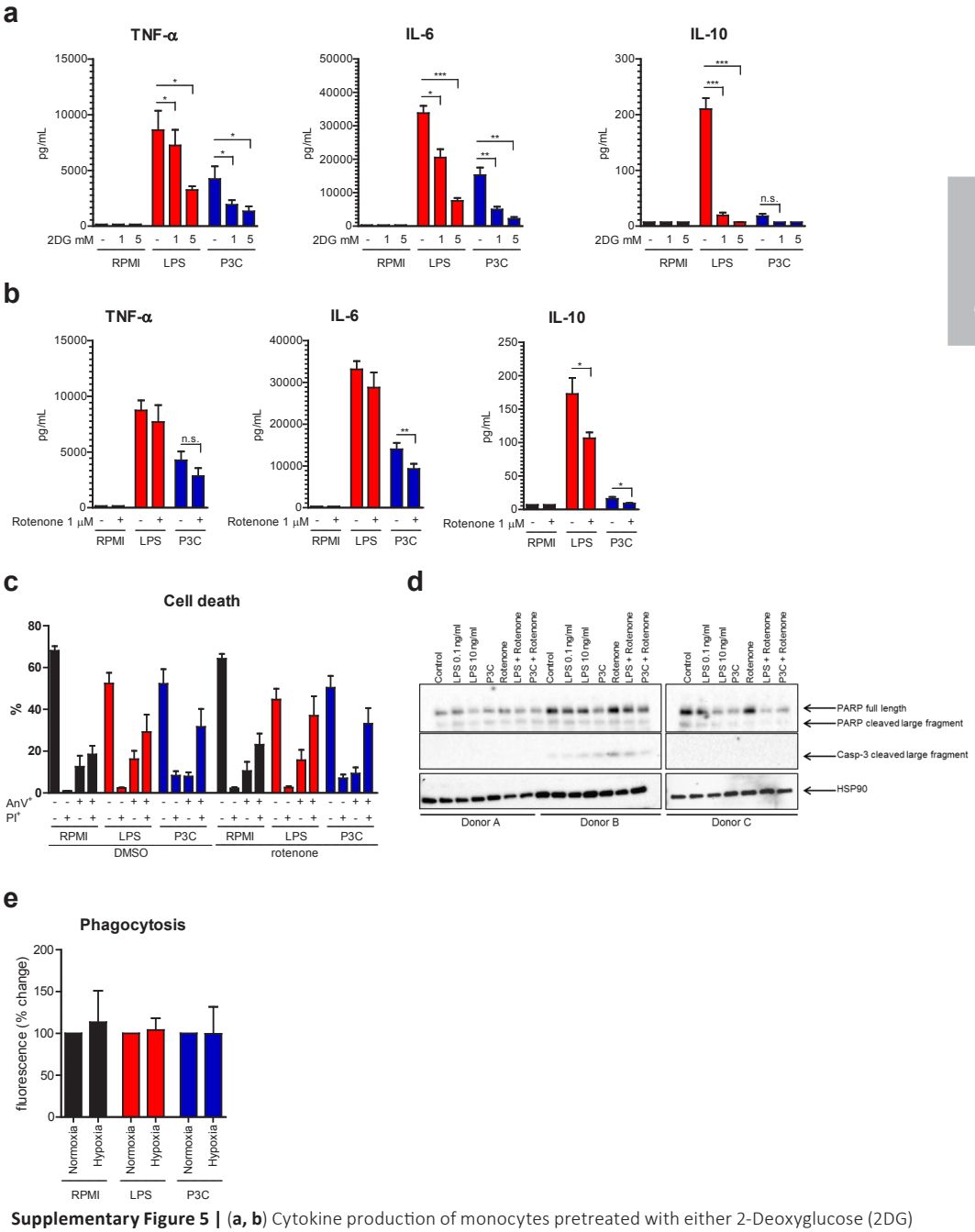
Supplementary Figure 2 | (**a**) Basal oxygen consumption rate (OCR) of human monocytes pretreated with the PPARγ-inhibitor GW9662 for 1 h before addition of LPS or P3C for 24 h. (**b**) Cytokine production of human monocytes pretreated with GW9662 or the PPARγ-activator rosiglitazone for 1 h before addition of LPS or P3C for 24 h. (**c**) Basal extracellular acidification rate (ECAR), OCR and spare respiratory capacity (SRC) of human monocytes treated with various TLR ligands for 24 h. (**d**, **e**) Cytokine production (**d**) and gene expression (**e**) of human monocytes pretreated with either a TRIF-specific inhibitory peptide or a control peptide for 1 h before addition of LPS or P3C for 24 h. Data (mean ± s.e.m.) are from one experiment with five donors (**a**), one experiment with three to five donors (**b**), two experiments with a total of eight donors (**c**) or one experiment with three donors (**d**, **e**). * p<0.05, ** p<0.01 (paired two-tailed t-test).



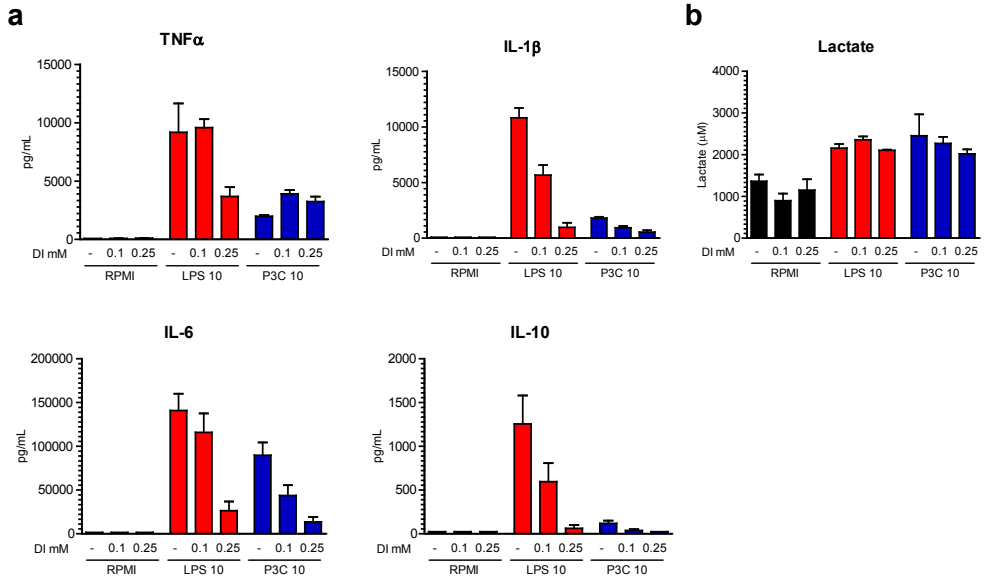
Supplementary Figure 3 | (**a**) Cytokine production of human monocytes treated with several doses of LPS and P3C for 24 h. (**b**) Basal extracellular acidification rate (ECAR) and lactate production of human monocytes stimulated with TLR ligands or whole pathogen lysates for 4 h. (**c**) Basal oxygen consumption rate (OCR) and real-time changes in OCR of human monocytes stimulated with TLR ligands or whole pathogen lysates for 4 h, assessed during the sequential treatment with oligomycin, FCCP and antimycin A/rotenone. (**d**, **e**) Microarray analysis of the following datasets: human monocytes stimulated with LPS (10 ng/mL) or P3C (10 µg/mL) for 24 h (*ex vivo: monocytes*), and whole blood stimulated with LPS (1 ng/mL) or P3C (200 ng/mL) for 1-24 h (*ex vivo: whole blood;* data retrieved from GSE55375). (**d**) PCA plot of the whole blood dataset based on genes part of selected KEGG-derived metabolic pathways. (**e**) Heat map of genes part of selected KEGG-derived metabolic pathways. Expression data are presented as signal log ratio (SLR) of LPS vs. P3C. For each pathway the top fifteen most differentially increased (red) or decreased (blue) genes in LPS- vs. P3C-stimulated monocytes are shown. Data (mean ± s.e.m.) are from one experiment with five donors (**a**) or from two experiments with a total of five to six donors (**b**-**c**). * p<0.05, ** p<0.01 (paired two-tailed t-test).



Supplementary Figure 4 | Maturation markers present on human monocytes stimulated with LPS or P3C for 24 h. (**a**) Representative flow cytometry plots from one donor. (**b**) Mean fluorescent intensity (MFI) representing the presence of maturation markers in monocytes of three donors. Data (mean ± s.e.m.) are from one experiment with three donors.



Supplementary Figure 5 | (**a, b**) Cytokine production of monocytes pretreated with either 2-Deoxyglucose (2DG) (**a**) or rotenone (**b**) for 1 h before addition of LPS or P3C for 24 h. (**c**, **d**) Cell death in monocytes pretreated with rotenone for 1 h prior to addition of LPS or P3C for 24 h, assessed by annexinV-PI-staining (**c**) and Western blot for PARP and cleaved caspase-3 (**d**). (**e**) Phagocytic capacity of monocytes stimulated with LPS or P3C for 24 h and held under normoxic or hypoxic conditions for 2 h prior to the start of the assay. Data (mean ± s.e.m.) are from two experiments with a total of five donors (**a**-**b**), or one experiment with three donors (**c**-**e**). * p<0.05, ** p<0.01 (paired two-tailed t-test).



Supplementary Figure 6 | Cytokine (**a**) and lactate (**b**) production of human monocytes pretreated with dimethyl itaconate (DI) for 1 h before addition of LPS or P3C for 24 h. Data (mean ± s.e.m.) are from one experiment with three donors. * p<0.05, ** p<0.01 (paired two-tailed t-test).

Supplementary Table 1 | Primer sequences used to examine gene expression levels by RT-qPCR.

	3' primer	5' primer
B2M	ATGAGTATGCCTGCCGTGTG	CCAAATGCGGCATCTTCAAAC
CD36	GTGCTGTCCTGGCTGTGTTT	TCACTTCCTGTGGATTTTGC
CPT1α	TCCAGTTGGCTTATCGTGGTG	TCCAGAGTCCGATTGATTTTTGC
FABP4	CAGGAAAGTCAAGAGCACCAT	GCATTCCACCACCAGTTTATC
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
HK2	TGCCACCAGACTAAACTAGACG	CCCGTGCCCACAATGAGAC
IDH1	GGAAAAGCTCGGACCTACCC	CGCTCACAAGCTCAGACTCA
IRG1	GTTCCTGGGAACCACTACGG	GATGTCTGGCTGACCCCAAA
LIPA	CCCACGTTTGCACTCATGTC	CCCAGTCAAAGGCTTGAAACTT
LPL	CATTCCCGGAGTAGCAGAGT	GGCCACAAGTTTTGGCACC
PDK4	GGAGCATTTCTCGCGCTACA	ACAGGCAATTCTTGTCGCAAA
PFKFB3	ATTGCGGTTTTCGATGCCAC	GCCACAACTGTAGGGTCGT
SDHa	TGCCATCCACTACATGACGG	GCTCTGTCCACCAAATGCAC
PPARγ	ATTGACCCAGAAAGCGATTCC	TCTTCCATTACGGAGAGATCCAC

3

Adipose tissue macrophages: going off track during obesity

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ABSTRACT

Inflammation originating from the adipose tissue is considered to be one of the main driving forces for the development of insulin resistance and type 2 diabetes in obese individuals. Although a plethora of different immune cells shapes adipose tissue inflammation, this review is specifically focused on the contribution of macrophages that reside in adipose tissue in lean and obese conditions. Both conventional and tissue-specific functions of adipose tissue macrophages (ATMs) in lean and obese adipose tissue are discussed and linked with metabolic and inflammatory changes that occur during the development of obesity. Furthermore, we will address various circulating and adipose tissue-derived triggers that may be involved in shaping the ATM phenotype and underlie ATM function in lean and obese conditions. Finally, we will highlight how these changes affect adipose tissue inflammation and may be targeted for therapeutic interventions to improve insulin sensitivity in obese individuals.

Highlights

- Macrophages play a significant role in regulating adipose tissue functioning during health and disease
- In addition to conventional functions such as clearing cellular debris and participating in tissue immune surveillance, lipid buffering is an important function of ATMs
- Obesity-induced inflammation, characterised by an elevated number of pro-inflammatory macrophages in adipose tissue, has been suggested to contribute to systemic insulin resistance
- Their origin, as well as a combination of peripheral changes and adipose tissue-derived stressors, probably contribute to ATM dysfunction and inflammatory traits during obesity
- Identification of transcriptional differences between ATMs from lean vs obese adipose tissue at several key points during the development of obesity and insulin resistance may reveal upstream triggers, regulatory factors and intracellular pathways that shape ATM function
- Targeting metabolic capacity rather than the inflammatory phenotype of ATMs may hold potential to restore ATM function and adipose tissue homeostasis in obese individuals

INTRODUCTION

Historically, adipose tissue is known to perform a number of functions including storage of excess energy, cold insulation and protection of vital organs. Intense research efforts over the last decades have greatly furthered our understanding of adipose tissue biology and led to the identification of various additional functions. The adipose tissue—composed of adipocytes and non-adipocyte cells, including endothelial cells, pre-adipocytes and various types of immune cells [1]—is known to produce and secrete a wide variety of so-called adipokines. The release of adipokines enables communication with other cells and tissues throughout the body involved in the regulation of energy metabolism, satiety and various other processes.

The central role of adipose tissue in homeostasis becomes apparent during the development of obesity, which is a major risk factor for the development of insulin resistance [2]. During the development of obesity, storage of excess amounts of triacylglycerol in adipose tissue is associated with altered release of adipokines and cytokines that control local and systemic inflammatory processes and interfere with insulin signalling [3]. At the cellular level, it has been well established that obesity promotes robust changes in adipose tissue morphology [4]. In particular, hypertrophy of adipocytes and significant alterations in the number and composition of immune cells promote a state of chronic low-grade inflammation during obesity that is strongly associated with the development of insulin resistance and type 2 diabetes [1].

Macrophages are immune cells that have gained much attention as important contributors to adipose tissue functioning. Whereas macrophages in lean mice and humans make up around 5% of the cells in adipose tissue, during obesity they constitute up to 50% of all adipose tissue cells [5]. As well as increasing in number, adipose tissue macrophages (ATMs) change their localisation and inflammatory features during obesity. Contrary to the lean state, in which ATMs are distributed throughout the adipose tissue exposing limited inflammatory properties, ATMs in obese adipose tissue are located around dead adipocytes and form so-called crown-like structures (CLSs) while displaying profound pro-inflammatory features [6–8]. Macrophage presence in CLSs within obese adipose tissue has been directly linked with insulin resistance [9, 10]. Notably, however, the importance of macrophage-mediated inflammation in determining insulin resistance is related to long-term exposure to a high-fat diet (HFD) whereas the initial stage of insulin resistance is independent of macrophage action [11].

In contrast to mice, inflammatory changes in human adipose tissue during obesity are somewhat less pronounced, although the presence of CLSs is positively correlated with a worsening in insulin resistance levels [12]. Furthermore, inter-individual differences in the degree of adipose tissue inflammation are likely to exist in humans and the importance of macrophage-mediated adipose tissue inflammation on the development of insulin resistance

will vary between obese individuals.

In addition to macrophages, numerous other types of immune cells populate the adipose tissue and affect its function [5, 13]. Indeed, dendritic cells [14], mast cells [15], neutrophils [16], B cells [17, 18] and T cells [19–21] have been found to reside in adipose tissue during obesity and contribute to the development of adipose tissue inflammation and insulin resistance. In particular, neutrophils and $CD8^+$ T-cells have recently gained considerable attention because of the observed early influx into adipose tissue upon HFD-feeding and their potential contribution either to attracting macrophages or affecting their phenotype [16, 19, 22]. On the other hand, forkhead box (FOX)P^+ regulatory $CD4^+$ T cells have been found to reduce in number prior to the accumulation of macrophages in obese adipose tissue [23]. In line with their well-established role in dampening pro-inflammatory signalling, reduced presence of regulatory $CD4^+$ T cells in adipose tissue might explain the accumulation and/or pro-inflammatory signalling of ATMs during obesity. Overall, these findings suggest a strong interplay between adaptive and innate immunity that together determine the inflammatory characteristics of obese adipose tissue. An excellent overview of the contribution of various immune cells to adipose tissue biology can be found elsewhere [24, 25]. This review will primarily focus on the function of macrophages in adipose tissue and their contribution to the inflammatory traits of obese adipose tissue.

Macrophages are unique in their capacity to quickly adapt to a changing environment, causing them to embrace a variety of phenotypes ranging from anti-inflammatory to pro-inflammatory. In virtually every tissue, macrophages are actively involved in maintaining tissue homeostasis by clearing cellular debris, participating in tissue immune surveillance and resolving inflammation [26]. Although ATMs have been extensively associated with the development of obesity and adipose tissue inflammation, they are known to populate lean adipose tissue as well, executing numerous functions crucial for maintaining adipose tissue homeostasis [27].

In this review, we will touch upon both well established and recently identified functions of macrophages in lean and obese adipose tissue. The origin of macrophages residing in lean and obese adipose tissue will be addressed and several triggers that may account for an increase in number and a phenotypical switch of ATMs during the development of obesity will be discussed. Finally, this knowledge is used to pinpoint relevant future research directions to establish potential therapeutic targets for shifting macrophage phenotype and function in order to promote adipose tissue health.

What are the functions of macrophages in adipose tissue?

ATMs and efferocytosis

In 1908, Elie Metchnikoff received the Nobel Prize for his important discovery of the phagocytic activity of macrophages. Nowadays phagocytosis is still considered to be the most prominent function of macrophages. Although Metchnikoff specifically described the engulfment of microbes, phagocytosis also encompasses the engulfment of apoptotic endogenous cells in a process termed 'efferocytosis' [28]. Efficient efferocytosis licenses anti-inflammatory removal of aged or damaged cells by macrophages and is crucial to maintain homeostasis in tissues where cellular turnover occurs [28, 29]. Cellular turnover is also active in adipose tissue, characterised by continuous removal of adipocytes and their replacement by new adipocytes. Depending on the study population, the adipose tissue depot studied and the methodology used, calculations of the rate of adipocyte turnover in adults range from 10% per year [30] to up to 58–106% per year [31]. Since the total number of adipocytes in adipose tissue is thought to be set during childhood and thus to remain stable in adults [30, 32], probably a continuous cycle of cell death and replenishment exists in which macrophages presumably play a key role. Indeed, studies in which excessive adipocyte death is induced through activation of caspase 8 [33] or ongoing stimulation of lipolysis [34] have revealed anti-inflammatory macrophage accumulation around dead adipocytes, pointing to the need for macrophages during removal of dead adipocytes and the capacity of ATMs to clear high numbers of dead adipocytes while maintaining an anti-inflammatory state. The importance of efficient efferocytosis becomes apparent in several chronic inflammatory diseases, such as cystic fibrosis, chronic obstructive pulmonary disease and asthma, where ineffective clearance of dead cells has been identified as important source of pro-inflammatory signalling [29]. In obese adipose tissue, macrophages surrounding dead adipocytes in CLSs demonstrate pro-inflammatory features [6–8]. Since efficient efferocytosis is known to be accompanied by an anti-inflammatory macrophage trait, the presence of pro-inflammatory ATMs surrounding dead adipocytes in the obese state might reflect futile adipocyte clearance contributing to adipose tissue inflammation.

ATMs, adipocyte lipolysis and lipid buffering

Building upon their unique role as professional phagocytes, recent data suggest that macrophages facilitate acute metabolic tasks executed by the adipose tissue. During lipolysis induced by either fasting or pharmacologic adrenergic activation, macrophages rapidly infiltrate the adipose tissue and adopt an anti-inflammatory phenotype [35]. In adipose tissue, macrophages buffer lipolysis by taking up and storing excessive amounts of adipocyte-released lipids, supervising gradual lipid release into the bloodstream [35–37]. Recently, macrophages have also been linked to controlling adipocyte lipolysis during cold exposure. Upon exposure to cold, anti-inflammatory macrophages have been reported to secrete catecholamines to stimulate adipocyte lipolysis in both inguinal and brown adipose

tissue [36, 38–40]. These acute challenges appear to promote macrophage mobility and quick adaptation to a changing environment facilitating tissue flexibility.

Interestingly, 7 days of continuous infusion of a β3-adrenergic receptor agonist to induce adipocyte lipolysis not only results in an influx of anti-inflammatory macrophages that adopt a lipid-laden cell appearance, it also increases the presence of dead adipocytes and the formation of CLSs. Within these CLSs, anti-inflammatory macrophages have been found to recruit and stimulate platelet-derived growth factor receptor (PDGFR)α^+ adipocyte progenitors to differentiate [34]. Intriguingly, these adipogenic clusters near CLSs point to a direct link between efferocytosis, lipid buffering and adipose tissue remodelling by macrophages in lean adipose tissue.

In obese adipose tissue, macrophages surrounding dead adipocytes in CLSs also form multiple intracellular lipid droplets and activate transcriptional programs involved in lysosomal lipolysis [41]. The presence of lipid-filled macrophages in CLSs of obese adipose tissue in both humans [42] and mice [8] is indicative of macrophages attempting to buffer and process excessive amounts of lipids originating from adipocytes in a similar fashion to that observed in lean adipose tissue. However, increased spilling of NEFAs during obesity is suggestive of unsuccessful lipid buffering by ATMs. Thus, although engulfment and storage of lipids by ATMs seems to hold functional relevance in both lean and obese adipose tissue, in obesity the lipid buffering capacity of ATMs appears to be insufficient.

ATMs and adipogenesis

The close correlation between adipocyte death and new cell formation through adipogenesis that has been observed in lean adipose tissue may not hold true during obesity. While macrophages in obese adipose tissue have been found to populate adipogenic clusters and facilitate angiogenesis and adipogenesis, adipogenic clusters are formed at sites away from CLSs [34, 43, 44]. This might relate to the pro-inflammatory state of macrophages in CLSs in obese adipose tissue. Although it has been proposed that pro-inflammatory signalling is needed for efficient adipogenesis to occur [45], multiple cell culture experiments have demonstrated that pro-inflammatory macrophages inhibit both proliferation and differentiation of adipogenitor cells [46–49]. The pro-inflammatory phenotype of macrophages in CLSs of obese adipose tissue may suppress adipogenesis and thus explain the appearance of adipogenic clusters away from inflammatory CLSs [34].

In contrast to studies postulating a role for macrophages in adipogenesis, other studies have argued that ATMs inhibit adipogenesis in obese adipose tissue [49, 50]. For example, it was found that exposure of adipocyte progenitors to conditioned medium of $CD14^+$ cells from obese adipose tissue blocks adipogenesis [49]. A prominent factor involved in suppressing adipogenesis is Wnt-5a, which is abundantly expressed in ATMs and circulating monocytes from individuals with obesity and type 2 diabetes compared with lean individuals [50]. Overall, the effect of macrophages on adipogenesis in obese adipose tissue is still being debated. One might hypothesise that the role of macrophages shifts during the progression of obesity

from a predominant role in stimulating adipogenesis at the start of adipose tissue expansion to inhibiting adipogenesis once obesity progresses. The observation that adipogenic clusters containing macrophages appear at an early stage of adipose tissue expansion during obesity, yet decline in number upon the progression of obesity, supports this hypothesis [43]. Reduced adipogenesis at later stages of obesity would promote hypertrophy of adipocytes to allow for storage of the excess amounts of lipids entering the adipose tissue, which is in turn linked to metabolic dysfunction such as adipocyte insulin resistance [51, 52]. Indeed, increased adipocyte size has been found to correlate with macrophage presence in obese adipose tissue [53, 54]. However, future in vivo studies will be needed to further unravel the role of ATMs in adipogenesis and adipocyte hypertrophy in both lean and obese adipose tissue. In conclusion, multiple functions of macrophages in lean adipose tissue, including the prevention of lipid spill over into the circulation and licensing adipose tissue remodelling, appear to be impaired in obese adipose tissue. It is likely that impairment of these functions executed by ATMs contributes to loss of adipose tissue homeostasis in obese individuals. Differential metabolic and inflammatory traits of ATMs residing in either lean or obese adipose tissue may underlie shifts in its functional output and thus total adipose tissue functioning. Hence, careful identification of ATM phenotypes in lean and obese individuals might provide evidence for functional rewiring of ATMs upon the development of obesity.

What are the effects of the adipose tissue environment on macrophage phenotype?

Macrophage phenotype in lean vs obese adipose tissue

Besides increasing in number, ATMs are known to dramatically change their phenotype during obesity. It has been suggested that the majority of the macrophage population in adipose tissue of obese mice consists of $F4/80^{+}CD11c^{+}$ or 'classically activated' M1 macrophages, characterised by increased expression levels of TNFα and inducible nitric oxide synthase (iNOS) [8]. By contrast, most of the macrophages in lean adipose tissue can be identified as $F4/80^{+}CD206^{+}CD301^{+}CD11c^{-}$ macrophages, resembling an alternatively activated M2 phenotype that is characterised by the expression of genes encoding anti-inflammatory proteins including Ym1, arginase 1 and IL-10 [8, 27]. A balanced 'M0' $F4/80^{+}CD206^{-}CD11c^{-}$ phenotype has also been reported [55], supporting an even greater diversity of inflammatory macrophage subtypes in the adipose tissue. However, in light of the metabolic functions performed by ATMs, a classification based on their inflammatory properties may not suffice. In line with this hypothesis, both M1 and M2 markers have been identified on a phenotypical heterogeneous population of macrophages in obese adipose tissue [56]. Interestingly, a metabolic classification has recently been put forward, characterised by increased lysosomal biogenesis and subsequent lipid catabolism as a hallmark of ATMs in obese adipose tissue [41]. Rather than cytokine-driven pro-inflammatory activation of ATMs, fatty acids (FAs) are the main trigger for metabolic activation of ATMs in obese adipose tissue. Importantly,

metabolic changes in lipid metabolism may predominantly underlie inflammatory activation of macrophages in adipose tissue, promoting an inflammatory phenotype of ATMs that may not fit into any of the classically defined inflammatory macrophage subtypes [57].

Macrophages in human vs mouse adipose tissue

In parallel with an increase in ATMs during obesity in mice, macrophage numbers have also been found to increase in human obese adipose tissue [5, 7]. In contrast to the pro-inflammatory phenotype of macrophages found in many animal studies, an 'M2-type' macrophage with remodelling capacity, yet still capable of secreting substantial quantities of pro-inflammatory cytokines, has been identified in adipose tissue of obese individuals [58]. The mixed inflammatory phenotype of ATMs in obese individuals is further illustrated by the presence of both CD206 and CD11c on the ATM membrane, markers generally used to distinguish between M2- and M1-type macrophages [59]. Despite their anti-inflammatory characteristics, this population of $CD11c^{+}CD206^{+}$ macrophages has been associated with insulin resistance [60]. Interestingly, the M2 macrophage marker CD163 has even been proposed to be the single macrophage marker that significantly correlates with HOMA-IR [61]. As mentioned before, less pronounced correlations between ATM numbers and insulin resistance have been found in humans, compared with mice [62–64]. It is likely that inter-individual differences in the development of adipose tissue inflammation during obesity exist and contribute to the inconsistency in reported correlations between ATMs, adipose tissue inflammation and the development of insulin resistance in humans.

Location: visceral vs subcutaneous adipose tissue

Adipose tissue is stored at various locations throughout the body, characterised by specific metabolic and inflammatory properties. Both subcutaneous (scAT) and visceral (vAT) storage depots exist, which are identified based on their anatomical location and differentially contribute to the development of metabolic abnormalities [65]. Epidemiological evidence demonstrates that vAT mass is a dominant risk factor for the development of metabolic abnormalities including insulin resistance [65]. Ex vivo experiments have shown that the inflammatory status, determined by the release of cytokines, is elevated in vAT compared with scAT [66]. This would imply that the number and/or phenotype of macrophages differ between the two adipose tissue depots. Indeed, various studies have shown an enhanced number of macrophages in vAT vs scAT [62, 67]. However, others have failed to report any differences in macrophage cell numbers [66]. In animal studies, bigger adipocytes were found in scAT compared with vAT, which negatively correlated with the number of CLSs in the adipose tissue depots [10]. Moreover, distinct rates of adipocyte turnover, adipocyte lipolysis and blood flow, with consequences for oxygen and nutrient availability, have been detected in scAT vs vAT [68, 69]. Although controversy about the different characteristics exists, distinct microenvironments in the adipose tissue depots might importantly affect macrophage phenotypes. Phenotypical differences in macrophages that populate either vAT

or scAT may be exemplified by the higher expression of pro-inflammatory cytokines by ATMs in vAT [67, 70, 71].

In general, most studies are mainly focused on determining the inflammatory phenotypes of ATMs. However, in line with the metabolic functions executed by ATMs, inflammatory markers may not suffice to distinguish functional from dysfunctional ATMs. It would be interesting to unravel whether macrophages within human adipose tissue display a metabolically activated phenotype, as has been identified recently in mice [57]. Moreover, deciphering what type of metabolic and inflammatory changes in ATMs finally result in functional differences in obese vs lean adipose tissue might improve our understanding of obesity-induced adipose tissue inflammation. Multiple triggers may underlie the macrophage phenotype, explaining the distinct responses of ATMs to several challenges in obese and lean adipose tissue. In order to shed light on such triggers, one should consider how and where the phenotype of the ATMs is being affected.

From where do adipose tissue macrophages originate?

Contrasting with the prevalent idea that all tissue macrophages are derived from circulating monocytes, novel techniques that allow fate mapping have revealed that not all tissue-resident macrophages originate from monocyte precursors [72]. Many resident tissue macrophages are established during early embryonic development and are maintained during adulthood independently of an influx of blood monocytes [73]. However, inflammation has been shown to drive the recruitment of blood monocyte-derived macrophages that replace embryonically established resident macrophage populations in the myocardium and other tissues [74]. Surprisingly little is known about the origin of adipose tissue-resident macrophages and the maintenance of this population.

Monocyte recruitment

It is well established that adipocytes are able to produce specific adipokines that function as chemoattractants for circulating monocytes. Monocyte chemoattractant protein-1 (MCP-1) is one such chemokine that is known to be produced in large amounts by adipocytes and is robustly increased in obesity [75]. Indeed, overexpression of MCP-1 in adipose tissue promotes macrophage accumulation [76]. Interestingly, the presence of the C–C chemokine receptor (CCR) type 2, which allows monocytes to respond to MCP-1, is a typical characteristic of newly recruited macrophages that is used to distinguish them from resident macrophages in a variety of tissues [74] including fat [7]. These observations imply that monocyte recruitment through MCP-1 is important in populating adipose tissue with macrophages, and that this pathway is enhanced in the presence of obesity.

In addition to increased secretion of MCP-1 by adipose tissue, obesity is characterised by a significantly increased number of CCR2 molecules on circulating monocytes [77]. Moreover, monocytes from obese individuals demonstrate a higher chemotactic activity, which is associated with both insulin resistance and CCR2 expression [77]. Interestingly, the

migratory behaviour itself might be an important influence on macrophage phenotype. This is illustrated by the finding that the inflammatory state is linked to the migratory capacity of macrophages into adipose tissue [6, 8, 78]. In addition to a higher degree of chemotaxis, $CCR2^+$ tissue macrophages may have an increased inflammatory phenotype compared with $CCR2^-$ tissue macrophages, which is illustrated by an induction of genes involved in the regulating of the NLRP3 inflammasome, at least in the myocardium [74]. The NLRP3 inflammasome allows processing and release of active IL-1β. IL-1β controls inflammatory responses and is involved in the development of insulin resistance [79, 80]. Similar differences appear to exist in ATMs isolated from obese animals shown by enhanced expression of CCR2 and inflammatory cytokines compared with the ATMs of lean animals [7]. However, monocyte recruitment to adipose tissue may not solely depend on MCP-1-controlled pathways, as the absence of MCP-1 does not completely curtail monocyte influx into adipose tissue [81]. Indeed, other chemokine receptor/ligand complexes have been shown to play a role in these processes, including CCR5 [82].

Bone marrow myelopoiesis

Recent evidence has shed light on mechanisms and signals that have a vital role in controlling the recruitment of monocytes during obesity and go beyond MCP-1 and other chemokine-signalling modules. Indeed, the bone marrow has been identified as an important contributor to the ATM pool [5, 83]. The development of obesity has been found to stimulate bone marrow myelopoiesis, which licenses the ongoing infiltration of monocytes into adipose tissue [84, 85]. Interestingly, it appears that signals from obese adipose tissue actively enhance bone marrow myelopoiesis partly via local activation of the alarmin S100A8/A9, which drives production of IL-1β [84].Other adipokines have also been shown to govern myelopoiesis, including leptin [85], further corroborating the observation that adipose tissue appears to determine bone marrow function via remote control. It is worth noting that fat residing within the bone marrow is known to express various cytokines and adipokines that change during the development of obesity and type 2 diabetes and might also be involved in the remodelling and activation of bone marrow cell populations [86]. Even though these observations shed some light on the mechanism of action involved, many issues concerning recruitment remain unresolved. In addition to adipose tissue-derived IL-1β, are haematopoietic factors involved in the control of myelopoiesis during obesity as well? Are signals from the adipose tissue also responsible for controlling bone marrow myelopoiesis in lean conditions? And are monocytes phenotypically different when originating from bone marrow during lean vs obese conditions? One line of evidence suggests that monocytes originating from bone marrow of lean or obese animals have a similar migration capacity. Using PKH26 staining to track monocytes, ATM accumulation was found to be enhanced in obese recipient mice compared with lean mice, independently of the origin of donor monocytes from either obese or lean animals [87]. These results imply that adipose tissue-derived signals determine local macrophage numbers. Nonetheless, it has been demonstrated

that bone-derived macrophages from HFD-fed mice display an enhanced inflammatory phenotype compared with macrophages derived from bone marrow of lean animals [88]. Moreover, haematopoietic stem cells from obese mice harbour an enhanced capacity to develop into inflammatory $CD11c^+$ ATMs after bone marrow transplantation [89]. Together these data suggest that adipose tissue-derived factors determine both the infiltration and differentiation of monocytes and that obesity affects the characteristics of the bone marrow cell population, ultimately translating into phenotypically different monocytes infiltrating lean and obese adipose tissue.

Local regulation of ATM numbers

In addition to recruitment during obesity, multiple lines of evidence suggest that adipose tissue is equipped with tools allowing for local regulation and proliferation of ATM pools independently of the influx of blood precursors. vAT that is part of human omentum displays enhanced myelopoiesis during acute inflammatory conditions, which is suggestive for in situ production of cell populations in the abdomen [90, 91]. In line with these results, the presence of haematopoietic stem/progenitor cell populations in mouse white adipose tissue has been reported. The capacity of these cells to differentiate into myeloid and lymphoid lineages was similar to that of bone marrow-derived haematopoietic cells [92], yet no evidence currently exists supporting a fundamental role for local myelopoiesis in determining ATM numbers in obese adipose tissue. Another pathway contributing to influx-independent pathways that nourish ATM numbers concerns the involvement of pre-adipocytes that have been assigned with characteristics that closely resemble macrophages. 3T3-L1 pre-adipocytes, a frequently used model to study adipogenesis in vitro, display macrophage-like functions that are lost upon differentiation towards adipocytes [93]. Moreover, pre-adipocytes isolated from adipose tissue carry out many macrophage-like functions including phagocytosis and express macrophage-specific antigens including F4/80, macrophage 1 antigen (Mac-1) and CD45 [94]. Overall, these results imply closely resembling phenotypes of pre-adipocytes and macrophages. When receiving the appropriate signals, pre-adipocytes may efficiently and rapidly adapt and execute ATM functions.

Besides the contribution of local precursors, local proliferation has been held responsible for increasing ATM numbers during obesity [95]. Moreover, ATMs in obese adipose tissue have been postulated to have reduced migration capacity through increased netrin-1 expression that traps them in adipose tissue [96]. Finally, reduced apoptosis of resident ATMs has been suggested to account for enhanced ATM numbers during obesity, independently of monocyte influx [97].

To summarise, the origin of ATMs in lean and obese adipose tissue is still a matter of debate. The presence of specific cell surface markers on ATMs might help us learn more about their origin. Moreover, lineage tracing of ATMs using time-lapse microscopy, cell labelling and/or genetic markers will help us understand the origin of ATMs. On the basis of the currently available data, ATMs appear to be mainly derived from bone marrow-

controlled pathways involving adipose-derived signals. Other lines of evidence suggest that local mechanisms may determine macrophage numbers in adipose tissue, including prolonged survival or increased retention of ATMs. Probably, various pathways at multiple levels are at work to set ATM numbers in adipose tissue. Whether the number of ATMs is primarily regulated at the level of the bone marrow or is determined by local factors in the adipose tissue will need further study.

Drivers of the adipose tissue macrophage phenotype

Both during myelopoiesis and in the circulation, monocytes can be primed by different factors that will eventually contribute to their migration and inflammatory fate after infiltration into the adipose tissue. It is likely that adipose tissue-derived factors will further determine the final ATM phenotype, which may differ between lean and obese adipose tissue. Some important triggers that shape the metabolic, inflammatory and functional traits of ATMs will be discussed below.

As explained earlier, other immune cells residing in the adipose tissue probably shape ATM phenotype as well, but these will not be discussed in this review. The same also applies to various adipokines that are known to be differentially secreted by lean rather than obese adipose tissue. Their role in affecting the phenotype of macrophages has been reviewed elsewhere [98, 99].

Hypoxia

Both in humans and mice, rapid adipose tissue mass expansion during the development of obesity occurs without a concurrent increase in blood flow towards the tissue [100]. Indeed, animal studies have demonstrated that obese adipose tissue is characterised by hypoxic areas and an increased expression of hypoxia-related genes including HIF1α [19, 101–103]. In humans, oxygen levels in the adipose tissue are much harder to measure, and this technical challenge likely contributes to the contradictory results that have been reported so far, ranging from decreased oxygen tension [104, 105], unaltered oxygen tension, and even to increased oxygen tension [106] in obese adipose tissue.

As a result of their profound increase in size upon the development of obesity, adipocytes are generally thought to suffer from hypoxia in obese adipose tissue. Hypoxic conditions in adipocytes promote angiogenesis and fibrotic remodelling and have often been associated with the development of adipose tissue insulin resistance [102, 103, 107].

Only recently has attention been directed to the effects of hypoxic conditions on macrophages in the adipose tissue. In line with adipocytes, ATMs from obese adipose tissue have increased expression levels of hypoxia-related genes [101, 108]. It is not unthinkable that prolonged hypoxia is one of the driving forces behind the pro-inflammatory phenotype of macrophages in obese adipose tissue. When $CD14^+$ cells from human obese vAT are exposed to hypoxic conditions, the secretion of pro-inflammatory cytokines is enhanced in

comparison with culturing under normoxic conditions [70]. Moreover, human macrophages derived from circulating monocytes held under hypoxic conditions show an increased inflammatory response when exposed to the saturated fatty acid (SFA) palmitate [109]. Interestingly, in vivo, M1-like macrophages accumulate more pimonidazole, a hypoxia probe, than do anti-inflammatory macrophages in the adipose tissue of obese mice, and display higher expression levels of hypoxia-related genes including Hif1α [101]. Together these data underline a direct link between the hypoxic state of ATMs and their inflammatory phenotype in obese adipose tissue.

Importantly, the response of macrophages to hypoxia might depend on their initial inflammatory state. Indeed, it has been proposed that stimulation of bone marrow-derived macrophages with IL-4 prior to hypoxia exposure decreases the expression of pro-inflammatory genes, while leaving hypoxia-related gene expression unaltered [101]. Alternatively, differential responses of macrophages to hypoxia could also relate to the presence of two different isoforms of hypoxia-inducible factor (HIF). HIF1α is the most well-known isoform and mediates a shift towards glycolysis, known as the Warburg effect [110]. Inflammatory stimuli are prominent stimulants of HIF1α activity, inducing rapid ATP production independent of oxygen [110, 111]. During hypoxic conditions HIF1α activity is also considered to be crucial to maintain energy levels [100], although other transcription factors probably play an additional role [109]. In addition to fuelling a metabolic switch, HIF1α enhances pro-inflammatory signalling via IL-1β transcription [110]. The control of IL1β expression might provide a direct link between HIF1α activity in obese ATMs, either activated via hypoxia or inflammatory signalling, and the presence of insulin resistance.

The isoform HIF2α is also upregulated under low oxygen levels. While HIF1α increases the glycolytic flux and pro-inflammatory signalling, HIF2α is associated with a more anti-inflammatory macrophage phenotype [112, 113]. Interestingly, in obese adipose tissue HIF2α is predominantly upregulated in M2-like macrophages [108] and peritoneal macrophages overexpressing HIF2α show a blunted increase in TNFα expression on co-culturing with obese adipose tissue [108]. Adipocyte-specific deletion of Hif2α has been linked to adipose tissue inflammation, glucose intolerance and insulin resistance [114] but so far no studies specifically targeting HIF2α in macrophages have been conducted.

Overall, the inflammatory state of macrophages might be linked to the relative presence of HIF isoforms that may influence the metabolic and inflammatory response of ATMs under hypoxic conditions. Importantly, in addition to hypoxia, one should not ignore the fact that HIF-dependent gene expression can also be regulated independently of oxygen availability, through the involvement of inflammatory-related signals. In addition to direct effects of hypoxia on macrophages, signals originating from hypoxic adipocytes may also shape the phenotype of ATMs in obese adipose tissue. Indeed, in vitro experiments have revealed that adipocytes exposed to hypoxic conditions release potential stressors that affect macrophage function.

Examples include the pro-inflammatory adipokines IL-6, visfatin, leptin and macrophage migration inhibitory factor (MIF) [100]. Importantly, prolonged exposure to hypoxia might also lead to adipocyte cell death and release of intracellular content which subsequently affects the phenotype of ATMs.

Adipocyte cell death

In animal studies, cell death in obese adipose tissue has been well established. Dead adipocytes that have lost membrane integrity are surrounded by macrophages that cluster in CLSs and together form pro-inflammatory, fibrotic lesions [115]. Signals released from dying cells are known to act as strong chemoattractants and affect immune responses [116, 117].One example is the nuclear protein high mobility group box 1 (HMGB1), which acts as a danger signal that attracts and activates immune cells [118]. Interestingly, HMGB1 levels are increased in obese adipose tissue and are associated with inflammation [119, 120]. In addition to HMGB1, other cellular proteins with intrinsic inflammatory features are released upon cell death and may skew ATM phenotypes in obese adipose tissue. Uric acid, adenosine, ATP and galectins are among some of these so-called 'danger signals' that affect inflammatory macrophage responses [116, 117, 121].

The type of adipocyte cell death occurring in adipose tissue may determine the inflammatory phenotype of macrophages as well. Apoptotic cells induce a predominant anti-inflammatory response, whereas necrotic cells stimulate pro-inflammatory cytokine secretion [117]. Dead cells that are not effectively cleared are known to turn into secondary necrotic cells releasing their noxious content to induce pro-inflammatory signalling, autoimmunity and tissue damage [122–125]. Although impaired clearance of dead cells has been causally linked to various chronic inflammatory diseases [29], to date there have been no extensive studies on the contribution of adipocyte death to inflammation during obesity. Some studies have reported necrotic adipocyte cell death in obese adipose tissue, with membrane rupture and release of cell content [9]. However, others postulate programmed necrotic cell death [126], programmed pyroptosis [127] or even apoptosis [128] of adipocytes in obese adipose tissue, making it difficult to draw conclusions on the effect of adipocyte cell death on the ATM phenotype. Considering the profound consequence for macrophage phenotype found in other organs, examination of the type of adipocyte cell death occurring in obese adipose tissue, but also during regular cell turnover in lean adipose tissue, is of relevance.

Importantly, adipocyte cell death is unique in the fact that it leads to the release of substantial amounts of lipids and one might hypothesise that prolonged exposure to excess lipids affects ATM phenotype.

Lipotoxicity

A lipid-rich environment during the development of obesity may represent an important metabolic stressor for ATMs. In general, there are three ways through which ATMs are exposed to lipids; via chylomicrons or VLDLs, by adipocyte lipolysis, or after adipocyte cell

death. Hyperlipidaemia seems not to lead to macrophage accumulation in obese adipose tissue [129], indicating that lipids resulting from adipocyte lipolysis and adipocyte cell death represent the main causes of excessive lipid accumulation in ATMs during obesity. However, the exact contribution of adipocyte lipolysis is also somewhat controversial. On the one hand higher basal rates of lipolysis have been observed in adipocytes of obese individuals [130]. In line with this, adipocyte hypertrophy and TNFα exposure, which are both elevated in obese adipose tissue, have been linked to increased adipocyte lipolysis [131–133]. On the other hand, decreased basal lipolytic rates [134] and reduced catecholamine-induced lipolysis by insulin resistant adipocytes in obese adipose tissue [135, 136] have been reported as well, and are indicative of lower lipolysis rates in obese adipose tissue. Nonetheless, although the exact source is still a matter of debate, macrophages in obese adipose tissue are exposed to excess lipids as illustrated by their lipid-laden appearance. Exposure to FAs is known to influence macrophage phenotype profoundly. Importantly, SFAs induce a pro-inflammatory macrophage phenotype via Toll-like receptor (TLR)-induced NF-kB activation [137]. The TLR-family member 4 (TLR 4) translates most of the pro-inflammatory effects of SFAs that trigger TLR4 activation via binding to its adaptor molecule fetuin-A [138, 139], although some argue that the effects of SFAs are partly TLR-independent [57, 140]. Another source of pro-inflammatory signalling finds it origin intracellularly, as an increase in intracellular FAs is associated with endoplasmic reticulum (ER) stress and oxidative stress, enhancing pro-inflammatory signalling and inducing insulin resistance in vivo [141]. Moreover, SFAs and their derivatives, including ceramides, are classified as danger-associated molecular patterns (DAMPs) that are recognised by the NLRP3 inflammasome and lead to IL-1β secretion via caspase 1 activation [142]. However, the response of macrophages upon lipid uptake does not necessarily have to be pro-inflammatory in nature [143]. Although it has been suggested that internalised palmitate, a very well-known SFA, promotes lipid metabolism and limits inflammation [57], in general only unsaturated FAs, particularly n-3 FAs, are recognised for their anti-inflammatory properties [139, 144]. Hence, FA species present in adipocytes may for a large part determine the degree of lipid toxicity in macrophages. Alternatively, one might speculate that the metabolic and inflammatory state of macrophages could also be involved in determining its response to different FAs. Overall, mechanisms by which intracellular FAs determine the inflammatory traits of macrophages are relatively unclear. Importantly, lipid-overloaded macrophages in obese adipose tissue [42] may trigger the production of pro-inflammatory cytokines such as TNFα and IL-1β, as has been observed in macrophages exposed to FAs in vitro [138, 145–147], and thus contributing to the development of insulin resistance. The type of FAs stored in the adipocyte, the timespan of lipid exposure, the inflammatory phenotype of the macrophage and the presence of various other inflammatory signalling molecules might explain the predominant anti-inflammatory response of ATMs in lean adipose tissue vs the pro-inflammatory response in obese adipose tissue following lipid exposure.

Hyperinsulinaemia

Obesity-induced adipose tissue inflammation is associated with systemic insulin resistance and hyperinsulinaemia in obese rodents and humans. Multiple lines of evidence suggest that insulin itself directly affects inflammatory processes. In fact, insulin has been assigned with both anti- and pro-inflammatory properties. In circulating mononuclear cells of obese individuals, insulin inhibits NFkB activity, suggesting an anti-inflammatory effect [148]. By contrast, other studies have shown that hyperinsulinaemia promotes pro-inflammatory responses by affecting T cell regulatory function [149].

It is difficult to pinpoint the sole effects of insulin in vivo during the development of obesity, as changes in many other circulating factors accompany hyperinsulinaemia. However, several lines of evidence suggest that insulin directly contributes to ATM homing. The start of insulin therapy in patients diagnosed with type 2 diabetes has been shown to promote the appearance of macrophages in scAT [150]. Intriguingly, in both humans and mice, circulating insulin positively correlates with inflammatory cytokine expression in the adipose tissue, independently of body weight differences [151]. Recently, a mechanism of action whereby insulin may control propagation of adipose tissue inflammation was uncovered. Even though adipocytes become insensitive to insulin-dependent glucose uptake during obesity, insulin is able to enhance adipocyte MCP-1 gene expression levels aggravating the influx of monocytes [151].

Insulin resistance has also been shown to negatively affect macrophage function itself, especially in the context of atherosclerotic disease. Although glucose uptake primarily takes place via insulin-independent pathways involving GLUT1, circulating monocytes express the insulin receptor machinery and develop resistance to the effects of insulin. Interestingly, insulin receptor deficiency on macrophages reduces their influx into the adipose tissue and alleviates the development of low-grade inflammation and systemic insulin resistance in mice on an HFD [152]. Functional consequences of insulin resistance include a failure to deal with ER stress, which enhances the risk of macrophage apoptosis and subsequent loss of function [153]. In addition to apoptosis, primary macrophages isolated from ob/ob mice have a defect in efferocytosis that was associated with defective phosphoinositide (PI)3 kinase activity, whereas cells lacking the insulin receptor were protected [154].

It remains to be determined whether insulin resistance develops in ATMs in obese adipose tissue. Moreover, the functional consequences and possible contributions to adipose tissue inflammation are currently unknown. Another interesting aspect involves the development of insulin resistance in circulating monocytes [155]. It is not unthinkable that insulin resistance in circulating monocytes may promote functional changes leading to altered migration and inflammatory properties. Studies involving monocytes lacking specific parts of the insulin signalling machinery would shed light on the role of this pathway in shaping ATM phenotypes both during lean and obese conditions.

Hyperglycaemia

Chronic hyperglycaemia is known to induce insulin resistance by mechanisms that may partly be conveyed by an enhanced inflammatory state. Indeed, there is a substantial amount of evidence demonstrating pro-inflammatory effects of prolonged periods of hyperglycaemia. In humans, hyperglycaemia is associated with inflammation in individuals with diabetes [156]. Similar data has been generated using various animal models where the induction of hyperglycaemia using streptozotocin (STZ)-mediated disruption of pancreatic beta cells drives monocyte recruitment to tissues and promotes inflammation [157]. Other approaches to induce short-term periods of hyperglycaemia, including clamping, trigger pro-inflammatory responses in adipose tissue of rats as well [158].

In both adipocytes and macrophages, harmful effects of prolonged exposure to high levels of glucose have been reported. In vitro approaches in which adipocytes are cultured in medium with 25 mmol/l glucose for prolonged periods of time revealed increased production of various pro-inflammatory cytokines including IL-6 [158], and similar observations have been made in immune cells [159]. Moreover, hyperglycaemia has been shown to interfere with IL-4 action to polarise macrophages towards an alternatively activated state illustrated by decreased expression of M2 markers and a reduction in arginase functional activity, compared with cells treated under normoglycaemic conditions [160]. It has been postulated that the induction of reactive oxygen species (ROS) as a consequence of hyperglycaemia evokes inflammatory responses. An important molecular switch that translates the presence of hyperglycaemia into pro-inflammatory conditions is the thioredoxin-interacting protein (TXNIP). TXNIP expression is enhanced by high levels of glucose and in the presence of diabetes via the transcription factor carbohydrate response element-binding protein (ChREBP) and regulates a variety of processes including redox state and inflammation [161]. Deletion of Txnip using small interfering (si)RNA approaches prevents hyperglycaemia-induced ROS generation and the induction of inflammation [162]. Moreover, TXNIP has been linked to inflammasome activation in macrophages [163], a pathway known to contribute to adipose tissue inflammation and insulin resistance [79, 164]. In adipose tissue, glucose-induced activation of TXNIP mediates IL-1β mRNA expression levels and intracellular pro-IL-1β accumulation [66].

The consequences of hyperglycaemia may also be conveyed via changes in cellular metabolism leading to alterations in intermediate metabolites. It has been documented that cultured adipocytes exposed to high levels of glucose produce and secrete enhanced quantities of lactate [165]. Interestingly, diabetes is associated with markedly increased lactate production in adipocytes derived from obese adipose tissue [166]. Lactic acid, secreted from tumour cells as a by-product of aerobic or anaerobic glycolysis, has been shown to drive an M2like polarisation of tumour-associated macrophages [167]. Whether lactic acid secreted by adipocytes may alter ATM phenotype is currently unknown.

High levels of glucose may thus represent an important trigger that activates pro-

inflammatory changes in macrophages, either directly or indirectly. Interestingly, both shorter periods of hyperglycaemia, either during a clamp procedure or postprandially, and chronic periods in the presence of obesity and insulin resistance, appear to induce pro-inflammatory macrophage characteristics.

A future challenge: identification of key regulators of macrophage phenotype

Macrophages can be identified via specific transcription factors that control tissue-tailored transcriptional programs and allow the cells to adopt extremely tissue-specific functions. An elegant example is the identification of GATA-6 as master regulator of the peritoneal-specific gene transcriptional program. By comparing the transcriptome of various tissue macrophages including those from lung, liver, adipose tissue and peritoneum [90], the authors identified a set of genes controlled by GATA-6 specifically expressed in peritoneal macrophages. Using a similar approach, we have re-analysed their dataset to pinpoint ATM-specific genes leading to the identification of transforming growth factor (TGF)β and IL-4 as important upstream regulators. The identification of anti-inflammatory upstream regulators in ATMs isolated from lean animals is not surprising, yet it does demonstrate the usefulness of this approach. Comparing transcriptional regulators of ATMs during various conditions may bypass the rather complex quest for responsible triggers, and may help to unravel important pathways and molecules that underlie the distinct metabolic, inflammatory and functional traits of ATMs as a consequence of multiple triggers present in lean vs obese adipose tissue.

Because of the high versatility of macrophages, which have a phenotype largely dependent on their environment, conclusions solely based on in vitro studies should be taken with caution. Rather, in vivo profiling of macrophage phenotype via several techniques, including metabolomics and transcriptomics, and identification of key regulators of distinct macrophage phenotypes, residing in different adipose tissue depots and/or during the development of obesity and insulin resistance in both humans and mice, will advance our knowledge on adipose tissue biology. Determining a causal relationship between triggers, a switch in the metabolic and inflammatory status of ATMs and ATM function will be an important challenge. This information will be crucial for our understanding of initial triggers that ultimately link obesity to adipose tissue inflammation and the development of insulin resistance, and might provide us with future therapeutic targets. An overview of stressors that may determine the phenotype of ATMs in lean vs obese conditions and the subsequent consequences for adipose tissue function are shown in **Fig. 1**.

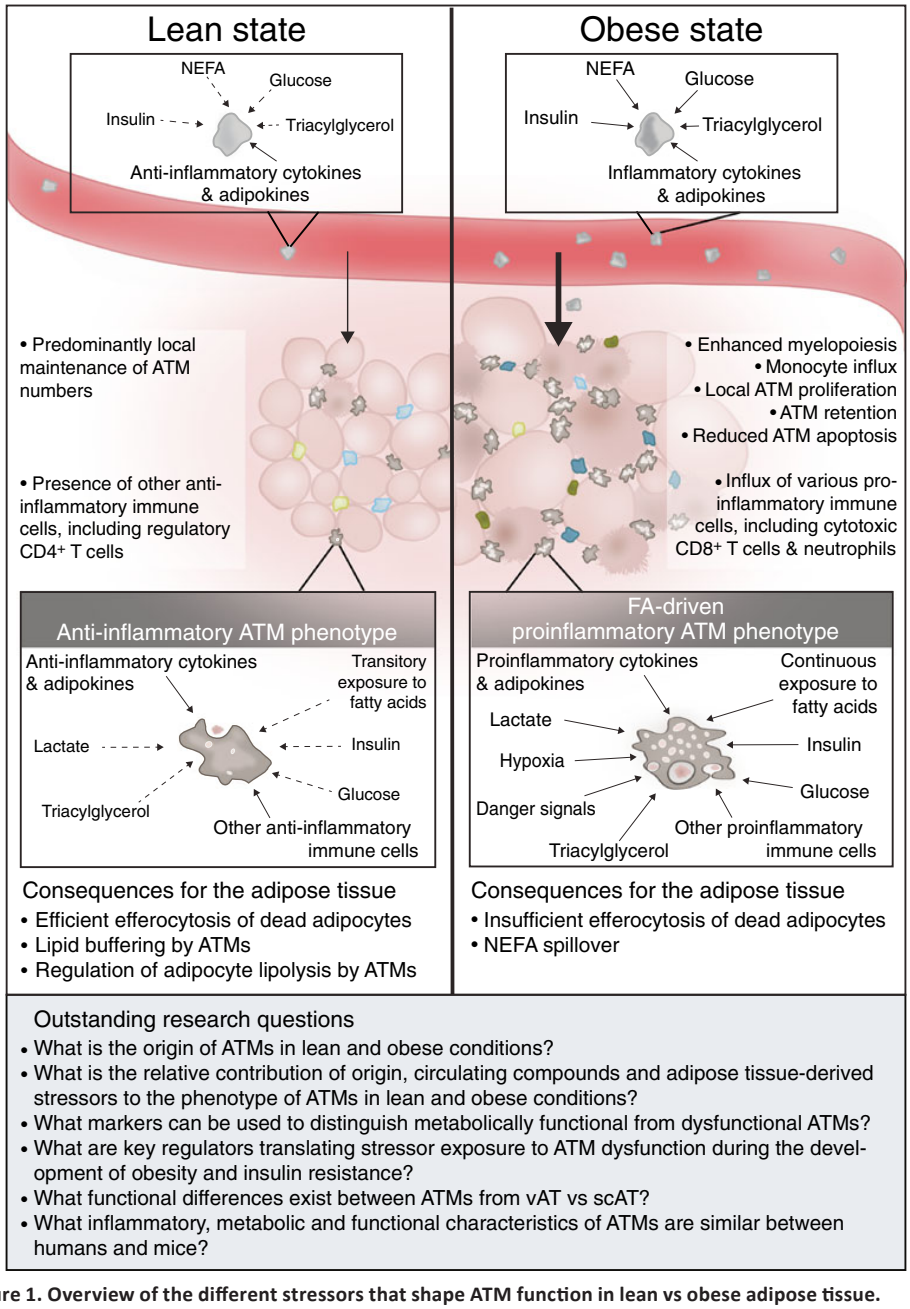

Figure 1. Overview of the different stressors that shape ATM function in lean vs obese adipose tissue.

Limiting adipose tissue inflammation by targeting macrophage metabolism

The presence of adipose tissue inflammation translates the development of obesity into insulin resistance and type 2 diabetes. Macrophages play a key role in maintaining adipose tissue homeostasis, yet fuel adipose tissue inflammation upon the development of obesity. Current efforts to unravel adipose tissue-specific functions of macrophages have revealed an essential role in lipid buffering for ATMs, alongside their more conventional functions such as clearing cellular debris and participating in tissue immune surveillance to maintain homeostasis. Importantly, both in lean and obese adipose tissue, lipid storage by macrophages prevents spill over into the circulation. However, the metabolically activated macrophage that resides in obese adipose tissue appears to be continuously exposed to an overabundance of lipids. Chronic lipid overloading of ATMs translates into a pro-inflammatory phenotype and may drive dysfunction of ATMs, ultimately fuelling the inflammatory traits of obese adipose tissue.

This new knowledge might shift intervention approaches away from targeting the inflammatory traits of ATMs, towards targeting their metabolic programming. Interventions aimed at increasing metabolic capacity might be used to reprogram macrophage metabolism, allowing macrophages to cope with metabolic challenges during obesity, in order to maintain adipose tissue homeostasis.

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4

Unique metabolic activation of adipose tissue macrophages in obesity promotes inflammatory responses

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ABSTRACT

Aims/hypothesis Recent studies have identified intracellular metabolism as a fundamental determinant of macrophage function. In obesity, proinflammatory macrophages accumulate in adipose tissue and trigger chronic low-grade inflammation that promotes the development of systemic insulin resistance, yet changes in their intracellular energy metabolism are currently unknown. We therefore set out to study metabolic signatures of adipose tissue macrophages (ATMs) in lean and obese conditions.
Methods F4/80-positive ATMs were isolated from obese vs lean mice. High-fat feeding of wild-type mice and myeloid-specific *Hif1α*$^{-/-}$ mice was used to examine the role of hypoxia-inducible factor-1α (HIF-1α) in ATMs part of obese adipose tissue. In vitro, bone marrow-derived macrophages were co-cultured with adipose tissue explants to examine adipose tissue-induced changes in macrophage phenotypes. Transcriptome analysis, real-time flux measurements, ELISA and several other approaches were used to determine the metabolic signatures and inflammatory status of macrophages. In addition, various metabolic routes were inhibited to determine their relevance for cytokine production.
Results Transcriptome analysis and extracellular flux measurements of mouse ATMs revealed unique metabolic rewiring in obesity characterised by both increased glycolysis and oxidative phosphorylation. Similar metabolic activation of CD14^{+} cells in obese individuals was associated with diabetes outcome. These changes were not observed in peritoneal macrophages from obese vs lean mice and did not resemble metabolic rewiring in M1-primed macrophages. Instead, metabolic activation of macrophages was dose-dependently induced by a set of adipose tissue-derived factors that could not be reduced to leptin or lactate. Using metabolic inhibitors, we identified various metabolic routes, including fatty acid oxidation, glycolysis and glutaminolysis, that contributed to cytokine release by ATMs in lean adipose tissue. Glycolysis appeared to be the main contributor to the proin- flammatory trait of macrophages in obese adipose tissue. HIF-1α, a key regulator of glycolysis, nonetheless appeared to play no critical role in proinflammatory activation of ATMs during early stages of obesity.
Conclusions/interpretation Our results reveal unique metabolic activation of ATMs in obesity that promotes inflammatory cytokine release. Further understanding of metabolic programming in ATMs will most likely lead to novel therapeutic targets to curtail inflammatory responses in obesity. Data availability Microarray data of ATMs isolated from obese or lean mice have been submitted to the Gene Expression Omnibus (accession no. GSE84000).

INTRODUCTION

In obesity, macrophages accumulate in adipose tissue and trigger chronic low-grade inflammation which promotes the development of systemic insulin resistance [1–3]. Based on transcriptional profiles and expression markers derived from in vitro experiments, macrophages are generally classified as classically/inflammatory (M1) or alternatively/anti-inflammatory (M2) activated [4, 5]. Applying this phenotypical classification to adipose tissue macrophages (ATMs) has led to the identification of M2 macrophages in lean adipose tissue vs M1 macrophages in obese adipose tissue [6]. In recent years, however, the two-dimensional M1/M2 spectrum has been challenged and macrophages in different tissue environments have been shown to adopt a variety of inflammatory phenotypes that fall outside this classification [7, 8]. Indeed, macrophages in obese adipose tissue display surface-proteins that resemble neither classical nor alternative activation, but rather represent a state of metabolic activation [9]. Intracellularly, ATMs in obese adipose tissue are characterised by lysosomal activity [10], suggestive of robust changes in intracellular energy metabolism of ATM in obesity.

Recent developments in the field of immunology have identified macrophage intracellular energy metabolism as a fundamental determinant of its functional response. M1 macrophages are characterised by a high glycolytic rate whereas M2 macrophages rely mainly on oxidative phosphorylation (OXPHOS) [11, 12]. A central role in driving macrophage polarisation has been appointed to hypoxia-inducible factor-1α (HIF-1α), a master regulator of glycolysis that is critically involved in the development of the M1 phenotype [13, 14]. Profiling of intracellular metabolism in ATMs, as well as identifying key regulators involved, is expected to further the understanding of their metabolic functions and may ultimately bring forward targets for modulating their inflammatory traits. Using various approaches, we identified unique metabolic activation of ATMs in obesity that does not resemble M1 or M2 macrophages. Metabolic activation of macrophages, characterised by increased OXPHOS and glycolysis, was dose-dependently induced during a co-culture with adipose tissue and translated into increased cytokine secretion. Although various metabolic pathways contributed to cytokine release by ATMs, glycolysis accounted mostly for the higher cytokine production by ATMs from obese mice. Inflammatory activation of ATMs during early stages of obesity, however, appeared to be independent of HIF-1α. Further understanding of the functional consequences of metabolic programming in macrophages in lean adipose tissue and metabolic activation in ATMs residing in obese adipose tissue is expected to lead to novel therapeutic targets to curtail inflammatory responses that will ultimately reduce obesity-induced metabolic complications.

METHODS

Mice

Male C57Bl/6 mice (Harlan, Horst, Germany) were on a high-fat diet (HFD) containing either 45% (D12451) or 60% energy derived from fat (D12492), or on a low-fat diet (LFD) containing 10% energy derived from fat and matching most other components present in either the 45% HFD (D12450B) or 60% HFD (D12450J), for 16 weeks. Mice were stratified based upon body weight at the start of the LFD or HFD intervention. All procedures were approved by the ethics committee for animal experiments at Wageningen University.

For studying the role of HIF-1α in ATMs during the development of obesity, 9- to 12-week-old male C57/Bl6 mice with floxed *Hif-1α* (also known as *Hif1a*) (exon 2) crossed into a background of lysozyme M-driven cre recombinase (LysM *Hif-1α*$^{-/-}$) or C57/Bl6 controls not carrying lysozyme M-driven cre recombinase (LysM *Hif-1α*$^{+/+}$) [15] were exposed to an HFD (D12492) for 8 weeks. After 7 weeks, an insulin tolerance test was performed in mice fasted for 5 h, by injecting insulin (1 U/kg body weight) intraperitoneally. Blood was taken from the tail at specific time points and glucose was measured using Accu-check glucose meters (Roche Diagnostics, Almere, the Netherlands). The study was carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The protocol was approved by the Dartmouth IACUC.

All mice were individually housed and had ad libitum access to food and water. All diets were obtained from Research Diets (New Brunswick, NJ, USA). Experimenters were not blinded to group assignment and outcome assessment.

Cell culture

ATMs and peritoneal macrophages were isolated from male, wildtype C57Bl/6 mice (Harlan). For details of tissue and cell collection, see ESM Methods.

ATMs

Freshly isolated ATMs were cultured in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% (vol./vol.) FCS and 1% (vol./vol.) penicillin/streptomycin (PS) (RPMI/FCS/PS) for 24 h (200,000 cells/well). The contribution of various metabolic pathways to cytokine release was examined by providing 5.5 mmol/l 2-deoxy glucose (2-DG) (Merck, Darmstadt, Germany), 50 µmol/l Etomoxir (Merck), 10 µmol/l UK5099 (Merck) or 10 µmol/l BPTES (Merck) 2 h after plating until the end of the 24 h culture period.

Bone marrow-derived macrophages

Bone marrow cells were cultured in DMEM (Lonza) supplemented with 10% (vol./ vol.) FCS and 1% (vol./vol.) PS (DMEM/FCS/PS) and 5% (vol./vol.) L929-conditioned medium (L929). After 3–4 days, adherent bone marrow-derived macrophages (BMDMs) were re-plated and exposed for 3 days to an insert containing 25 mg or 100 mg of minced epididymal adipose

tissue collected from LFD-fed or HFD-fed mice. Control BMDMs were held in DMEM/FCS/PS containing 5% (vol./vol.) L929 with an empty insert for the same length of time. M1 and M2 macrophages were generated by 24 h incubation with 10 ng/ml lipopolysac- charide (LPS) (M1) or 25 ng/ml IL-4 (M2). For measuring cytokine and lactate production of BMDMs after adipose tis- sue priming, inserts were removed and BMDMs were held in fresh DMEM/FCS/PS for an additional 24 h. For extracellular flux analysis, BMDMs were scraped, counted and re-plated in a 96-well Seahorse microplate (Seahorse Bioscience, Santa Clara, CA, USA).

Adipose tissue

Epididymal adipose tissue was brought into culture and exposed to 17.5 nmol/ml insulin in DMEM/PS for 20 min to measure insulin sensitivity. The tissue was kept in DMEM/FCS/PS with or without LPS (10 ng/ml) for 24 h to measure IL-6 release or was cultured in DMEM/FCS/PS for 3 days for leptin and lactate measurements.

Extracellular flux analysis

The real-time oxygen consumption rate (OCR) and extracel- lular acidification rate (ECAR) of ATMs and BMDMs were analysed using an XF-96 Extracellular Flux Analyzer (Seahorse). See ESM Methods for further details.

Cytokine and lactate measurements

Levels of IL-6, chemokine (C-X-C motif) ligand-1 (KC), TNF-α, IL-1β, IL-10 and leptin in cell culture supernatant fractions were measured with murine DuoSet ELISA Development kits (R&D Systems, Abingdon, UK). An enzymatic assay adapted from the Lactate Assay kit (Merck) was used to determine lactate levels.

Immunohistochemistry

Paraffin-embedded sections of epididymal adipose tissue were stained with an F4/80 antibody (Bio-Rad, Veenendaal, the Netherlands) and counterstained with haematoxylin. Macrophages were visualised with 3,3-diaminobenzidene (Merck).

Western blot

Primary antibodies for actin (Merck), AMP-activated protein ki- nase (AMPK) (no. 2532L), phospho-AMPK Thr172 (no. 2531) and p-Akt Ser473 (no. 4060) (Cell Signalling, Leiden, the Netherlands) were all used at a ratio of 1:1000 according to manufacturers' instructions, and incubated overnight at 4°C. See ESM Methods for further details.

RNA isolation and qRT-PCR

RNA from ATMs and peritoneal macrophages isolated from HFD-fed or LFD-fed mice, or from BMDMs, was used for quantitative reverse-transcription PCR analysis (qRT-PCR). The following genes were measured: *Cd11c* (also known as *Itgax*), *Cd206* (also known as *Mrc1*),

Cd36, *Cd68*, *Glut1*, *Hk2*, *Hif-1α*, *Ldhα* (also known as *Ldha*), *Lipa*, *Pdk4*, *Plin2* and *Vegfα* (also known as *Vegfa*), normalised against *36b4* (also known as *Rplp0*) (**ESM Table 1**). For further details, see ESM Methods.

Microarray analysis and interpretation

Four pools of ATMs isolated from epididymal adipose tissue of male C57Bl/6 mice fed an LFD or HFD in four separate exper- iments were subjected to expression profiling by microarray. In addition, raw transcriptome data from various tissue macro- phages, including ATMs (GEO accession no. GSE56682), from LPS-stimulated BMDMs (GSE53986) and from obese diabetic and obese non-diabetic humans (GSE54350) were obtained from the Gene Expression Omnibus. Details of the microarray analysis and interpretation are in the ESM Methods. Microarray data have been submitted to the Gene Expression Omnibus (accession number GSE84000).

Statistical analysis

Results are shown as mean ± SEM. Statistically significant differences between two groups were calculated using Student's t test. For comparisons between more than two groups, a one-way ANOVA and post hoc Bonferroni's multi- ple comparison test was done. When comparing diet and treatment effects within one experiment, data were analysed with a two-way ANOVA with post hoc Bonferroni test (treatment vs control). A p value ≤0.05 was considered significant.

RESULTS

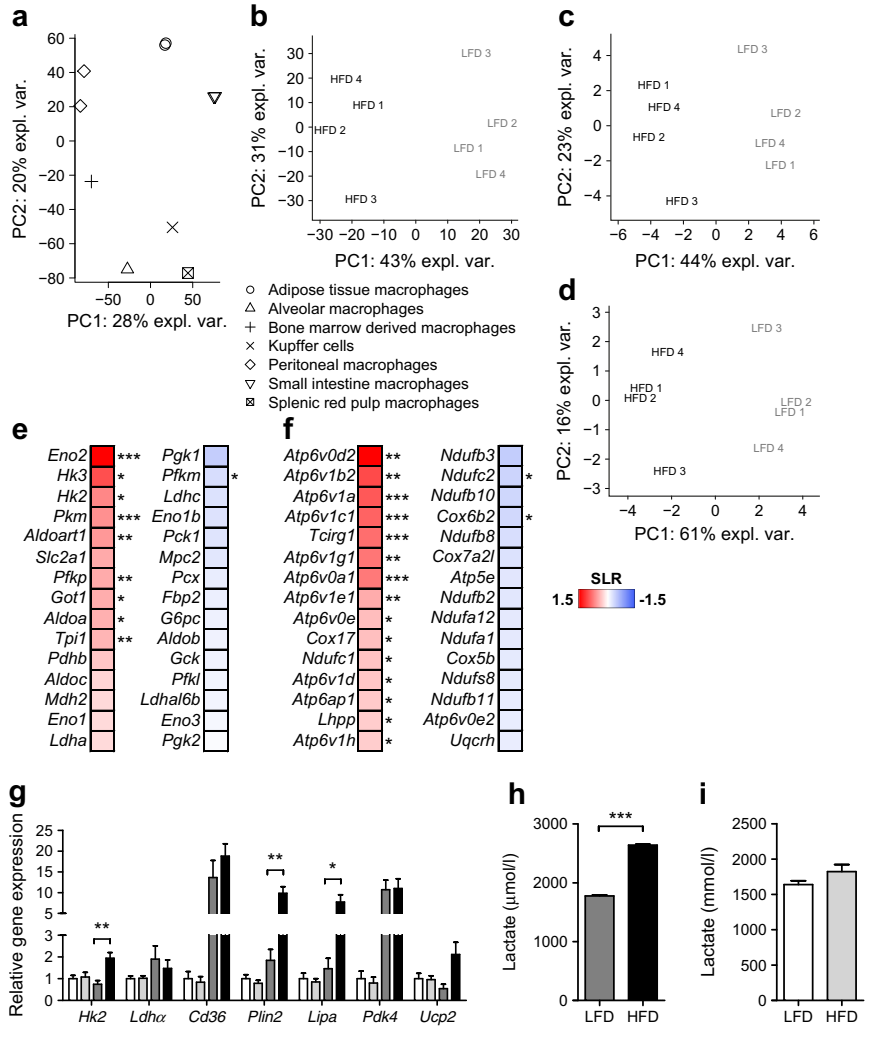

Fig. 1 Unique metabolic and inflammatory activation in ATMs during obesity.
(**a**) PCA of various tissue macrophages, separated based on their transcriptome. n = 2 for macrophages from peritoneum, small intestine and adipose tissue; n = 1 for other macrophages. Data are derived from GEO accession no. GSE53986. Percentage of explained variance (expl. var.) is shown. (**b**–**d**) PCA plot of ATMs from obese (HFD-fed) vs lean (LFD-fed) mice (four pools of 4–7 HFD- or LFD-fed mice), separated based on the complete transcriptome (**b**) or on expression levels of inflammatory genes (**c**) or metabolic genes (**d**). (**e**, **f**) Heat maps reflecting expression levels of the top 15 most differentially higher (red) or lower (blue) expressed genes involved in glycolysis (**e**) or OXPHOS (**f**) in ATMs from obese vs lean mice, presented as $\log_2$ ratio difference (SLR). (**g**) Expression levels of metabolic genes in peritoneal macrophages and ATMs. The $2^{-\Delta\Delta C_t}$ method was used to determine fold change inductions normalised to *36b4*. Diet-induced changes in expression levels (HFD vs LFD) were tested for significance. (**h**, **i**) Lactate production over 24 h by ATMs (**h**) or peritoneal macrophages (**i**) from lean (LFD) or obese (HFD) mice. Peritoneal macrophages: n = 7 (LFD, white bars) vs n = 6 (HFD, light grey bars) were used for qRT-PCRs and lactate measurements (**g**, **i**). ATMs: four pools (LFD, dark grey bars) vs six pools (HFD, black bars) of 4–7 mice were used for qRT-PCR (**g**) and a pool of 9 LFD-fed mice vs a pool of 5 HFD-fed mice was used for lactate measurements (n = 3) (**h**). Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, as shown by brackets, or for obese vs lean mice in (**e**, **f**)

Unique metabolic and inflammatory activation of ATMs in obesity

To examine whether macrophages residing in adipose tissue are transcriptionally distinct from other tissue macrophages, we performed a principal component analysis (PCA) using publicly available gene expression profiles of macrophages isolated from the peritoneal cavity, liver, spleen, lung, intestine and adipose tissue [16]. Indeed, ATMs exhibited unique transcriptomes (**Fig. 1a**). The presence of obesity clearly affected complete transcriptomes of ATMs further as demonstrated by distinct clustering of ATMs sorted from obese vs lean mice by the pan macrophage-membrane marker Emr1-F4/80 [17, 18] (**Fig. 1b**). Traditionally, macrophages in obese adipose tissue are characterised by an enhanced inflammatory state [2]. Using inflammatory genes (**ESM Table 2**) as input for PCA confirmed distinctive inflammatory activation of macrophages residing in obese adipose tissue (**Fig. 1c**). Interestingly, expression data for genes involved in glycolysis, OXPHOS and amino acid metabolism (**ESM Table 2**) were also sufficient to distinguish ATMs of obese mice from ATMs of lean mice (**Fig. 1d**), suggestive of robust changes in energy metabolism of ATMs in obesity. In fact, many genes involved in glycolysis and OXPHOS were upregulated in ATMs from obese vs lean mice (**Fig. 1e**, **f**). Noticeably, strong metabolic rewiring upon obesity was specific for ATMs, as these changes were not observed in peritoneal macrophages (**Fig. 1g**, **i**). Indeed, metabolic genes were expressed at much higher levels in ATMs than in peritoneal macrophages and were upregulated in obesity (**Fig. 1g**). In support of our gene expression data, ATMs derived from obese vs lean animals produced more lactate ex vivo (**Fig. 1h**), reflective of higher glycolytic rates. By contrast, no robust difference in lactate secretion was found in peritoneal macrophages isolated from obese vs lean mice (**Fig. 1i**).

Metabolic and inflammatory activation of macrophages in obese adipose tissue is distinct from classical activation by LPS and associates with the presence of type 2 diabetes in obese humans

To gain further insight into functional properties of metabolic and inflammatory rewiring found in ATMs, gene set enrichment analysis (GSEA) was performed using metabolic and inflammatory gene sets as input (**ESM Table 3**). Overall, far more gene sets were significantly ($p < 0.01$) enriched than depleted (30 vs 2) in ATMs from obese vs lean mice and none of the depleted gene sets reflected metabolic pathways. Interestingly, metabolic gene sets including glycolysis and OXPHOS were strongly enriched in ATMs upon obesity (**Fig. 2a**). Noticeably, transcriptional regulation in ATMs in obesity was very distinct from classical macrophage activation by LPS. Although some pathways were similarly regulated, most metabolic routes, including OXPHOS, glycolysis and the pentose phosphate pathway, were less or inversely regulated in LPS-activated macrophages compared with ATMs from obese vs lean mice (**Fig. 2b**). In line with different metabolic regulation in ATMs vs classically activated macrophages, we found diverse regulation of various pro- and anti-inflammatory genes, including *Cd11c*, *Myd88*, *Arg1* and *Il-10* (also known as *Il10*) in ATMs from obese vs lean mice not resembling M1 (nor M2) macrophages (**Fig. 2c**).

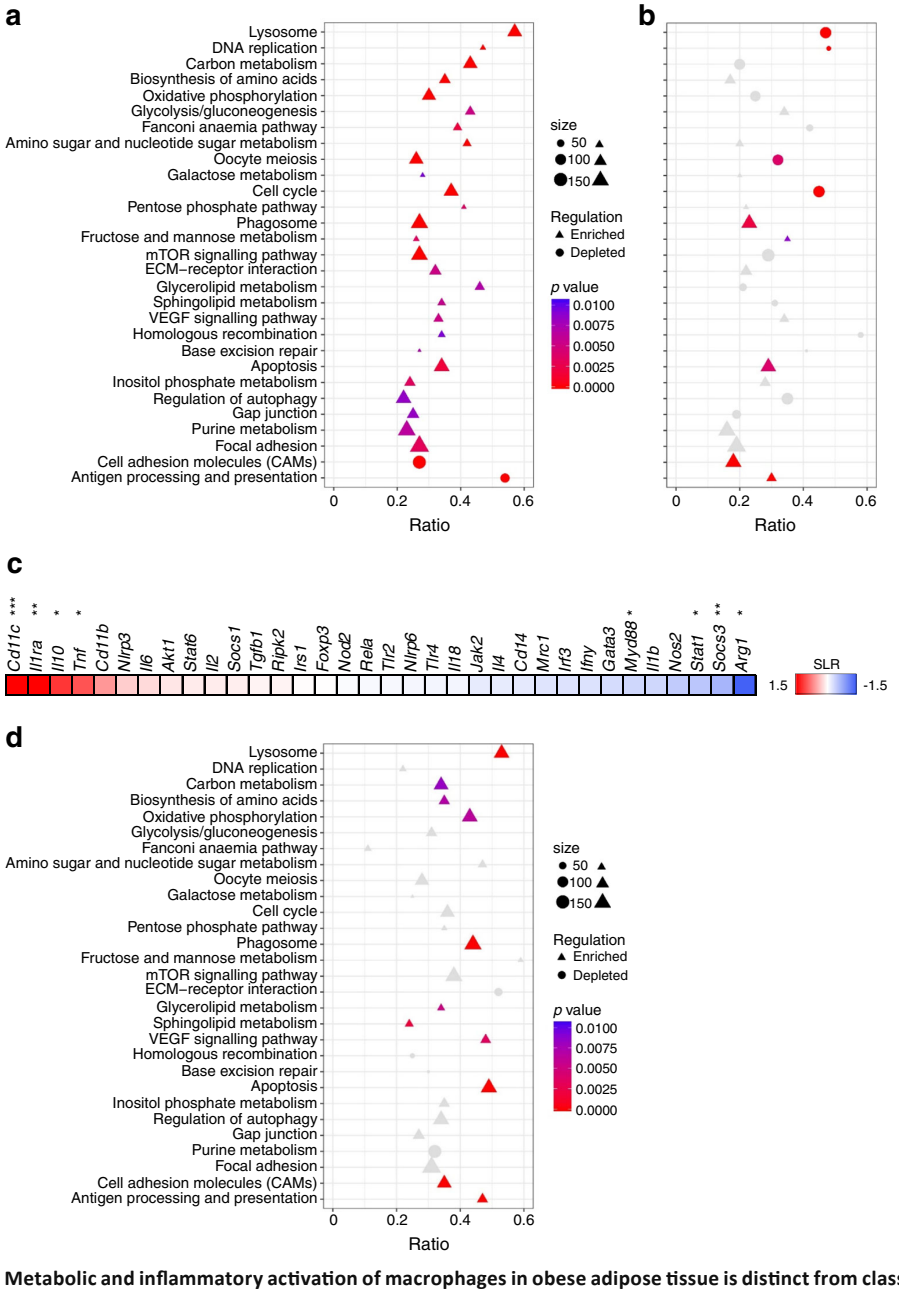

Fig. 2 Metabolic and inflammatory activation of macrophages in obese adipose tissue is distinct from classical activation by LPS and associates with the presence of type 2 diabetes in obese humans.
(**a**) Enriched or depleted ($p < 0.01$) KEGG-derived gene sets in ATMs of obese (HFD; n = 4 pools) vs lean (LFD; n = 4 pools) mice. (**b**, **d**) Regulation of these enriched or depleted gene sets in LPS-activated (n = 4) vs untreated BMDMs (n = 4) (**b**) or in CD14 cells from obese diabetic (n = 6) vs obese non-diabetic individuals (n = 6) (**d**). Data presented in (**b**) are derived from GEO accession no. GSE53986 and in (**d**) from GSE54350. (**c**) Heat map showing expression of genes involved in inflammation in ATMs from obese vs lean mice presented as $\log_2$ ratio (SLR) difference with red representing upregulation and blue representing downregulation in ATMs of HFD- vs LFD-fed mice; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for obese vs lean mice

Interestingly, metabolic activation as found in the ATM part of obese adipose tissue was also apparent in $CD14^+$ cells isolated from visceral adipose tissue of obese humans with diabetes compared with obese non-diabetic individuals (**Fig. 2a**, **d**).

Functional consequences of metabolic activation of ATMs in obesity

Next, we evaluated whether transcriptional changes in ATMs translated into differences in energy metabolism. To that end, OXPHOS and glycolysis rates of freshly isolated ATMs were examined by measuring OCR and ECAR. In line with increased expression of metabolic genes, ATMs from obese mice displayed higher rates of OXPHOS (**Fig. 3a**). Moreover, a higher glycolytic rate was seen in ATMs from obese mice (**Fig. 3b**). Although this difference did not reach statistical significance, it corroborated the significantly higher levels of lactate in supernatant fractions of ATMs from obese vs lean mice after a 24 h culture period (**Fig. 1h**). As expected based on previous findings [2, 19–21], ATMs from obese mice produced more IL-6 and KC than macrophages isolated from lean adipose tissue (**Fig. 3c**, **d**). In contrast, less TNF-α was secreted by ATMs from obese vs lean mice (**Fig. 3e**), even though expression of Tnf was increased in ATMs (**Fig. 2c**). Of note, neither IL-1β nor IL-10 could be detected in the supernatant fractions of ATMs.

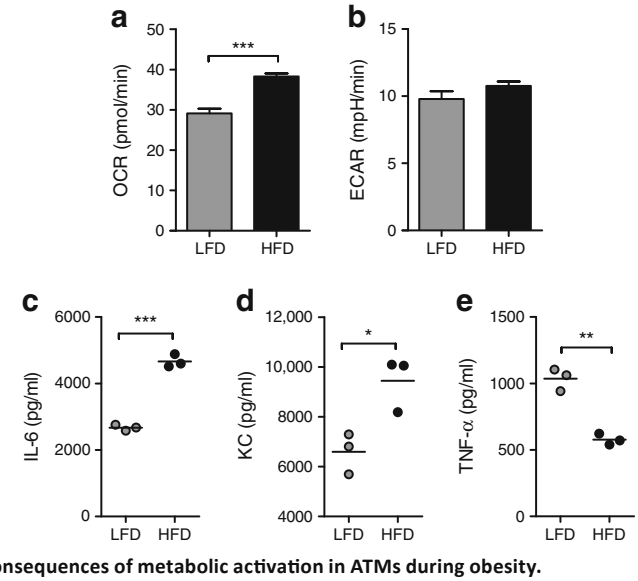

Fig. 3 Functional consequences of metabolic activation in ATMs during obesity.
(**a**, **b**) Basal OCR (**a**) or ECAR (**b**) of freshly isolated ATMs from obese (HFD) or lean (LFD) mice. (**c**–**e**) IL-6 (**c**), KC (**d**) or TNF-α (**e**) secretion over 24 h by ATMs from HFD- or LFD-fed mice. The ATMs (n ≥ 3) were plated from a pool of nine (LFD) or five (HFD) mice. Data are presented as means (**c**, **d**, **e**) ± SEM (**a**, **b**). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

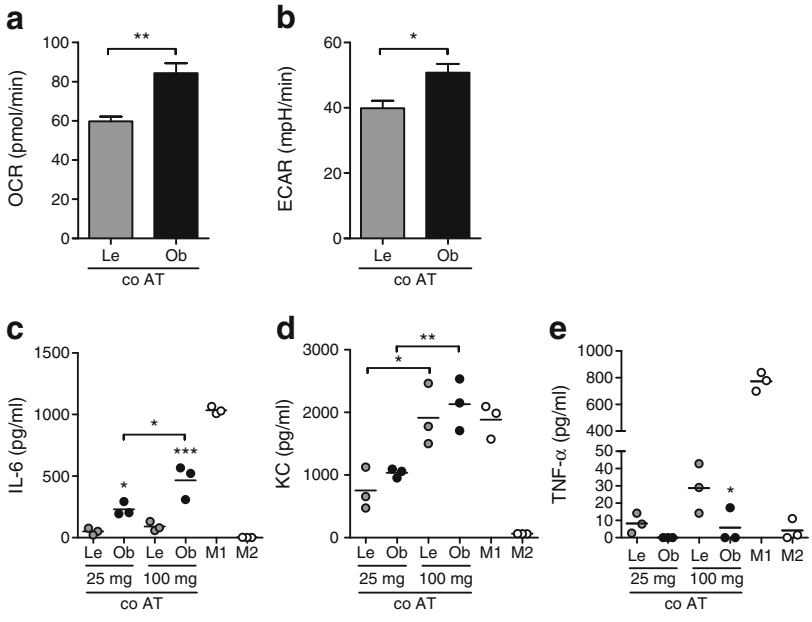

Fig. 4 Dose-dependent adipose tissue-induced activation of macrophages ex vivo.
(**a**, **b**) Basal OCR (**a**) and ECAR (**b**) of BMDMs co-cultured (co AT) with 100 mg of obese (Ob) or lean (Le) adipose tissue for 3 days. (**c**–**e**) Secretion of IL-6 (**c**), KC (**d**) or TNF-α (**e**) by BMDMs exposed to lean or obese adipose tissue (AT), or activated by LPS (M1) or IL-4 (M2). Effects of diet (obese vs lean) and dose of adipose tissue (25 mg vs 100 mg) were tested for significance. n ≥ 3 for all experiments. Data are presented as means (**c**, **d**, **e**) ± SEM (**a**, **b**); *$p < 0.05$, **$p < 0.01$

Exposing macrophages to adipose tissue explants ex vivo dose-dependently induces metabolic activation and cytokine release

To examine whether changes in the adipose tissue environment drive metabolic and inflammatory activation of ATMs in obesity, we co-cultured BMDMs with lean or obese adipose tissue for 3 days, removed the adipose tissue and examined the adipose tissue-induced activation of BMDMs in comparison with classically (LPS) or alternatively (IL-4) activated BMDMs both directly (metabolism) and after 24 h in fresh medium (cytokines). As observed with the ATMs from obese mice, BMDMs exposed to obese adipose tissue exhibited enhanced glycolysis and OXPHOS compared with BMDMs exposed to lean adipose tissue, reflected by increased OCR (**Fig. 4a**) and ECAR (**Fig. 4b**). Moreover, BMDMs exposed to obese adipose tissue secreted more IL-6 and KC and less TNF-α than BMDMs exposed to lean adipose tissue (**Fig. 4c**–**e**), like ATMs from obese vs lean mice (**Fig. 3c**–**e**). Interestingly, inflammatory activation was found to be dose-dependently induced by the adipose tissue explants and appeared distinct from either LPS- or IL-4-activated macrophages (**Fig. 4c**–**e**). It is worth noting that metabolic rewiring followed a similar trend, with a dose-dependent increase in glycolysis in macrophages exposed to either lean or obese adipose tissue (**ESM Fig. 1a**) and higher OXPHOS specifically in macrophages exposed to 100 mg of obese adipose tissue (**ESM Fig. 1b**). Our observation of dose-dependent adipose tissue-induced

activation of macrophages, different from classical activation, points to a role for specific environmental cues in driving the unique ATM signatures. Adipose tissue is a source of leptin [22] and lactate [23] which are known to affect immune cell metabolism and function [24–26]. Despite higher levels of both lactate and leptin in the supernatant fractions of adipose tissue explants isolated from obese vs lean mice (**ESM Fig. 1c**,**d**), application of neither leptin nor lactate could induce metabolic activation comparable with that seen in macrophages exposed to obese adipose tissue (**ESM Fig. 1e**,**f**). Of note, as with ATMs, neither IL-1β nor IL-10 were detected in supernatant fractions of BMDMs after exposure to adipose tissue.

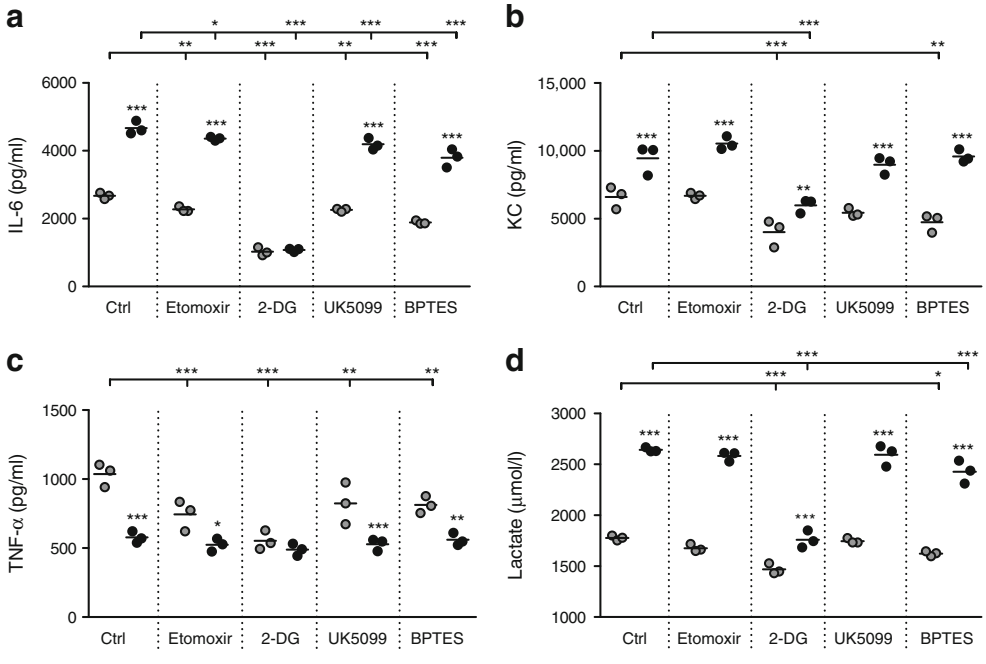

Fig. 5 Glycolysis largely controls cytokine release by ATMs during obesity.
Secretion of IL-6 (**a**), KC (**b**), TNF-α (**c**) or lactate (**d**) by ATMs isolated from lean (grey circles) or obese (black circles) mice. Cytokines were measured basally (Ctrl) or upon stimulation of ATMs with various metabolic inhibitors for 24 h. Effects of diet (obese vs lean) and treatment (metabolic inhibitors vs control) were tested for significance. Secretion of TNF-α by ATMs from obese mice tended to be reduced upon treatment with 2-DG ($p = 0.06$). ATMs (n = 3) were plated from a pool of 9 LFD-fed mice and 5 HFD-fed mice. Data are presented as means; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for indicated comparisons (vs Ctrl obese or lean as shown) or for obese vs lean within each treatment

Glycolysis largely controls cytokine release by ATMs in obesity

To examine the contribution of metabolic routes for cytokine production by ATMs we measured IL-6, KC and TNF-α upon inhibition of glucose uptake (2-DG), fatty acid oxidation (Etomoxir), glucose oxidation (UK5099) or glutamine influx into the tricarboxylic acid cycle (BPTES). Minimal morphological changes were observed in ATMs after treatment with these compounds. Although several metabolic routes appeared to contribute to cytokine release in ATMs from lean mice, overall, 2-DG treatment had the most profound effects

on cytokine release in ATMs from both lean and obese mice (**Fig. 5a**–**c**). Importantly, inhibition of glycolysis with 2-DG abolished basal differences in cytokine release by ATMs from obese vs lean mice, demonstrating that glycolysis makes an important contribution to the increased inflammatory cytokine production by ATMs in obese adipose tissue (**Fig. 5a**–**c**). By competing with glucose, 2-DG may reduce both lactate production and glucose oxidation. Because UK5099 affected cytokine release by ATMs to a lesser extent than 2-DG, likely anaerobic glycolysis and not glucose oxidation is important for cytokine secretion by ATMs, especially in the obese state. Indeed 2-DG was the only inhibitor that strongly reduced lactate secretion by ATMs (**Fig. 5d**), supporting a link between lactate production and cytokine release by ATMs, particularly in obese conditions.

Myeloid-specific Hif-1α does not affect adipose tissue inflammation in HFD-fed mice

Key metabolic regulators such as AMPK and HIF-1α link intracellular metabolism to inflammatory activation [14, 27]. Metabolic stress-reactive AMPK enhances OXPHOS and fatty acid oxidation upon its activation through phosphorylation and was clearly induced in macrophages in an obese adipose tissue environment (**ESM Fig. 2a**). The transcription factor HIF-1α, a regulator of genes involved in glycolysis, was predicted to be activated in ATMs part of obese adipose tissue as well (Ingenuity Pathway Analysis: *z* score 2.460, *p* value overlap <0.01). Moreover, *Hif-1α* itself and its target genes were upregulated in macrophages in an obese adipose tissue environment, with higher levels in BMDMs ex- posed to obese vs lean adipose tissue (**Fig. 6a**). Unexpectedly, however, deletion of *Hif-1α* in myeloid cells did not affect the inflammatory state of obese adipose tissue after 8 weeks of HFD feeding, as demonstrated by equal IL-6 secretion by complete adipose tissue isolated from LysM *Hif-1α*$^{-/-}$ and LysM *Hif-1α*$^{+/+}$ mice, both basally (**Fig. 6b**) and upon LPS stimulation (**Fig. 6c**). Analysis of macrophage and inflammatory marker expression (*Cd68*, *Cd11c*, *Cd206*) in the adipose tissue revealed no protection against adipose tissue inflammation in HFD-fed mice lacking *Hif-1α* in the myeloid compartment (data not shown). Moreover, no robust differences were found in either total body insulin sensitivity (**Fig. 6d**) or in adipose tissue-specific insulin sensitivity (**Fig. 6e**) in LysM *Hif-1α*$^{-/-}$ vs LysM *Hif-1α*$^{+/+}$ mice after 8 weeks of HFD feeding. We did observe slightly higher glucose levels, as well as a steeper rebound phase of the insulin tolerance test, in LysM *Hif-1α*$^{-/-}$ vs LysM *Hif-1α*$^{+/+}$ mice (**Fig. 6d**), pointing to impaired fasting glucose. However, the LysM *Hif-1α*$^{-/-}$ mice were heavier than the LysM *Hif-1α*$^{+/+}$ mice (**ESM Fig. 2b,c**), resulting in higher adipose tissue weight (**ESM Fig. 2d**), which may have accounted for the higher fasting glucose. It is worth noting that differences in body weight could not be explained by food intake, which was similar in both genotypes (data not shown), and may rather be due to differences in locomotor activity or energy absorption, although this requires further study.

We observed increased spare respiratory capacity (SRC) of macrophages in an adipose tissue environment compared with control macrophages (**Fig. 6f**), indicative of enhanced cellular flexibility and capacity to manage stressful situations [28, 29]. Interestingly, macrophages lacking *Hif-1α* had lower SRC than wild-type BMDMs (**Fig. 6g**) and appeared to be less glycolytic (**Fig. 6h, i**). The absence of compensation for lower glycolytic rates by *Hif-1α*$^{-/-}$ BMDMs (i.e. by increasing oxygen consumption) (**Fig. 6j**), is suggestive of reduced metabolic capacity and flexibility in the absence of *Hif-1α* and this may outweigh the potential anti-inflammatory effects of lower glycolysis in an adipose tissue environment.

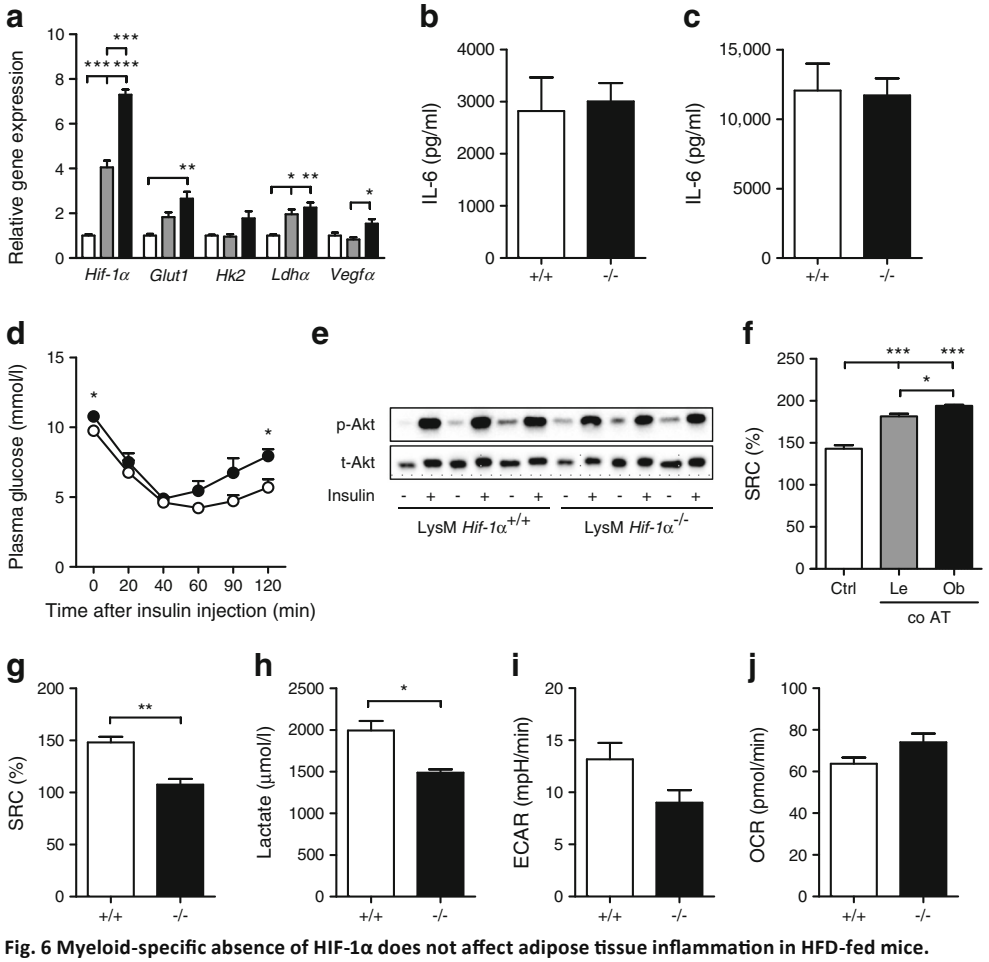

Fig. 6 Myeloid-specific absence of HIF-1α does not affect adipose tissue inflammation in HFD-fed mice. (**a**) Fold change expression of *Hif-1α* and its target genes in BMDMs held in L929-conditioned medium (white bars) or exposed to 100 mg lean adipose tissue explant (grey bars) or obese adipose tissue explant (black bars) for 3 days. Starting quantities were used for normalisation against *36b4*. (**b**, **c**) IL-6 production by epididymal adipose tissue isolated from LysM *Hif-1α*$^{+/+}$ (+/+) or LysM *Hif-1α*$^{-/-}$ (–/–) mice, either unstimulated (**b**) or stimulated with 10 ng/ml LPS for 24 h (**c**). (**d**) Glucose measured in plasma of LysM *Hif-1α*$^{+/+}$ (white circles) or LysM *Hif-1α*$^{-/-}$ (black circles) mice upon the injection of insulin at 0 min. (**e**) Total Akt (t-Akt) and p-Akt protein levels in epididymal adipose tissue from LysM *Hif-1α*$^{+/+}$ or LysM *Hif-1α*$^{-/-}$ mice unstimulated (–) or stimulated with insulin (+) for 20 min. (**f**) SRC as percentage increase from basal OCR in BMDMs in 5% (vol./vol.) L929 (Ctrl) or upon 3 days of co-culture (co AT) with 100 mg adipose tissue isolated from lean (Le) or obese (Ob) mice. (**g**) SRC as percentage increase from basal OCR in BMDMs isolated from LysM *Hif-1α*$^{+/+}$ (+/+) or LysM *Hif-1α*$^{-/-}$ (–/–) mice. (**h**) Lactate secretion over 24 h by BMDMs isolated from LysM *Hif-1α*$^{+/+}$ (+/+) or LysM *Hif-1α*$^{-/-}$ (–/–) mice. (**i**, **j**) Basal ECAR (**i**) and OCR (**j**) in BMDMs from LysM *Hif-1α*$^{+/+}$ (+/+) or LysM *Hif-1α*$^{-/-}$ (–/–) mice. Basal ECAR were lower in BMDMs of LysM *Hif-1α*$^{-/-}$ vs LysM *Hif-1α*$^{+/+}$ mice (although the difference did not reach statistical significance, $p < 0.051$). For the in vivo study, n = 8 animals per genotype/diet were included. n = 3 for all in vitro experi- ments. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

DISCUSSION

In obesity, macrophages fuel adipose tissue inflammation, promoting the development of insulin resistance and type 2 diabetes [30]. The inflammatory state of ATMs has been studied extensively. To our knowledge, however, we are the first to measure real-time metabolic fluxes in freshly isolated ATMs. Interestingly, our data strongly point to unique metabolic activation that drives cytokine release by ATMs in obesity, resembling neither the metabolic nor inflammatory signatures seen in M1- or M2-primed macrophages or peritoneal macrophages.

Cumulative evidence from the field of immunology shows that robust metabolic rewiring fuels differential inflammatory activation of macrophages. On the one hand, M2 macrophages require OXPHOS for responses, whereas M1 macrophages rely on aerobic glycolysis [11, 12]. In contrast to these two extremes, we found that macrophages in an adipose tissue environment adopt a unique metabolic profile in obesity, characterised by activation of various metabolic routes including both OXPHOS and glycolysis. Metabolic and inflammatory adaptations in obesity were specific for ATMs, as no metabolic rewiring was found in peritoneal macrophages. In line with our finding of unique metabolic rewiring in ATMs and supportive of various studies reporting diverse inflammatory activation of ATMs in obese adipose tissue [9, 16, 31, 32], we found inflammatory activation of ATMs to be different from that of classically activated macrophages.

Interestingly, macrophages co-cultured with obese adipose tissue developed similar phenotypical adaptations in a dose-dependent manner, suggestive of obesity-induced changes in the adipose tissue microenvironment shaping the ATM phenotype. Indeed, the composition of adipose tissue is importantly affected in obesity, with resultant adipocyte hypertrophy and both accumulation and phenotypical changes of immune cells including macrophages. In our co-culture system we have used lean and obese adipose tissue explants of equal weight. This may not have accounted for all the shifts in relative cell numbers occurring in obese adipose tissue, yet strongly points toward the existence of divergent factors secreted by obese vs lean adipose tissue that may critically influence the macrophage phenotype in a dose-dependent manner. Potential factors may include adipokines, cytokines, fatty acids or other metabolites [30]. Interestingly, leptin [24, 25] and lactate [26] have been shown capable of remodelling intracellular metabolism and changing the inflammatory state of macrophages. We found both to be secreted more by obese adipose tissue than by lean adipose tissue, yet neither leptin nor lactate induced metabolic rewiring similar to that seen in macrophages in an adipose tissue environment. Additionally, differences in cell death between lean and obese adipose tissue in vivo may have an effect on metabolic rewiring in macrophages. Most likely, a mixture of signals is responsible for shaping ATM metabolic phenotypes in the lean and obese state, although this needs further investigation.

Importantly, metabolic activation of ATMs contributes to their inflammatory cytokine release. First, metabolically active ATMs from obese mice secreted far more IL-6 and KC than the less metabolically active ATMs isolated from lean mice. Second, we found that interference with metabolic routes directly affected cytokine release by ATMs. Especially in ATMs from lean mice, several metabolic routes including fatty acid oxidation, glycolysis and glutaminolysis contribute to cytokine release. Glycolysis appears to play a dominant role in fuelling the inflammatory trait of ATMs from obese adipose tissue, since inhibiting glycolysis with 2-DG almost completely abolished the greater basal cytokine secretion by ATMs from obese vs lean mice.

Our finding of lower TNF-α secretion by ATMs from obese vs lean mice was unexpected, as was the lower level of TNF-α in supernatant fractions of macrophages exposed to obese vs lean adipose tissue. Despite lower cytokine levels, we found *Tnf* upregulated at the mRNA level. A similar discrepancy between mRNA and protein level has been reported in ATMs before [10]. In obese adipose tissue, however, macrophage influx and proliferation as well as an increase in other immune cell populations likely overrules lower TNF-α secretion per macrophage and might be responsible for higher TNF-α levels found in the adipose tissue and circulation of obese individuals [6, 33–35]. Alternatively, enhanced autocrine TNF signalling in ATMs may explain the lower levels of TNF-α measured in ATM supernatant fractions.

Next to cytokine release, intracellular metabolism most likely controls several other macrophage functions. For example, OXPHOS has been found to contribute to phagocytosis by human monocytes [36], and lysosomal biogenesis and function in T cells [37]. Both phagocytic and lysosomal genes were found to be strongly upregulated in ATMs of obese mice and in obese individuals with type 2 diabetes. Phagocytosis of dead adipocytes by macrophages [38–40] and lysosomal function of ATMs [10, 39] are considered to be important for maintaining adipose tissue homeostasis. Hence, the obesity-induced increase in OXPHOS might fuel ATM functions in expanding adipose tissue, not directly related to inflammatory cytokine release yet may greatly affect adipose tissue function.

Our data show that interfering with metabolic routes alters the inflammatory phenotype of ATMs and that glycolysis importantly contributes to inflammatory cytokine release by ATMs. Unexpectedly, however, myeloid-specific deletion of a previously identified key regulator of glycolysis, *Hif-1α*, did not alleviate inflammatory activation of ATMs during the early stages of obesity. Because mice lacking *Hif-1α* in myeloid cells were significantly heavier yet did not display increased adipose tissue inflammation or insulin resistance, one could speculate that HIF-1α may be partly protective for the development of obesity-induced adipose tissue inflammation, as has been reported before in mice fed an HFD for 18 weeks [41]. One might also hypothesise that during the earlier stages of HFD-induced obesity HIF-1α is important for controlling other metabolic properties of macrophages not related to cytokine production.

For example, our data revealed a decreased SRC in BMDMs from *Hif-1α*$^{-/-}$ mice, suggestive of a role for HIF-1α in maintaining metabolic flexibility of macrophages. Indeed, despite decreased capability of using glycolysis as an energy source, *Hif-1α*$^{-/-}$ macrophages lack the flexibility to increase their oxidative capacity [15]. Metabolic flexibility is probably needed for ATMs, as we observed enhanced SRC in macrophages in an adipose tissue environment which was even further in- creased upon exposure to obese adipose tissue. Reduced metabolic flexibility in macrophages lacking *Hif-1α* in expanding adipose tissue might have overruled effects on cytokine release during the development of obesity.

In conclusion, we identified unique metabolic activation of ATMs in obesity, characterised by increased OXPHOS and glycolysis. Blocking metabolic routes in isolated ATMs led to the identification of glycolysis as a main contributor to their proinflammatory trait, especially in obesity. Interestingly, metabolic signatures, similar to those found in ATMs upon obesity, including the induction of OXPHOS and lysosomal genes, were observed in human macrophages isolated from adipose tissue of obese individuals with type 2 diabetes. Further understanding of metabolic programming in ATMs will most likely lead to novel therapeutic targets to modulate macrophage metabolism and curtail inflammatory responses that drive insulin resistance and type 2 diabetes in obese individuals.

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SUPPLEMENTARY METHODS (ESM)

Collection of adipose tissue and macrophages

After cervical dislocation, epididymal adipose tissue was removed and fixated for immunohistochemistry, fresh-frozen for RNA isolation, or processed sterile to separate the stromal vascular fraction (SVF) from adipocytes using collagenase digestion as described previously [1]. Adipose tissue macrophages (ATMs) were purified from the stromal vascular fraction by F4/80+ positive selection with magnetic beads (Milteny Biotec, Leiden, The Netherlands). Cell pellets were frozen directly for RNA isolation, or were re-suspended and brought into culture in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% vol./vol. FCS and 1% vol./vol. PS (RPMI/FCS/PS). Peritoneal macrophages were obtained by infusion and subsequent collection of ice-cold PBS from the abdominal cavity, and frozen directly or brought into culture in Dulbecco's modified eagle's medium (Lonza) supplemented with 10% vol./vol. FCS and 1% vol./vol. PS (DMEM/FCS/PS). Bone marrow cells were isolated from femurs and tibia of low-fat diet- or chow-fed mice following a standard protocol.

Extracellular flux analysis

Basal metabolic rates of ATMs or bone marrow-derived macrophages (BMDMs) seeded in quintuplicate were determined during four consecutive measurements in unbuffered Seahorse medium (8.3 g DMEM powder, 0.016 g phenol red and 1.85 g NaCl in 1 l milli-Q, pH set at 7.4 at 37 °C; sterile-filtered) containing 11 mmol/l glucose (ATMs) or 25 mmol/l glucose (BMDMs) and 2 mmol/l L-glutamine (ATMs & BMDMs). After basal measurements, three consecutive measurements were taken upon the addition of each 1.5 µmol/l oligomycin, 1.5 mmol/l FCCP, and 2 µmol/l antimycin together with 1 µmol/l rotenone. Pyruvate (1 mmol/l) was added together with FCCP to fuel maximal respiration. Spare Respiratory Capacity was calculated as percentage increase in OCR after FCCP injection, with basal OCR set at 100%. All compounds used during the Seahorse runs were acquired from Merck. Signals were normalized to relative DNA content in the wells using the Quant-iT™ dsDNA Assay Kit (ThermoFisher Scientific).

Western blot

Cells were lysed in RIPA buffer (ThermoFisher Scientific, Landsmeer, The Netherlands) enriched with protease and phosphatase inhibitors (Roche Diagnostics). Total protein was determined using a BCA protein assay (ThermoFischer Scientific) and for each sample 15 µg of protein was loaded on a 4-15% Mini-PROTEAN TGX Precast gel (Bio-Rad). Proteins were separated by SDS gel electrophoresis and transferred to a PVDF membrane (Bio-Rad) using the Transblot Turbo System (Bio-Rad). All incubations were in Tris-buffered saline with 0.1% vol./vol. Tween-20 (TBS-T), containing 5% weight/vol. dry milk (β-actin) or 5% weight/vol. bovine serum albumin (AMPK, phospho-AMPK, phospho-Akt). HRP-conjugated goat-anti-rabbit secondary Ab (Merck) was used 1:5000 in TBS-T with 5% weight/vol. dry milk. Blots

were visualized using the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad).

RNA isolation and qPCR

Total RNA of ATMs and peritoneal macrophages was isolated with a RNeasy mini- or microkit (Qiagen, Venlo, The Netherlands). RNA of BMDMs was isolated using phenol/chloroform extraction. The RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR was carried out with a SensiMix SYBR kit (Bioline, Alphen aan den Rijn, The Netherlands) in a CFX384 system (Bio-Rad). Relative expression levels were calculated after normalization against the housekeeping gene *36b4* using the 2^{-C_t} method (ATMs and peritoneal macrophages) or starting quantities (BMDMs). Primer sequences can be found in **ESM Table 1**. For qPCR analysis, four pools of ATMs from LFD-fed, and six pools of ATMs from HFD-fed mice were used. Peritoneal macrophages of seven individual mice on a LFD and six mice on a HFD were included.

Microarray analysis and interpretation

Purified total RNA (100 ng per sample) was labelled with the Whole-Transcript Sense Target Assay (Affymetrix, Santa Clara, CA, USA; P/N 900652) and hybridized to whole-genome Affymetrix Mouse Gene 1.1 ST arrays (Affymetrix). Quality control and data analysis pipeline have been described in detail previously [2]. Briefly, normalized expression estimates of probe sets were computed by the robust multiarray analysis (RMA) algorithm [3, 4] as implemented in the Bioconductor library AffyPLM. Probe sets were redefined according to Dai et al. [5] based on annotations provided by the Entrez Gene database, which resulted in the profiling of 21,187 unique genes (custom CDF v20). Differentially expressed probe sets (genes) were identified by using linear models (library limma) and an intensity-based moderated t-statistic [6, 7]. Probe sets that satisfied the criterion of $p<0.01$ were included for Ingenuity Pathway Analysis. The Affymetrix dataset (GSE53986) was reanalysed as described above. The Illumina BeadChip experiments (GSE56682 and GSE54350) were reanalysed by background correction followed by quantile normalization (neqc) [8], removal of unspecific probes [9], where after differentially expressed probes were identified using limma [6, 7]. Principle component analysis (PCA) was performed in R using the library mixOmics [10]. Changes in gene expression were related to biologically meaningful changes using gene set enrichment analysis [11]. Only gene sets comprising more than 15 and fewer than 500 genes were taken into account. For each comparison, genes were ranked on their t-value that was calculated by the moderated t-test. Statistical significance of GSEA results was determined using 1000 permutations. The library clusterProfiler was used for visualization and interpretation of the GSEA results [12].

GSE56682

Peritoneal macrophages: F4/80+ and CD11b+ selection
Alveolar macrophages: CD11b+ and CD11c+ selection
Splenic macrophages: F4/80+ and CD11b+ selection

Kupffer cells: F4/80+ and CD11b+ selection
Intestinal macrophages: F4/80+ CD11b+ selection

GSE54350

Adipose tissue macrophages: CD14+ selection
A total of *n*=6 subjects were used per group. The obese individuals part of each study group were age- and sex-matched.

GSE53986

Untreated (*n*=4) versus LPS-stimulated (*n*=4) (10 ng/mL for 24 h) bone marrow-derived macrophages.

SUPPLEMENTARY TABLES AND FIGURES (ESM)

ESM Table 1
Primer sequences used to measure gene expression levels with qPCR.

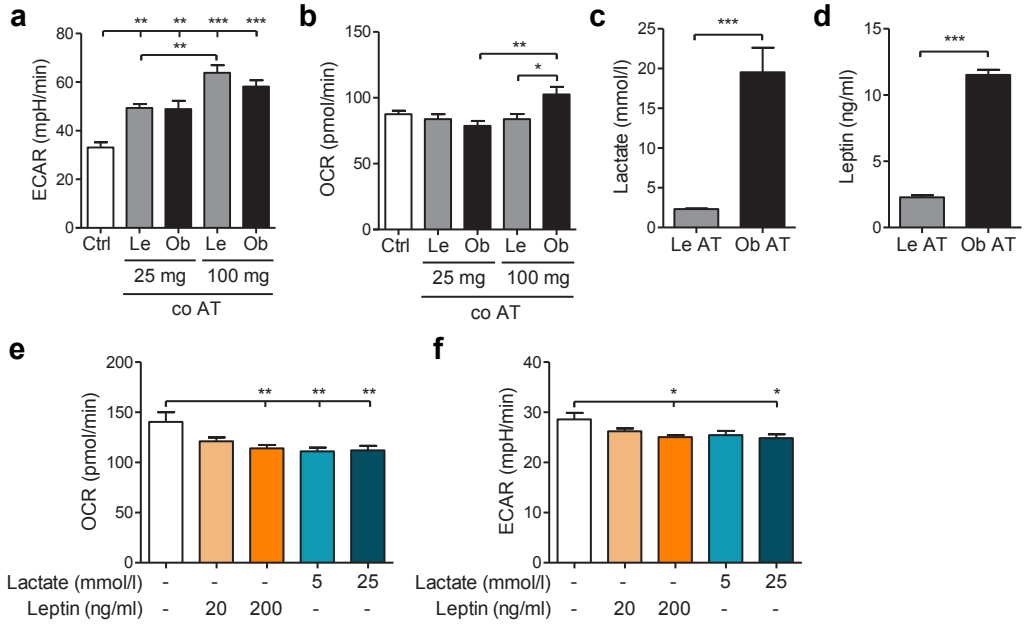
Gene	3' primer	5' primer
Cd11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTCA
Cd206	GCTTCCGTCACCCTGTATG	GTGTGTCATTCTTACACTCCCAT
Cd36	TCCAGCCAATGCCTTTGC	TGGAGATTACTTTTCAGTGCAGAA
Cd68	CCAATTCAGGGTGGAAGAAA	CTCGGGCTCTGATGTAGGTC
Glut1	GGTCACCATCTTGGAGCTGTTC	ACCTGCCTTCTCGAAGATGCT
Hk2	CGTGTCCCTACCTTTGGGTT	CCAGGTCAAACTCCTCTCGC
Hif-1α	ACCTTCATCGGAAACTCCAAAG	CTGTTAGGCTGGGAAAAGTTAGG
Ldhα	CATTGTCAAGTACAGTCCACACT	TTCCAATTACTCGGTTTTTGGGA
Lipa	TGTTCGTTTTCACCATTGGGA	CGCATGATTATCTCGGTCACA
Pdk4	TCTACAAACTCTGACAGGGCTTT	CCGCTTAGTGAACACTCCTTC
Plin2	GCCTCTCAACTGGCTGGTAG	ACAGCAAAAGGGGTCATCTG
Vegfα	CGGGCCTCGGTTCCAG	CTGGGACCACTTGGCATGG
36B4	ATGGGTACAAGCGCGTCCTG	GCCTTGACCTTTTCAGTAAG

ESM Table 2
KEGG-derived biosystems used for principle component analyses.

Gene set	BSID
Inflammatory activation	511699 & 511700
OXPHOS	83142
Glycolysis	198376
Amino acid metabolism	101155 & 83157

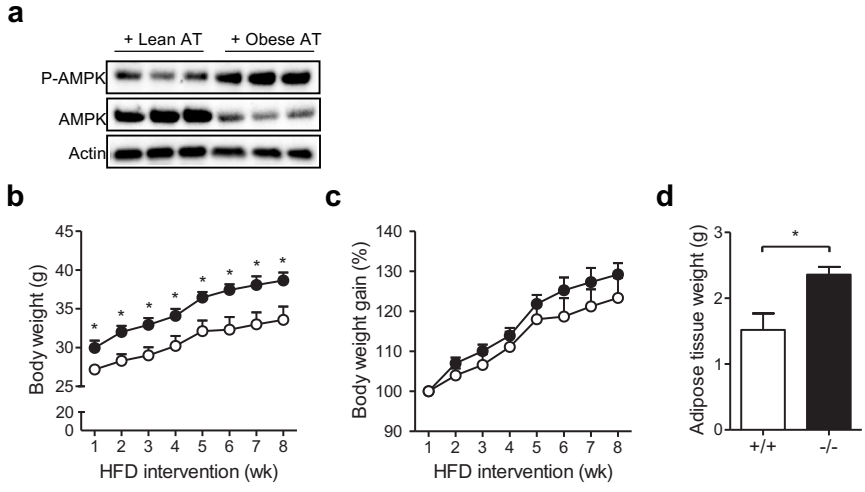
ESM Table 3
KEGG networks used as input for gene set enrichment analyses.

KEGG pathway	Network
Metabolism	1.0-1.8
Genetic information processing	2.3-2.4
Environmental information processing	3.1-3.2
Cellular processes	4.1
Organismal systems	5.1-5.2
Human diseases	6.7



ESM Fig. 1 Leptin and lactate are secreted by obese adipose tissue yet do not skew macrophage metabolism towards the adipose tissue-induced phenotype

(**a,b**) Basal extracellular acidification rate (ECAR) (**a**) or oxygen consumption rate (OCR) (**b**) measurements of BMDMs in 5% vol./vol. L929 (Ctrl) or exposed to 25 mg or 100 mg lean (Le) or obese (Ob) adipose tissue (AT) for 3 days. (**c,d**) Leptin (**c**) and lactate (**d**) measured in supernatants of a 3-day culture of 100 mg lean or obese adipose tissue. (**e,f**) OCR (**e**) and ECAR (**f**) of BMDMs exposed to leptin or lactate for 24 h were compared to BMDMs exposed to 5% vol./vol. L929 (-). n=3 for all experiments. Data are presented as means ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.01$.



ESM Fig. 2 Higher bodyweight and adipose tissue weight in LysM *Hif-1α*$^{-/-}$ vs LysM *Hif-1α*$^{+/+}$ mice fed a HFD for 8 weeks

(**a**) Presence of phospho-AMPK (P-AMPK) and AMPK protein levels in BMDMs exposed to 100 mg of obese or lean adipose tissue (AT) for 3 days. Actin serves as loading control. (**b,c**) Bodyweight (**b**) and bodyweight gain (**c**) of LysM *Hif-1α*$^{+/+}$ and LysM *Hif-1α*$^{-/-}$ mice over the 8-week high-fat diet (HFD) intervention period. (**d**) Epididymal adipose tissue weight of LysM *Hif-1α*$^{+/+}$ and LysM *Hif-1α*$^{-/-}$ mice after 8 weeks on a HFD. White bars or circles; LysM *Hif-1α*$^{+/+}$, black bars or circles; LysM *Hif-1α*$^{-/-}$. Data are presented as means ± SEM. * $p<0.05$.

5

Uptake of triglyceride-derived and free fatty acids by macrophages favours inflammatory responses in lipid-rich environments: Involvement of ANGPTL4

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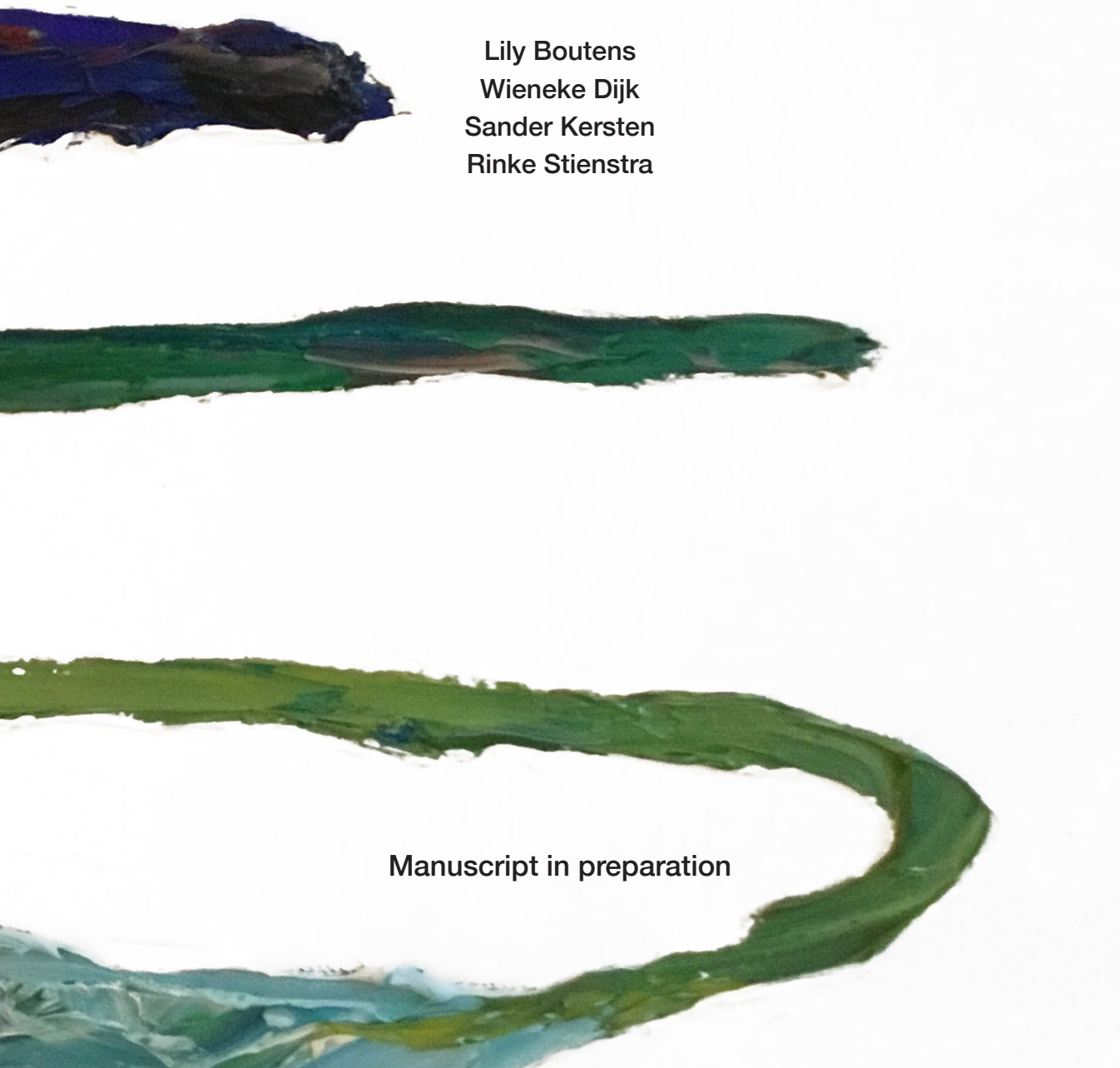

Manuscript in preparation

ABSTRACT

Macrophages are key players in the development of adipose tissue inflammation during obesity. In addition to free fatty acids (FFAs) present in the adipose tissue that can be readily taken up, adipose tissue macrophages (ATMs) are also exposed to triglycerides (TGs) that require hydrolysis by the enzyme lipoprotein lipase (LPL) to allow for FA uptake. To learn more about the relevance of this pathway we studied macrophages in lipid-rich environments including the adipose tissue, and particularly examined inflammatory responses. In addition, we explored the relevance of angiopoietin-like 4 (ANGPTL4), a well-known endogenous inhibitor of LPL, for macrophages in a lipid-enriched environment.

Compared to other tissue macrophages, ATMs displayed enhanced expression of various genes involved in lipid uptake and handling, including *Lpl*. High fat diet-feeding promoted further upregulation of *Lpl* accompanied by the presence of ER stress and increased expression of various pro-inflammatory genes. In vitro, culturing BMDMs in adipose tissue-conditioned medium induced a similar pro-inflammatory response and led to metabolic activation of macrophages, a specific phenotype of macrophages residing in obese adipose tissue. Interestingly, *Angptl4*, the endogenous inhibitor of LPL, was also strongly enhanced in BMDMs residing in lipid-rich environments suggestive of the presence of a negative feedback mechanism to limit LPL activity and thus excessive uptake of FAs from TGs. Indeed, absence of *Angptl4* in macrophages led to increased inflammatory responses during exposure to adipose tissue-conditioned medium. However, these effects appeared to be independent from lipid uptake. To further evaluate the specific contribution of TGs, we treated macrophages with Intralipid, a TG emulsion. Intralipid boosted *Angptl4* expression and led to metabolic activation of macrophages similarly to adipose tissue-conditioned medium. In macrophages exposed to Intralipid, however, absence of *Angptl4* did increase lipid loading but only partially prevented pro-inflammatory responses.

In conclusion, our results demonstrate that TGs drive metabolic activation of macrophages. Although ANGPTL4 is involved in modulating inflammatory activation of macrophages, its mode of action appears to be independent of LPL-inhibition and control of lipid uptake.

INTRODUCTION

Macrophages populate various tissues throughout our body adopting different phenotypes and performing different functions driven by local environmental cues (1). The adipose tissue encompasses a microenvironment dominated by lipids. In addition to high levels of free fatty acids (FFAs) released by adipocytes during lipolysis (2, 3), adipose tissue macrophages (ATMs) are also exposed to triglycerides (TGs) either originating from circulating lipoproteins or from adipocytes undergoing turnover and clearance (4-7). During health, ATMs actively participate in maintaining tissue homeostasis by performing several tasks including the direct regulation of adipocyte lipolysis and lipid buffering for gradual release into the bloodstream (3, 8).

In contrast to their contribution to adipose tissue homeostasis in the lean state, ATMs populating obese adipose tissue promote inflammation that ultimately contributes to the development of metabolic abnormalities including Type 2 Diabetes (9). Lipotoxicity as a consequence of lipid overloading in obese adipose tissue may be the culprit for the inflammatory trait of ATMs residing in obese adipose tissue (7). Indeed, the foam-cell-like appearance of macrophages in obese adipose tissue is similar to that seen in macrophages residing in atherosclerotic plaques in which excessive oxLDL uptake drives inflammatory activation (10).

Fatty acids activate multiple inflammatory pathways in macrophages. Extracellularly, they have been shown to activate Toll-like receptor (TLR)-signalling either via fetuin A-dependent binding of TLR4 (11), or through binding to other receptors that may stimulate TLR signalling, as has been shown for CD36 in atherosclerosis (12, 13). Intracellularly, FAs can induce ROS accumulation (14) and ER stress (15). As a result, the NLRP3 inflammasome is activated to induce caspase-1 cleavage and promoting inflammatory responses in macrophages and adipose tissue (15-17).

Although the effects of FFAs on macrophage activation are well-studied, much less is known about TGs, that are unable to pass the cellular membrane and first require hydrolysis. This process is tightly controlled by the enzyme lipoprotein lipase (LPL) that facilitates TG hydrolysis in various cell types including macrophages. In the vessel wall, LPL present in macrophages has been shown to be involved in the uptake of atherogenic lipoproteins (18-20). Absence of LPL in macrophages protects against atherosclerotic lesion development whereas overexpression enhances atherosclerosis progression (18-20).

Angiopoietin-like 4 (ANGPTL4) controls LPL activity via mechanisms involving dimer-to-monomer conversion of the LPL protein and competitive inhibition (21, 22). Indeed, ANGPTL4 has been shown to be involved in preventing lipid loading of macrophages in the arterial wall (23, 24), as well as in macrophages residing in mesemteric lymph nodes (25, 26).

We set out to study the contribution of TG-mediated lipotoxicity in macrophages residing in in a lipid-rich environment including the adipose tissue, and examined the involvement of ANGPTL4. Our results demonstrate profound regulation of various genes involved in lipid uptake, including *Lpl* and the fatty acid transporter *Cd36*, in macrophages in an adipose tissue environment. Pro-inflammatory ATMs of obese mice displayed further regulation of these genes involved in lipid handling and activation of ER stress, pointing to lipid-induced toxicity and inflammatory activation of ATMs during obesity. Interestingly, *Angptl4* was robustly upregulated in macrophages in lipid-rich environments including that of the adipose tissue, suggestive of a role for ANGPTL4 in limiting aberrant lipid uptake by ATMs. However, in contrast to macrophages in a pure TG-enriched environment in which ANGPTL4 inhibited lipid loading, we observed no differences in lipid loading or handling between *Angptl4*$^{-/-}$ and wild-type macrophages exposed to adipose tissue-conditioned medium. Interestingly, ANGPTL4 appeared to modulate inflammatory responses in an adipose tissue environment independent of its role in controlling macrophage FA uptake from TGs.

MATERIALS AND METHODS

Mice

All cells or tissues were isolated from four to six months old male *Angptl4* knock-out (*Angptl4*$^{-/-}$) (27) or wild-type (*Angptl4*$^{+/+}$) mice that had been on a C56BL/6J background for more than 10 generations.

Twelve weeks old male wild-type and *Angptl4*$^{-/-}$ mice were intraperitoneally injected with 10 µg/gram bodyweight of ultrapure lipopolysaccharide (LPS) (Sigma-Aldrich, Darmstadt, Germany) or an equal volume of PBS. Mice were euthanized 48 hours after LPS injection, and the adipose tissue and liver were collected for qPCR analysis. The study protocol was approved by the Animal Ethics Committee at Wageningen University.

Cell culture

Bone marrow cells were isolated from femurs of wild-type or *Angptl4*$^{-/-}$ mice following standard protocol and differentiated into macrophages (BMDMs) during 6-8 day culturing in Dulbecco's modified eagle's medium (DMEM; Lonza, Verviers, Belgium) containing 10% Foetal Calf Serum (FCS) and 1% Penicyllin Streptomycin (PS) (DMEM/FCS/PS) supplemented with 10% L929-conditioned medium (L929). After 6-8 days non-adherent cells were removed and adherent cells were washed and plated in 6-, 12-, 24-, or 48-wells plates in DMEM/FCS/PS + L929. After 24 hours, the cells were washed with PBS and received their treatment in culture medium without L929 and FCS (DMEM/PS).

Intralipid

Bone marrow-derived macrophages were exposed to 1-2 mM Intralipid (Sigma-Aldrich) in DMEM/PS for 6 hours as indicated at each figure. Orlistat (Sigma-Aldrich) was used at a concentration of 25 µM and was co-incubated with Intralipid for the same period of time.

Adipose tissue-conditioned medium

Epididymal adipose tissue from wild-type mice was isolated, minced in sterile conditions, and brought into culture for 48 hours in DMEM/PS containing 1% free fatty acid-free BSA (Sigma-Aldrich) to generate conditioned medium (CM AT). BMDMs were exposed to 75% of CM AT in DMEM/PS, or DMEM/PS containing 0.75% BSA (Cntrl).

Adipocytes

Human Simpson-Golabi-Behmel syndrome (SGBS) cells were maintained in DMEM/F12 (Lonza) supplemented with 10% FCS, 1% PS, 33 µM Biotin (Sigma-Aldrich) and 17 µM panthotenat (Sigma-Aldrich), and differentiated into adipocytes over a period of 21 days as has been described previously (28). Differentiated SGBS cells were exposed to 25 mJ/cm^2 UV-C light using a Stratalinker 1800 UV cross linker (Stratagene (Agilent), Middelburg, The

Netherlands) to induce cell death, and left in culture for 18 hours before they were collected using trypsin, pelleted with centrifugation, dissolved in DMEM/FCS/PS and co-cultured with BMDMs for 24 hours in a 1:1 ratio (for more information see Chapter 6).

LPS

Bone marrow-derived macrophages treated with Intralipid or CM AT (or corresponding controls) were washed with PBS and subsequently challenged with 1 ng/mL LPS (Sigma-Aldrich) in fresh culture medium (DMEM/FCS/PS) for an additional 24 hours.

Microscopy

For visualization of lipid droplets, treated BMDMs of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ mice were washed with PBS, fixated in 4% formaldehyde, and subsequently stained with 1 ng/mL bodipy (ThermoFisher Scientific, Landsmeer, The Netherlands). Lipid droplets in adipose tissue macrophages from low-fat (LFD) versus high-fat diet (HFD)-fed mice were visualized using bright light.

Extracellular flux analysis

Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) of wild-type or *Angptl4*$^{-/-}$ BMDMs were analysed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience, Santa Clara, CA, USA). Basal metabolic rates of BMDMs seeded in quintuplicate were determined during three consecutive measurements in unbuffered Seahorse medium (8.3 g DMEM powder, 0.016 g phenol red and 1.85 g NaCl in 1 L milli-Q, pH set at 7.4 at 37 °C; sterile-filtered) containing 25 mmol/l glucose and 2 mmol/l L-glutamine. After basal measurements, three consecutive measurements were taken upon the addition of each 1.5 µmol/l oligomycin, 1.5 mmol/l carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 2 µmol/l antimycin together with 1 µmol/l rotenone. Pyruvate (1 mmol/l) was added together with FCCP to fuel maximal respiration. All compounds used during the Seahorse runs were acquired from Merck. Signals were normalized to relative DNA content in the wells using the Quant-iT™ dsDNA Assay Kit (ThermoFisher).

Cytokine measurements

Levels of TNF-α, IL-6 and IL-10 were measured in cell culture supernatants with murine DuoSet ELISA Development kits (R&D Systems, Abingdon, UK).

RT-qPCR

Total RNA was isolated using TRIzol (ThermoFisher), either via phenol/chloroform extraction (BMDMs), or with the RNeasy microkit (Qiagen, Venlo, The Netherlands) (epididymal adipose tissue and liver of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ mice). Reverse transcription was executed using the iScript cDNA synthesis kit (Bio-rad, Veenendaal, The Netherlands). Real-time quantitative PCR was carried out with a SensiMix SYBR kit (Bioline, Alphen aan den Rijn) in a CFX384

system (Bio-rad). Relative expression levels were calculated after normalization against the housekeeping gene 36B4 using the ΔΔC(t) method. Primer sequences can be found in supplementary Table 1.

Western blot

Cells were lysed in RIPA buffer (ThermoFisher) enriched with protease and phosphatase inhibitors (Roche Diagnostics). Total protein was determined using a BCA protein assay (ThermoFischer) and for each sample 15-60 µg of protein was loaded on a 10% Criterion TGX Precast Midi protein gel (Bio-Rad). Proteins were separated by SDS gel electrophoresis and transferred to a PVDF membrane (Bio-Rad) using the Transblot Turbo System (Bio-Rad). Primary antibodies for spliced XBP1 (AB_2562960; BioLegend, London, UK) and CHOP (Ab11419; Abcam, Cambridge, UK) were used at a ratio of 1:1000 and incubated overnight at 4 ^{o}C. All incubations were in Tris-buffered saline with 0.1% v/v Tween-20 (TBS-T), containing 5% w/v dry milk. HRP-conjugated rabbit-anti-mouse secondary antibody (Merck) was used 1:5000 in TBS-T with 5% w/v dry milk. Blots were visualized using the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad).

Microarray analysis

Four pools of ATMs isolated from epididymal adipose tissue of male C57Bl/6 mice fed a LFD or HFD diet in four separate experiments (ATMs HFD vs. LFD) were subjected to expression profiling by microarray. Moreover, complete RNA from CD14+ monocytes isolated from the blood, and subcutaneous (SAT) or visceral adipose tissue (VAT) of five healthy donors was used for microarray analysis, in which expression profiles of monocytes in the adipose tissue (SAT+VAT) was compared to the expression profile of circulating monocytes using paired analysis (ATMs vs. monocytes). Publically available raw transcriptome data from various tissue macrophages including ATMs (GSE56682; ATMs vs. other tissue macrophages) and from THP-1 macrophages incubated with LPL hydrolysis versus non-hydrolysed end products of lipoproteins (GSE84791) were obtained from the Gene Expression Omnibus. Details of the microarray analysis and interpretation can be found in the supplementary material and methods section of chapter 4.

Statistical analysis

Results are shown as mean + SEM. Statistically significant differences between two groups were calculated using Student's t-tests. For evaluating the effect of treatment (treatment vs. control) and genotype (*Angptl4*$^{-/-}$ vs. *Angptl4*$^{+/+}$), a two-way ANOVA and Bonferroni post hoc test was done. $P < 0.05$ were considered significant.

RESULTS

Adipose tissue-specific regulation of genes involved in lipid uptake and handling, and in inflammation

The adipose tissue provides a unique environment that is highly enriched in lipids. Compared to other tissue macrophages, adipose tissue macrophages (ATMs) display overall higher expression of various genes well-known for their role in lipid uptake (**Figure 1A**). Interestingly, comparing human ATMs with circulating monocytes reveals a similar pattern with strong regulation of genes involved in lipid uptake in adipose tissue-based macrophages (**Figure 1B**). During obesity, macrophages are increasingly exposed to lipids and develop a lipid-laden appearance (5, 7, 29). Expression patterns of genes involved in lipid uptake (**Figure 1C**), as well as lipid droplet formation (**Figure 1D**) in ATMs isolated from mice fed a high-fat diet (HFD) versus mice fed a low-fat diet (LFD) corroborate increased lipid uptake and storage by ATMs during obesity. Moreover, in line with previous studies reporting inflammatory activation of ATMs during obesity that is different from classical macrophage activation, we found a diverse expression pattern of several marker genes involved in inflammatory cytokine secretion in ATMs from obese (HFD) versus lean mice (LFD) (**Figure 1E**). Our finding of an enrichment of pathways involved in ER stress in ATMs isolated from obese versus lean mice (**Figure 1F**), is suggestive of the existence of a link between increased lipid exposure, ER stress and inflammatory activation of ATMs part of obese adipose tissue, as has been previously shown in macrophages residing in atherosclerotic plaques (30, 31).

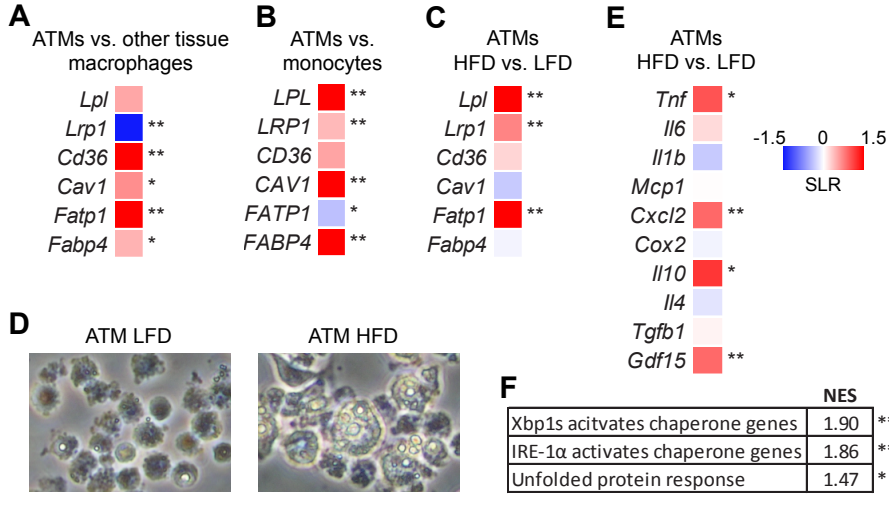

Figure 1. Expression signatures of adipose tissue macrophages reflect lipid uptake, ER stress and inflammation. Heat map reflecting the expression of selected well-known genes involved in lipid uptake in mouse adipose tissue macrophages (ATMs) vs. other tissue macrophages (**A**), human ATMs versus circulating monocytes (**B**), and ATMs isolated from mice fed a high-fat diet (HFD) vs. low-fat diet (LFD) (**C**). **D** Representative picture of lipid droplet formation in ATMs isolated from mice on a HFD vs. LFD. **E** Regulation of several marker genes for pro- vs. anti-inflammatory activation in ATMs from mice on a HFD vs. LFD. **F** Predicted activation of pathways representing ER stress in ATMs from HFD- vs. LFD-fed mice upon analysis of their complete transcriptomes using Ingenuity Pathway Analysis, presented as a Normalized Enrichment Score (NES).

Gene expression data are presented as log 2 ratio difference (SLR) with higher expressed genes in red and lower expressed genes in blue. * $p < 0.05$, ** $p < 0.01$.

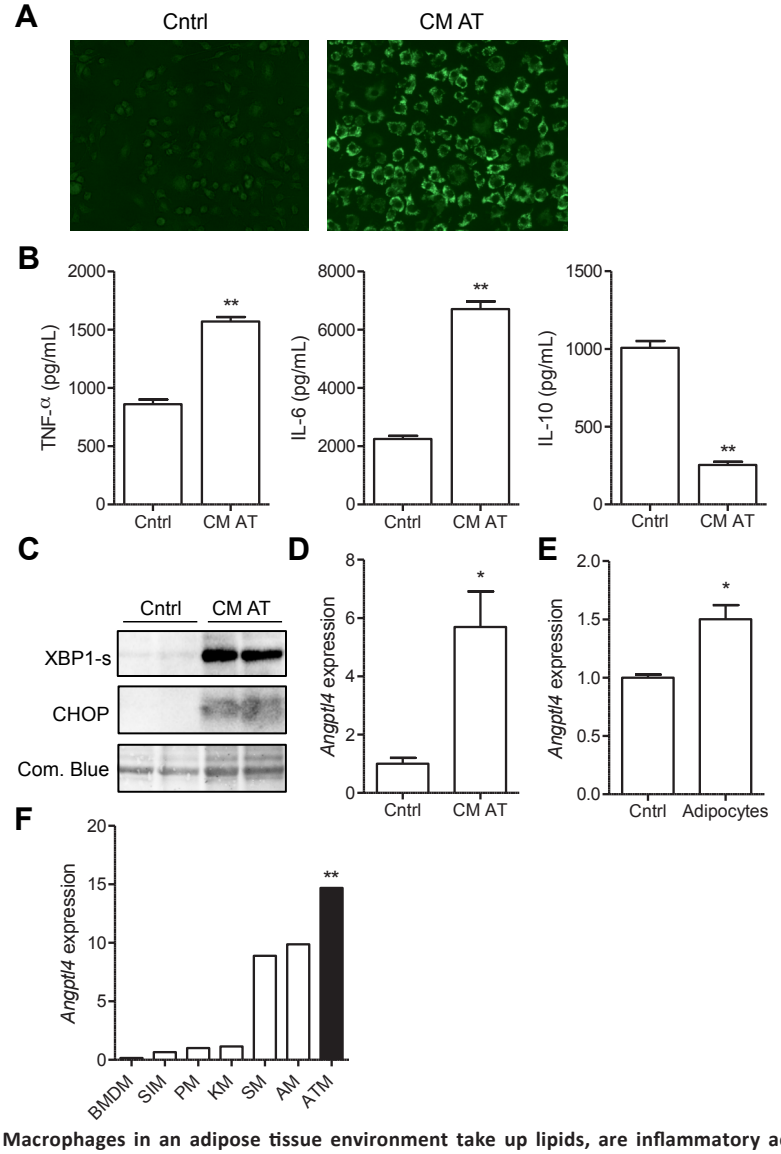

Figure 2. Macrophages in an adipose tissue environment take up lipids, are inflammatory activated and increase *Angptl4* expression.
A Representative picture of bone marrow-derived macrophages (BMDMs) stained for neutral lipids using Bodipy after exposure to regular culture medium (Cntrl) or adipose tissue-conditioned medium (CM AT) for 24 h. **B** Pro- (TNF-α, IL-6) and anti- (IL-10) inflammatory cytokines in supernatants of BMDMs pre-exposed to regular culture medium or CM AT for 24 h and subsequently challenged with 1 ng/mL LPS in fresh culture medium for an additional 24 h. Cytokines were measured after the 24 h LPS challenge. **C** Western blot for markers of ER stress, being XBP1-s (spliced form) and CHOP, in BMDMs in regular culture medium or CM AT for 24 h. Expression levels of *Angptl4* in: BMDMs cultured in CM AT (**D**) or co-cultured with dying adipocytes (**E**), and various tissue macrophages including adipose tissue macrophages (**F**). SIM: Small intestine macrophages, PM: Peritoneal macrophages, KM: Kupffer cells, SM: Splenic red pulp macrophages, AM: Alveolar macrophages, ATM: Adipose tissue macrophages. Data are presented as means + SEM. * $p<0.05$, ** $p<0.01$.

Macrophages in an adipose tissue environment in vitro resemble ATMs in vivo and display enhanced expression of *Angptl4*

Next, we mimicked ATMs in vitro by culturing BMDMs in adipose tissue-conditioned medium (CM AT) for 24 h to further examine the link between lipid loading and inflammatory activation of macrophages in an adipose tissue environment. As expected, BMDMs exposed to CM AT take up lipids and display profound lipid droplet formation (**Figure 2A**). Moreover, CM AT induced pro-inflammatory activation of BMDMs, characterized by enhanced secretion of TNF-α and IL-6, two pro-inflammatory cytokines, yet reduced secretion of the anti-inflammatory cytokine IL-10 (**Figure 2B**). In line with our finding in ATMs in vivo, BMDMs exposed to CM AT in vitro display signs of ER stress, with increased presence of the ER stress marker proteins XBP1 (spliced form) and CHOP (**Figure 2C**). Macrophages in an adipose tissue environment may either be exposed to lipids in the form of triglycerides (TGs) or as free fatty acids (FFAs). Interestingly, we found profound upregulation of *Angptl4*, a well-known endogenous inhibitor of lipoprotein lipase (LPL), in BMDMs cultured in CM AT (**Figure 2D**). In addition, *Angptl4* expression was also enhanced in BMDMs co-cultured with dying adipocytes that release their lipid content (**Figure 2E**) and appeared to be specifically higher in ATMs compared to other tissue macrophages (**Figure 2F**). Upregulation of *Angptl4* in ATMs in vivo and upon exposure to CM AT in vitro is suggestive of the presence of a negative feedback mechanism to limit LPL activity and thus excessive uptake of TG-derived FAs by macrophages in an adipose tissue environment.

Macrophages in an adipose tissue environment display enhanced lipid uptake, storage and metabolic activation regardless of ANGPTL4

To learn more about the relevance of TG-derived FAs for activation of macrophages in an adipose tissue environment we compared BMDMs from wild-type (*Angptl4*$^{+/+}$) with BMDMs from *Angptl4*$^{-/-}$ mice. No gross differences in lipid droplet formation between *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ mice was observed (**Figure 3A**). This was further supported on gene expression level. Both *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs display pronounced upregulation of *Plin2* and *Cd36* (**Figure 3B**,**C**), previously identified as marker genes of metabolically activated ATMs during obesity (32), yet no differences between the genotypes were found. Of note, expression of *Fabp4* was enhanced in wild-type BMDMs as well, fitting the ATM-specific phenotype found in vivo (**Figure 1A**,**B**). To check whether intracellular lipid handling was affected by ANGPTL4 in macrophages, we examined the expression of genes involved in lipid export (*Abcg1*, *Abca1*), autophagy (*Atg7*), lipid breakdown (*Lipa*) and FA oxidation (*Cpt1α*) in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs. Whereas no differences in expression of genes involved in export nor in *Lipa* were observed (**Supplementary Figure 1**), *Atg7* and *Cpt1α* appeared slightly higher expressed in *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs (**Figure 3D**). Because an increase in *Atg7* and *Cpt1α* may be suggestive of enhanced FA oxidation in the mitochondria, we examined real time metabolic fluxes using the Seahorse machine. Both BMDMs expressing and lacking *Angptl4* displayed enhanced metabolic activity of both glycolysis (ECAR measurement) and oxidative

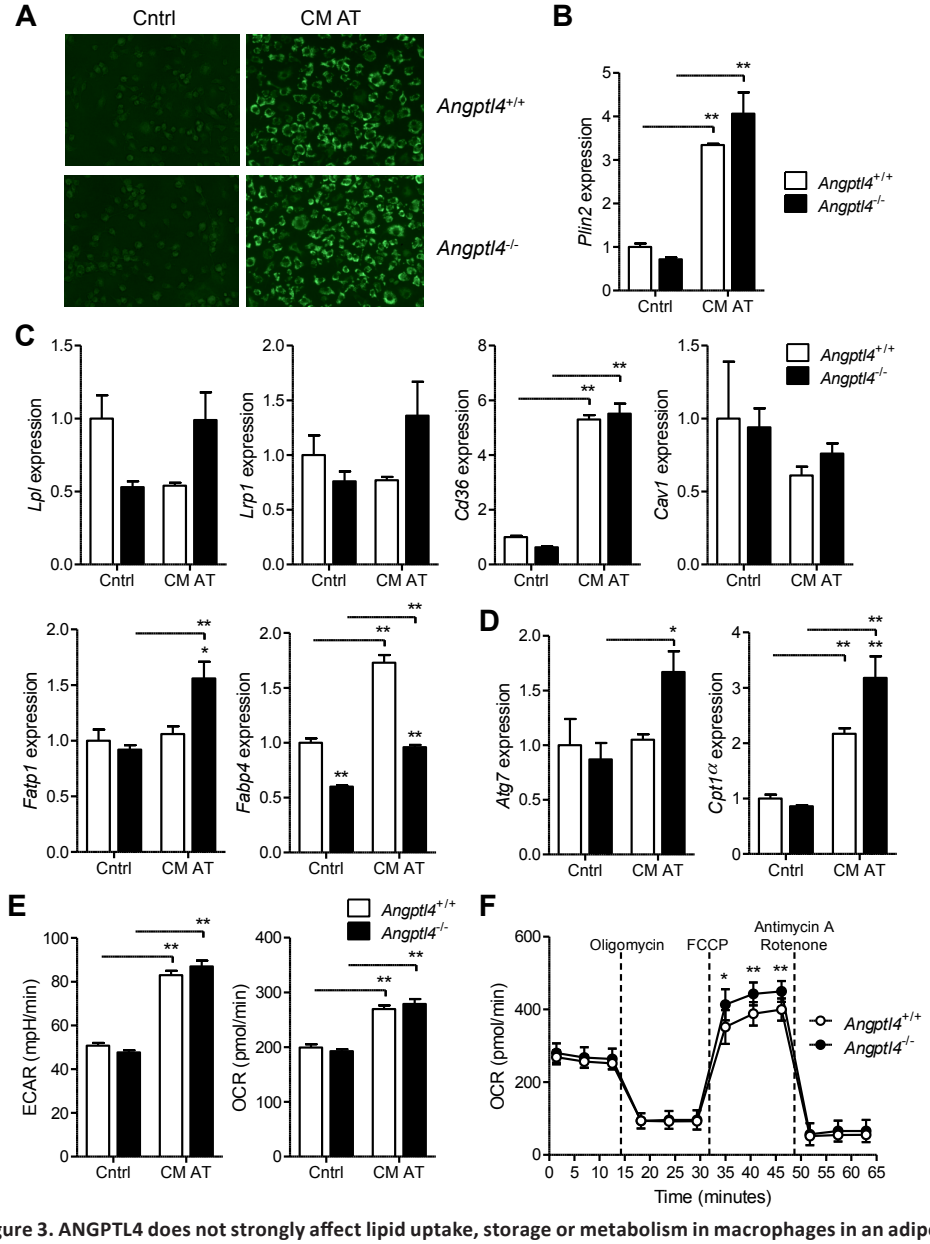

Figure 3. ANGPTL4 does not strongly affect lipid uptake, storage or metabolism in macrophages in an adipose tissue environment.

A Representative picture of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ bone marrow-derived macrophages (BMDMs) stained with Bodipy after 24 h in regular culture medium (Cntrl) or in adipose tissue-conditioned medium (CM AT). Expression levels of the lipid droplet protein *Plin2* (**B**) various genes involved in lipid uptake (**C**), or in autophagy and lipid breakdown (**D**) in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in control conditions and after exposure to CM AT for 24 h. **E** Basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in regular culture medium or CM AT for 24 h. Measurements were done in unbuffered medium. **F** Real time changes in OCR of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs exposed to CM AT for 24 h, assessed in unbuffered medium during sequential injections with Oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and Antimycin A + rotenone. Data are presented as means + SEM. * p<0.05, ** p<0.01.

phosphorylation (OCR measurement) (**Figure 3E**), again corroborating metabolic activation of macrophages in an adipose tissue environment (Chapter 4). The absence of differences in basal oxidative phosphorylation (OXPHOS) and only minor differences in maximal OXPHOS between *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs (**Figure 3F**), together with modest alterations on gene expression level between the two genotypes, however, are suggestive of similar intracellular lipid handling by *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ macrophages.

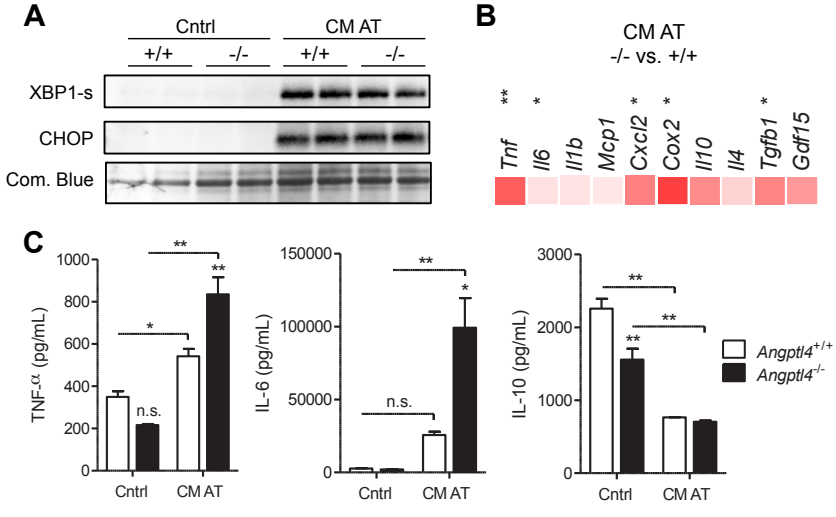

Figure 4. *Angptl4*$^{-/-}$ macrophages display increased inflammatory activation in an adipose tissue environment. **A** Western blot for the ER stress markers XBP1-s (spliced form) and CHOP in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ bone marrow-derived macrophages (BMDMs) held in control medium (Cntrl) or in adipose tissue-conditioned medium (CM AT) for 24 h. **B** Expression levels of various pro- and anti-inflammatory genes in *Angptl4*$^{-/-}$ vs. *Angptl4*$^{+/+}$ macrophages exposed to CM AT for 24 h, presented in a heat map as log 2 ratio difference (SLR) with higher expressed genes in red and lower expressed genes in blue. **C** Pro- (TNF-α, IL-6) and anti- (IL-10) inflammatory cytokines in supernatants of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs pre-exposed to regular culture medium or CM AT for 24 h and subsequently challenged with 1 ng/mL LPS in fresh culture medium for an additional 24 h. Cytokines were measured after the 24 h LPS challenge. Data are presented as means + SEM. * p<0.05, ** p<0.01.

ANGPTL4 protects against inflammatory activation in an adipose tissue environment

In line with substantial lipid loading by macrophages in an adipose tissue environment yet relatively similar lipid handling by BMDMs expressing or lacking *Angptl4*, we measured enhanced levels of proteins representative for ER stress both in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ macrophages cultured in CM AT without observing any differences in these markers between the two genotypes (**Figure 4A**). Interestingly, however, several genes involved in inflammatory activation and cytokine secretion were higher expressed by *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs during culturing in CM AT (**Figure 4B**). Supportive of increased inflammatory activation of BMDMs lacking *Angptl4* in an adipose tissue environment, the pro-inflammatory cytokines TNF-α and IL-6 were enhanced in supernatants of *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs stimulated with LPS specifically upon pre-exposure to CM AT (**Figure 4C**). Hence, although lipid uptake and intracellular handling appears relatively similar for BMDMs

with or without ANGPTL4, macrophages lacking *Angptl4* favour pro-inflammatory activation in an adipose tissue environment.

ANGPTL4 reduces lipid uptake by macrophages in a TG-enriched environment without affecting intracellular metabolism

To learn more about the consequences of absence of *Angptl4* during uptake of TG-derived FAs by macrophages, we cultured *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in a synthetic TG emulsion called Intralipid. Firstly, *Angptl4* was strongly induced in BMDMs exposed to Intralipid (**Figure 5A**). Induction of *Angptl4* expression appeared to be a consequence of LPL activity, since hydrolysis products liberated from lipoproteins by LPL appeared to enhance *Angptl4* levels in a human macrophage cell line (THP-1) as compared to non-hydrolysis products of lipoproteins (33) (**Figure 5B**). As expected, ANGPTL4 inhibits lipid loading of macrophages exposed to Intralipid. Most probably this was achieved via inactivation of LPL because Orlistat, an inhibitor of lipases including LPL, could abrogate increased lipid loading of *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs exposed to Intralipid (**Figure 5C**). Similar to macrophages in an adipose tissue environment, BMDMs exposed to Intralipid display enhanced expression of *Plin2*, *Cd36* and *Fabp4* (**Figure 5D**,**E**). Apart from *Cav1*, which was found higher expressed in *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs (**Figure 5E**), no differences between the two genotypes were found (**Figure 5E**, **Supplementary Figure 2**). In line with this finding and supportive of lipid-induced metabolic activation of macrophages, both *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs display increased glycolysis (ECAR) and OXPHOS (OCR) rates when exposed to the TG emulsion, similarly to what we found in ATMs (Chapter 4) and BMDMs exposed to AT CM (**Figure 3F**).

Macrophages lacking *Angptl4* in a TG-enriched environment tend to react stronger to a subsequent pro-inflammatory stressor

Despite increased lipid droplet formation in BMDMs lacking *Angptl4*, no signs of increased ER stress in *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs was found in a TG-enriched environment, as based on the presence of spliced XBP1 (**Figure 6A**) (CHOP could not be detected). Similar to macrophages in an adipose tissue environment, *Angptl4*$^{-/-}$ BMDMs exposed to Intralipid do display enhanced mRNA levels of *Tnf* and *Cxcl2* (**Figure 6B**). Pre-exposure to Intralipid, however, did not translate in enhanced inflammatory activation of *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ BMDMs upon stimulation with LPS (**Figure 6C**). Interestingly, on gene expression level *Angptl4*$^{-/-}$ BMDMs pre-exposed to Intralipid did display strong inflammatory activation upon stimulation with LPS compared to *Angptl4*$^{+/+}$ BMDMs (**Figure 6D**). Of note, Orlistat could rescue the enhanced expression of pro-inflammatory genes in *Angptl4*$^{-/-}$ macrophages, both directly after exposure to Intralipid and upon subsequent stimulation with LPS (data not shown), in line with a role for ANGPTL4 in inhibition of lipid uptake via LPL. It is noteworthy that Intralipid in general appeared to trigger anti-inflammatory macrophage responses, exemplified by reduced ER stress markers (**Figure 6A**) and cytokine secretion (**Figure 6C**) in or by macrophages of both genotypes.

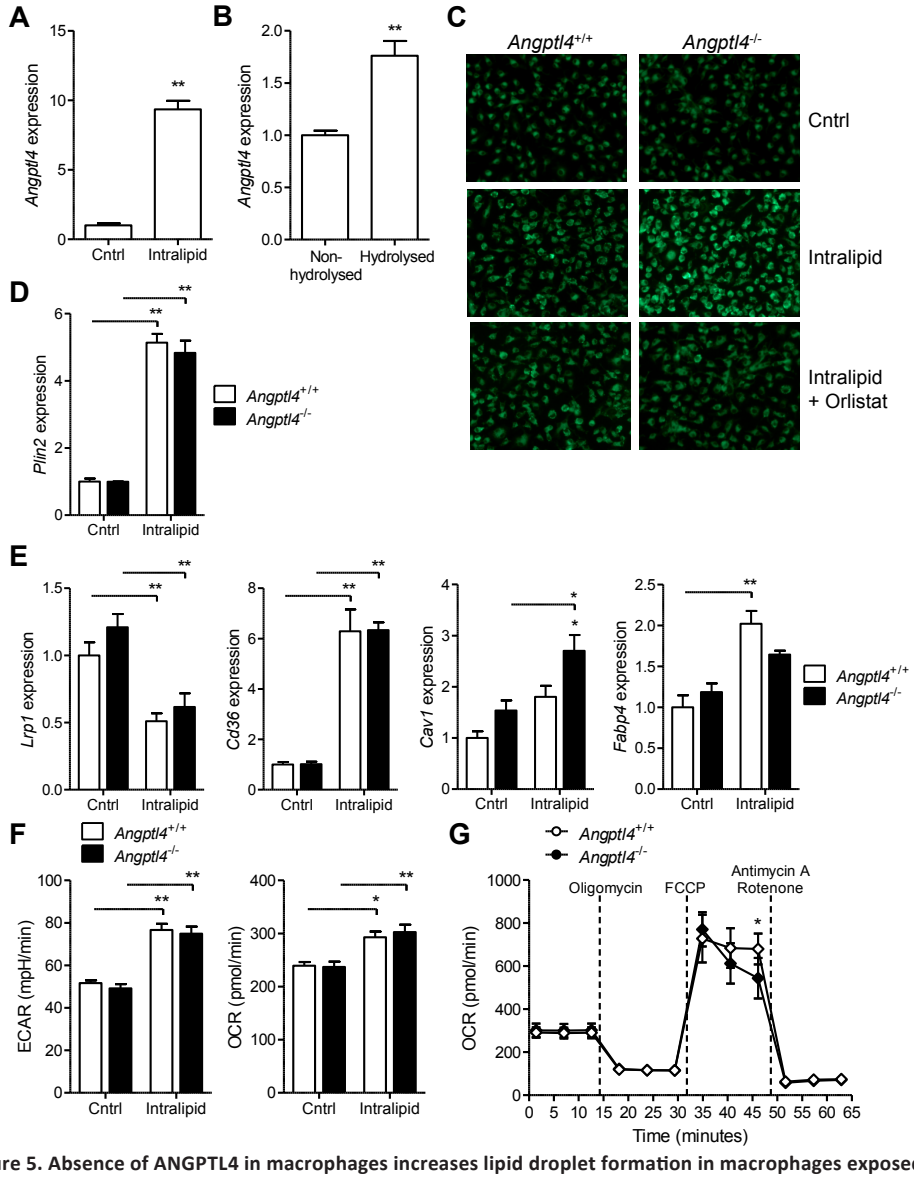

Figure 5. Absence of ANGPTL4 in macrophages increases lipid droplet formation in macrophages exposed to triglycerides without affecting lipid handling on mRNA level or real-time metabolism.
Expression of *Angptl4* in bone marrow-derived macrophages (BMDMs) cultured in 2 mM Intralipid; an emulsion of triglycerides (**A**), and BMDMs exposed to products formed upon lipoprotein lipase-mediated hydrolysis of lipoproteins vs. non-hydrolysed lipoprotein products (**B**). **C** Representative picture of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in regular culture medium (Cntrl), or after a 6 h culture in 1 mM Intralipid or 1 mM Intralipid + Orlistat. Neutral lipids were visualised using Bodipy. Expression levels of the lipid droplet protein *Plin2* (**D**) or various genes involved in lipid uptake (**E**) in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in control conditions and after exposure to 2 mM Intralipid for 6 h. **F** Basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs exposed to regular culture medium or medium containing 1 mM Intralipid for 6 h. Measurements were done in unbuffered medium. **G** Real time changes in OCR of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs exposed to 1 mM Intralipid for 6 h, assessed in unbuffered medium during sequential injections with Oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and Antimycin A + rotenone. Data are presented as means + SEM. * p<0.05, ** p<0.01.

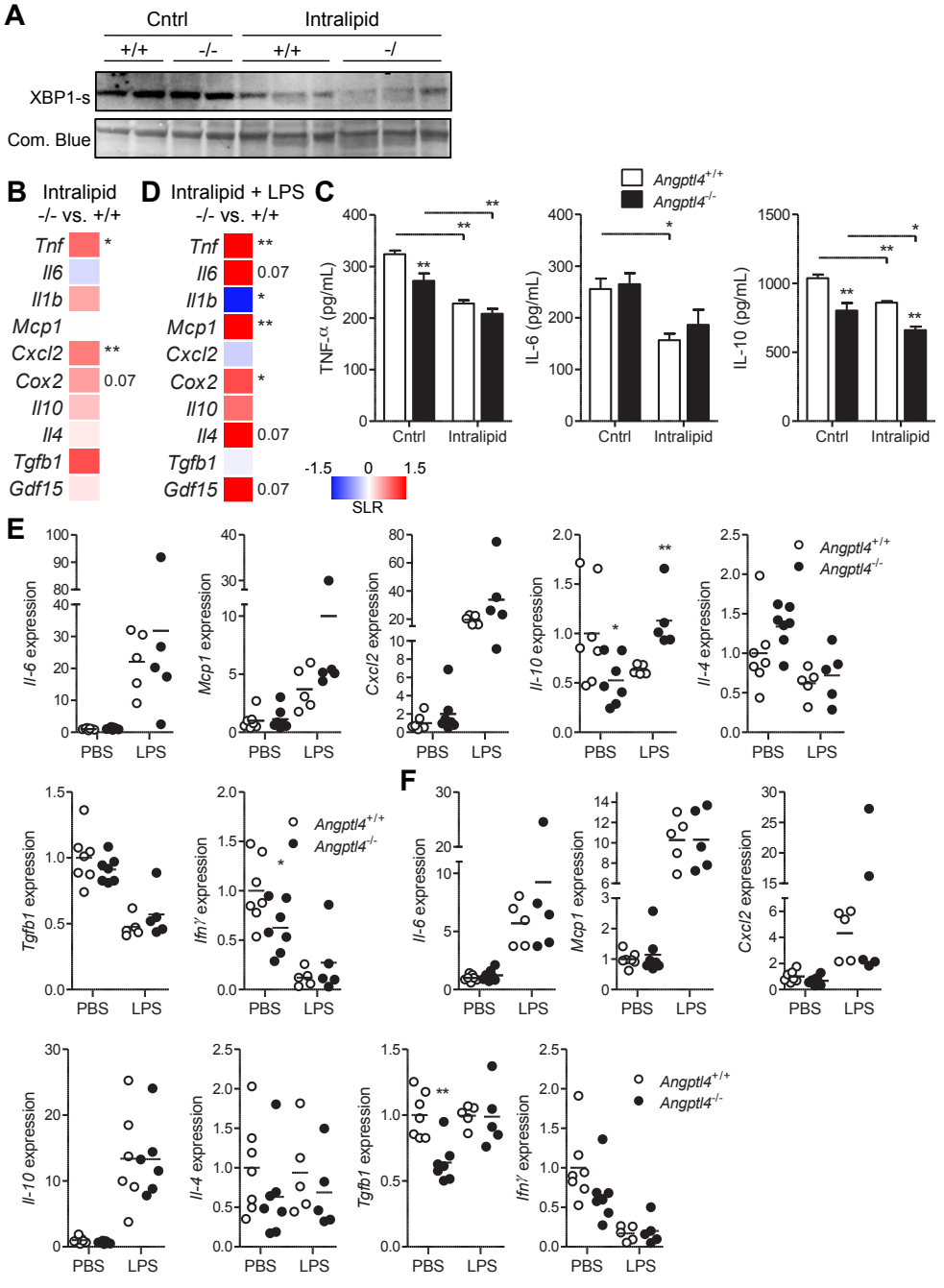

Figure 6. Macrophages lacking ANGPTL4 display no signs of increased ER stress in a triglyceride-rich environment but appear more prone to react pro-inflammatory to a second stressor.

A Western blot for the ER stress marker XBP1-s (spliced form) in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ bone marrow-derived macrophages (BMDMs) held in control medium (Cntrl) or in 1 mM Intralipid with or without Orlistat for 6 h. **B** Expression of various pro- and anti-inflammatory genes in *Angptl4*$^{-/-}$ vs. *Angptl4*$^{+/+}$ BMDMs exposed to 2 mM Intralipid for 6 h.

C Pro- (TNF-α, IL-6) and anti- (IL-10) inflammatory cytokines in supernatants of *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs pre-exposed to regular culture medium or 1 mM Intralipid for 6 h and subsequently challenged with 1 ng/mL LPS in fresh culture medium for an additional 24 h. Cytokines were measured after the 24 h LPS challenge. **D** Expression of various pro- and anti-inflammatory genes in *Angptl4*$^{-/-}$ vs. *Angptl4*$^{+/+}$ BMDMs pre-exposed to 2 mM Intralipid for 6 h and subsequently challenged with 1 ng/mL LPS for 24 h. Expression of various cytokines in the adipose tissue (**E**) or liver (**F**) of lean *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ mice 48 hours after i.p. injection of PBS or LPS (10 µg/kg bodyweight). Expression levels in **C** and **D** are presented in a heat map as log 2 ratio difference (SLR) with higher expressed genes in red and lower expressed genes in blue. Data are presented as means + SEM. * p<0.05, ** p<0.01.

To examine the relevance of *Angptl4* for the inflammatory trait of ATMs in vivo, we measured inflammatory markers in the adipose tissue, both basally and upon challenging the mice by injecting LPS, and compared the inflammatory state of the adipose tissue with that of the liver; a tissue-environment not enriched in lipids. Overall no gross differences between lean *Angptl4*$^{-/-}$ versus *Angptl4*$^{+/+}$ mice were detected in the adipose tissue or liver (**Figure 6E**,**F**), not basally (PBS) nor upon LPS injection. However, a trend toward differential inflammatory responses between the genotypes was observed in the adipose tissue (**Figure 6E**), whereas differences appeared smaller in the liver (**Figure 6F**). The expression of several other inflammatory marker including *Gdf15*, *Cox2*, *Il-1ß*, *Il-5* and *Il-15* was not changed (data not shown).

DISCUSSION

Adipose tissue macrophages (ATMs) are confronted with large amounts of lipid species that both include free fatty acids (FFAs) and triglycerides (TGs) released during adipocyte lipolysis or cell death (2, 3, 5). In contrast to FFAs that can readily be taken up into the cell by designated transporters, TGs require the action of LPL to allow for the uptake of FAs. We show that macrophages in an adipose tissue environment in vitro develop a metabolically activated and pro-inflammatory phenotype resembling the phenotype of ATMs residing in obese adipose tissue. Interestingly, macrophages exposed to TGs become metabolically activated as well, suggesting that TGs may represent an important environmental cue contributing to adipose tissue inflammation. However, even though both *Lpl* and *Angptl4* are expressed in ATMs and macrophages in an adipose tissue environment in vitro, we detected no difference in lipid uptake, processing, or metabolism between *Angptl4*$^{-/-}$ and wild-type bone marrow-derived macrophages (BMDMs). Intriguingly, macrophages lacking *Angptl4* do display enhanced inflammatory cytokine production in an in vitro adipose tissue environment, suggestive of a role for ANGPTL4 in cytokine production independently of its inhibition of LPL.

As well-known inhibitor of lipoprotein lipase (LPL), ANGPTL4 plays a crucial role in lipid metabolism in various tissues (21). In macrophages, ANGPTL4 is involved in regulation of uptake of oxidized LDL (oxLDL) and lipids from chyle and accordingly protects against the development of atherosclerosis (25). Although ANGPTL4 and LPL have been shown to be co-produced and interact in various cell types and tissues (22, 34), so far this has not been studied in macrophages residing in lipid-rich environments. Interestingly, our data reveal that endogenous ANGPTL4 represses lipid-loading by macrophages in a TG-rich environment.

The upregulation of *Angptl4* in macrophages in an adipose tissue environment is suggestive of a role for ANGPTL4 in controlling FA uptake from TGs and ultimately determining inflammatory traits of macrophages. Fatty acids, primarily saturated, are known to promote inflammatory responses in macrophages via pathways involving ER stress. Activation of such route involves several mechanisms, including effects on the lipid composition thus structure, motility and integrity of membranes (15, 17, 35-38), and reactive oxygen species (ROS) formation in mitochondria, peroxisomes, or the cytosol (14, 39). Interestingly, adipose tissue-conditioned medium promoted ER stress in macrophages, however no differences between wild-type versus macrophages lacking *Angptl4* were observed. Since the amount of lipids taken up by the macrophages is most likely closely related to the degree of ER stress, this may suggest that long-term exposure to an adipose tissue-like environment results in similar lipid uptake in wild-type and *Angptl4*$^{-/-}$ macrophages. This indeed was confirmed by the equal appearance of lipid droplets in *Angptl4*$^{-/-}$ and *Angptl4*$^{+/+}$ macrophages.

The enhanced inflammatory responses in *Angptl4*$^{-/-}$ subsequently argues effects of ANGPTL4 independently of the amount of lipids taken up by the cell. Indeed, it has been shown that the function of *Angptl4* is not limited to inhibition of LPL but also inhibits other lipases including pancreatic lipase or hormone sensitive lipase (40, 41). Moreover, ANGPTL4 exerts various functions in different cell types, and is involved in angiogenesis and tumour metastases (42). In myeloid cells, *Angptl4* is a target gene of HIF-1α (23), a transcription factor known to be involved in pro-inflammatory responses (43). Accordingly, ANGPTL4 may function as part of a negative feedback loop preventing excessive inflammatory responses. To what extent such a role for ANGPTL4 is completely independent of its control of lipid uptake via LPL inhibition will need more study.

Equal lipid loading of *Angptl4*$^{-/-}$ and wild-type macrophages exposed to adipose tissue-conditioned medium is suggestive of a primary role for FFAs that are released by the adipose tissue in vitro (data not shown) and do not require LPL for uptake. Free FAs in adipose tissue-conditioned medium may have fuelled differences between wild-type and *Angptl4*$^{-/-}$ macrophages by potently upregulating *Angptl4* expression in wild-type macrophages via peroxisome proliferator-activated receptors (PPARs) (24, 25). In addition, other compounds present in adipose tissue conditioned medium may impact on LPL or ANGPTL4 action. For example, adipocytes and the adipose tissue have previously been shown to secrete exosomes rich in lipids (44), proteins including enzymes involved in lipid metabolism (45, 46), RNA, and DNA (47), that have been shown to pro-inflammatory activate macrophages (48) and may have also contributed to the differential response of *Angptl4*$^{-/-}$ versus wild-type macrophages. Hence, a combination of lipids and other factors produced by the adipose tissue may have enhanced the pro-inflammatory phenotype of *Angptl4*$^{-/-}$ versus wild-type macrophages in an adipose tissue environment in vitro. Most likely, FFAs play a primary role in our in vitro system, yet we cannot exclude a role for TGs. For example, ANGPTL4 derived from adipocytes or other cell types may have been present in the adipose tissue-conditioned medium, and may have compensated for the absence of ANGPTL4 in *Angptl4*$^{-/-}$ macrophages thereby equalizing LPL-facilitated TG hydrolysis in *Angptl4*$^{-/-}$ and wild-type macrophages.

The role for TGs might be more important in cell-cell contact frequently occurring in adipose tissue. Our in vitro results demonstrate that co-culturing of macrophages and adipocytes also promotes *Angptl4* expression. This may particularly be relevant during clearance of damaged/dying adipocytes to tightly control engulfment of its cellular content including TGs by macrophages. To evaluate the role of TGs hence importance of the LPL-inhibitory actions of ANGPTL4 to protect against inflammatory responses, wild-type and *Angptl4*$^{-/-}$ macrophages were exposed to a TG emulsion. This led to robust upregulation of *Angptl4* in macrophages and promoted metabolic activation of macrophages. Hence, one might speculate that enhanced exposure during obesity, for example by adipocyte death and the release of adipocyte TGs, may drive this particular ATM phenotype. However, we were

unable to observe differences in inflammatory cytokine production between *Angptl4*$^{-/-}$ and wild-type macrophages. This implies that TGs alone are unable to drive pro-inflammatory responses, yet it is important to note that Intralipid is mostly composed of unsaturated fatty acids that are known to have much lower pro-inflammatory or even anti-inflammatory effects compared to saturated fatty acids. This may have blunted potential differences between *Angptl4*$^{-/-}$ and wild-type macrophages.

Because in vivo TGs together with FFAs and various other compounds will contribute to the ATM phenotype, we hypothesized absence of *Angptl4* to aggravate the inflammatory trait of ATMs. Although there appeared to be a trend toward increased inflammatory differences in adipose tissue of *Angptl4*$^{-/-}$ versus wild-type mice after exposure to LPS, profound variation within the treatment groups hindered differences from reaching significance. Interestingly, however, differences between *Angptl4*$^{-/-}$ and wild-type mice appeared less pronounced in the liver, suggestive of particular importance of ANGPTL4 in lipid-enriched environments. Accordingly, one may speculate that absence of *Angptl4* is of particular importance in an obese adipose tissue environment. Indeed, our in vitro adipose tissue environment – in which *Angptl4*$^{-/-}$ macrophages displayed increased inflammatory activation compared to wild-type macrophages – appeared to induce an obese ATM-alike phenotype, characterized by profound lipid droplet formation and metabolic and inflammatory activation of macrophages (Chapter 4). Therefore, future studies should be aimed at identifying the role of *Angptl4* in ATMs part of obese adipose tissue, when accumulation of lipids increasingly demands for effective lipid handling by macrophages.

In conclusion, our results identify LPL-dependent TG breakdown as an important contributor to lipid accumulation in macrophages driving metabolic activation of these cells. In addition, our results suggest a dual mode of action of ANGPTL4 in controlling macrophage inflammatory responses that involves both its well-known inhibitory action on LPL, yet also seems to be accomplished through other pathways that do not involve direct control of lipid uptake. Previous studies have pointed to the importance of careful control of LPL in ATMs part of obese adipose tissue, by revealing increased concentrations of circulating glucose when LPL is silenced in ATMs specifically (49). Because the anti-inflammatory effects of ANGPTL4 in macrophages in an adipose tissue environment appeared independent of lipid uptake inhibition, further research into the role of ANGPTL4 in abating inflammatory responses of ATMs in obese individuals may well be worthwhile.

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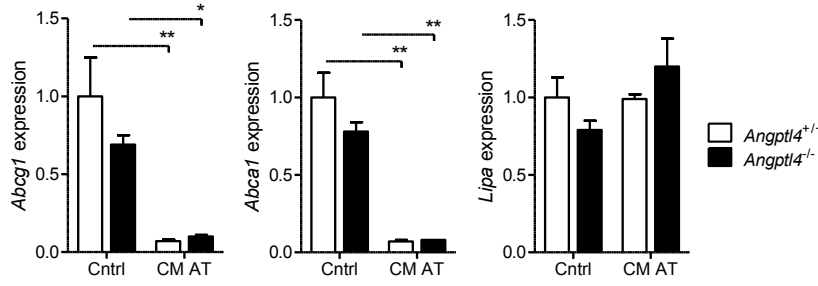
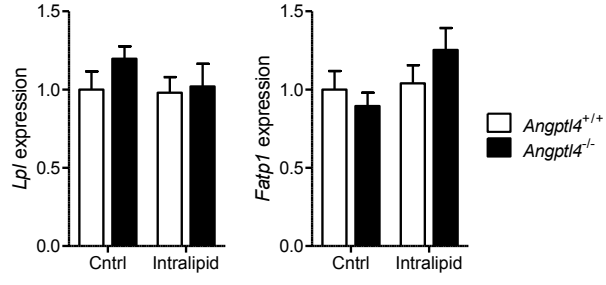
SUPPLEMENTARY FIGURES AND TABLE



Supplementary Figure 1. Expression of genes involved in lipid export or lipolysis.
Expression levels of genes involved in lipid export (*Abcg1, Abca1*) or lysosomal lipolysis (*Lipa*) in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in control conditions and after exposure to adipose tissue-conditioned medium (CM AT) for 24 h. Data are presented as means + SEM. * p<0.05, ** p<0.01.



Supplementary Figure 2. Expression of genes involved in lipid uptake and trafficking.
Relative expression of *Lpl* and *Fatp1* in *Angptl4*$^{+/+}$ and *Angptl4*$^{-/-}$ BMDMs in control conditions and after exposure to adipose tissue-conditioned medium (CM AT) for 24 h. Data are presented as means + SEM. * p<0.05, ** p<0.01.

Supplementary Table 1.
Sequences of primers used for examining mRNA levels using RT-qPCR.

Gene	3' primer	5' primer
36B4	ATGGGTACAAGCGCGTCCTG	GCCTTGACCTTTTCAGTAAG
Abca1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Abcg1	CCTACCACAACCCAGCAGACT	CGAGGTCTCTCTTATAGTCAG
Angptl4	GTTTGCAGACTCAGCTCAAGG	CCAAGAGGTCTATCTGGCTCTG
Atg7	GTTCGCCCCCTTTAATAGTGC	TGAACTCCAACGTCAAGCGG
Cav1	AACATCTACAAGCCCAACAACAAGG	GGTTCTGCAATCACATCTTCAAAGTC
Cd36	TCCAGCCAATGCCTTTGC	TGGAGATTACTTTTCAGTGCAGAA
Cox2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
Cpt1α	CTCAGTGGGAGCGACTCTTCA	GGCCTCTGTGGTACACGACAA
Cxcl2	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
Fabp4	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC
Fatp1	CGCTTTCTGCGTATCGTCTG	GATGCACGGGATCGTGTCT
Gdf15	GAACTGCGCTTACGGGTAG	CTGCACAGTCTCCAGGTGA
Ifnγ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Il10	CTGGACAACATACTGCTAACCG	GGGCATCACTTCTACCAGGTAA
Il1b	TGGTGTGTGACGTTCCCATT	CAGCACGAGGCTTTTTTGTTG
Il4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTG
Il6	AGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Lipa	TGTTCGTTTTCACCATTGGGA	CGCATGATTATCTCGGTCACA
Lpl	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG
Lrp1	ACTATGGATGCCCCTAAAACT	GCAATCTCTTTCACCGTCACA
Mcp1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTG
Plin2	GCCTCTCAACTGGCTGGTAG	ACAGCAAAAGGGGTCATCTG
Tgfb1	CTTCAATACGTCAGACATTCGGG	GTAACGCCAGGAATTGTTGCTA
Tnf	CAACCTCCTCTCTGCCGTCAA	TGACTCCAAAGTAGACCTGCCC

6

Efferocytosis of adipocytes by macrophages in obese versus lean adipose tissue

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Jan Willem Borst
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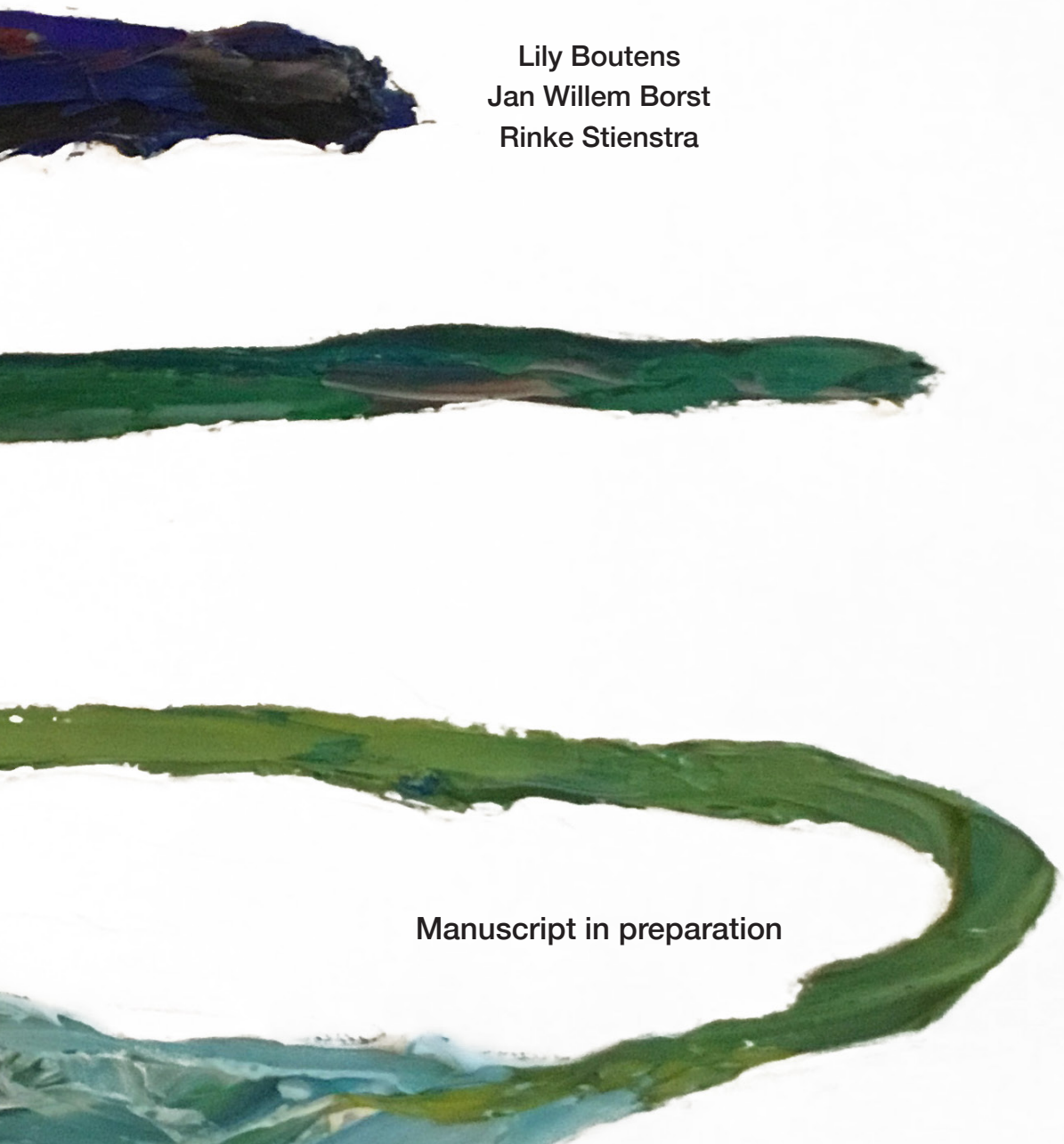

Manuscript in preparation

ABSTRACT

The adipose tissue is a highly dynamic organ. Adipocyte death and removal as part of regular tissue turnover or tissue expansion are crucial for adipose tissue functioning and, ultimately, whole body homeostasis. Persistent appearance of pro-inflammatory macrophages surrounding dead adipocytes in Crown-like structures is suggestive of ineffective adipocyte removal during obesity and considered to be a hallmark of chronic tissue inflammation that links obesity to insulin resistance. So far, however, the role of clearance (efferocytosis) of dead adipocytes by macrophages in obese or lean adipose tissue is incompletely understood. Here, we examined the relevance of dead adipocyte clearance by macrophages in obese adipose tissue, and compared inflammatory consequences of adipocyte clearance for macrophages in an obese versus lean adipose tissue environment. Interestingly, we found profound transcriptional regulation of the efferocytic machinery in obese versus lean adipose tissue macrophages (ATMs). Moreover, two weeks of daily oral administration of BMS-777607 to constrain Mer receptor tyrosine kinase-dependent efferocytosis in obese mice increased cell death markers in the adipose tissue. In vitro studies revealed enhanced inflammatory activation of macrophages primed by obese versus lean adipose tissue in response to dead adipocytes, linking adipocyte removal to inflammatory activation of macrophages in the obese state. Interestingly, our data point to a role for interferon (IFN) signalling during effective dead adipocyte clearance and link impaired IFN signalling to pro-inflammatory macrophage responses in an obese adipose tissue environment. Future studies aimed at unravelling regulation of efferocytosis in obese versus lean adipose tissue including IFN signalling are warranted and may provide new targets to improve the efferocytic capacity of macrophages in order to combat adipose tissue inflammation during obesity.

INTRODUCTION

Macrophages populate various tissues, supporting normal development and enabling tissue plasticity and turnover (1). Central to this role for macrophages in maintaining tissue homeostasis is the clearance of damaged or dead cells; a process called 'efferocytosis' (2, 3). The importance of this process becomes evident in conditions of impaired efferocytosis, when leakage from post-apoptotic remnants nourishes inflammation and auto-immunity. Indeed, impaired efferocytosis is identified to be the underlying cause of various diseases characterized by chronic inflammation including cystic fibrosis, chronic obstructive pulmonary disease and asthma (2, 3).

Adipose tissue is a highly flexible organ, responding to acute metabolic needs by storing fatty acids as triglycerides in lipid droplets or releasing fatty acids from triglycerides via lipid droplet breakdown. Prolonged demand for increased storage capacity during the development of obesity is associated with adipocyte hypertrophy and ultimately adipocyte cell death, which has been postulated to be one of the main driving factors of infiltration of macrophages in obese adipose tissue (4-8). Indeed, obese adipose tissue is characterized by Crown-like structures (CLS) formed by macrophages surrounding dying adipocytes (6, 7). The pro-inflammatory phenotype of accumulating macrophages in obese adipose tissue fuels inflammation that is thought to link obesity with metabolic disturbances including insulin resistance (9, 10).

Although macrophages have long been hypothesized to play a role in clearance of dying adipocytes in CLS (6, 7, 9), it was not until recently that interactions between dying adipocytes and macrophages have been studied in detail. As such, live cell imaging revealed clearance of dying adipocytes by adipose tissue macrophages (ATM) in vitro that appeared to depend on arachidonate 15-lipoxygenase (Alox15) and could be enhanced by IL-4 (11). Interestingly, macrophages have been shown to actively liberate lipids from adipocytes by forming extracellular acidic compartments near dying adipocytes in which they initiate degradation of lipids by secreting lysosomal enzymes via exocytosis (12).

The regulation and consequences of efferocytosis in adipose tissue might differ between lean and obese conditions. So far, however, differences in efferocytosis of adipocytes by macrophages in lean or obese adipose tissue are incompletely understood. In lean animals, high levels of adipocyte cell death instigated via β-adrenergic stimulation (11, 13, 14) or targeted activation of caspase-8 (15) initiates accumulation of anti-inflammatory macrophages displaying a lipid-laden appearance in adipose tissue, reflective of immunologically silent clearance of dead adipocytes. In contrast, sustained presence of lipid-laden macrophages with pro-inflammatory characteristics in CLS part of obese adipose tissue (16-19) may be suggestive of ineffective efferocytosis of dead adipocytes during obesity. Interestingly,

macrophages of patients with Type 2 Diabetes are known to have reduced phagocytic capacity (20), contributing to plaque instability in atherosclerosis (21) and impaired wound healing (22).

To gain insight into the relevance of adipocyte clearance by ATMs in the context of obesity, we examined gene expression profiles of ATMs from obese versus lean mice, studied interactions between adipose tissue-primed macrophages and dead adipocytes in vitro, and interfered with the phagocytic machinery in obese mice. As such, we found increased expression of the efferocytic machinery in ATMs during obesity, and increased cell death in the adipose tissue upon interference with efferocytosis. In vitro, priming with obese versus lean adipose tissue enhanced inflammatory activation of macrophages co-cultured with apoptotic adipocytes despite equal lipid uptake; suggestive of impaired processing of the engulfed cargo. Interestingly, interferon (IFN) signalling appeared involved in adipocyte clearance in vitro and was impaired in ATMs part of obese adipose tissue. Further understanding of the regulation of adipocyte clearance in lean and obese adipose tissue might provide new targets to limit pro-inflammatory activation of macrophages during obesity by increasing their efferocytic capacity.

MATERIALS AND METHODS

Animal studies

Male C57Bl/6 mice (Harlan, Germany) had ad libitum excess to food and water. Mice (n=12) on a high-fat diet (HFD) containing 60% kcal from fat (D12492, Research diets) for 10 weeks were then randomly allocated over two groups (both n=6) and received a daily oral gavage either containing 25 mg/kg bodyweight BMS-777607 (Seleck Chemicals) dissolved in vehicle, or vehicle only (0.1% Tween80, 0.5% carboxymethylcellulose in H_2O) for two weeks while remaining on their HFD.

Epididymal adipose tissue explants for generation of ATM-like macrophages in vitro were isolated from mice on a low fat diet (LFD) containing 10% kcal from fat (D12450J, Research diets) or HFD containing 60% kcal from fat (D12492, Research diets) for 13-16 weeks. All procedures were approved by the ethical committee for animal experiments from Wageningen University.

In vitro co-culture systems

Bone marrow cells were isolated from femurs of lean mice following a standard protocol, and brought in culture in Dulbecco's modified eagle's medium supplemented (Lonza) with 10% FCS and 1% PS (DMEM/FCS/PS) and 5-10% L929-conditioned medium. For direct co-cultures with adipocytes, non-adherent cells were removed after 6-7 days and adherent cells were washed and plated. Upon adhesion, cells were washed and subsequently co-cultured with adipocytes in DMEM/FCS/PS for 24 hours.

For IFN-γ pre-treatment, plated bone marrow-derived macrophages (BMDMs) were cultured in DMEM/FCS/PS containing 10 ng/mL IFN-γ for 18-24 hours before starting the 24-hour co-culture with adipocytes in DMEM/FCS/PS.

For adipose tissue priming, non-adherent bone marrow cells were removed after 3-4 days and adherent cells were washed and plated in 12- or 24-wells plates. After adhesion, fresh DMEM/FCS/PS was added together with an insert containing 100 mg of minced live or dying epididymal adipose tissue explant collected from LFD-fed or HFD-fed mice. Inserts containing adipose tissue were removed after 3 days, after which the adipose tissue-primed BMDMs were co-cultured with adipocytes in DMEM/FCS/PS for 24 hours.

Mouse 3T3-L1 fibroblasts were maintained in DMEM/FCS/PS. At 2 days post-confluence, cells were switched to DMEM/FCS/PS containing 0.5 mM IBMX, 10 µM dexamethasone, and 5 µg/mL insulin (all Sigma) for 2 days to initiate differentiation into adipocytes. Then, the cells were maintained in DMEM/FCS/PS with 5 µg/mL insulin for 3 days and subsequently held in DMEM/FCS/PS for 5-7 days to complete differentiation into adipocytes. Medium was replaced once every 3 days. Differentiated 3T3-L1 cells were used in co-cultures with BMDMs for flow cytometry. Human Simpson-Golabi-Behmel syndrome (SGBS) cells were maintained in DMEM/F12 (Lonza) supplemented with 10% FCS, 1% PS, 33 µM Biotin (Sigma)

and 17 µM panthotenat (Sigma), and differentiated into adipocytes over a period of 21 days as has been described previously (23). Differentiated SGBS cells were used in co-cultures with BMDMs for isolation of RNA and collection of cytokines.

Adipocyte and adipose tissue death was induced with UV-C light using a Stratalinker 1800 UV cross linker (Stratagene). Adipocytes were collected using trypsin, pelleted with centrifugation, dissolved in DMEM/FCS/PS and co-cultured with BMDMs in a 1:1 (1) or 1:6 (1/6) ratio (adipocytes:macrophages). Unless otherwise specified, a 1:1 ratio was used.

Flow cytometry

For quantification of lipid uptake from adipocytes by macrophages, BMDMs were stained with PKH26 (Sigma) following manufacturer's protocol, and co-cultured with apoptotic adipocytes. After a 4-hour co-culture, culture medium was aspirated carefully and cells were collected in PBS. After minimizing cell clumps by pipetting up and down, cells were fixated in 4% formaldehyde for 30 min at 4 ºC. Upon centrifugation, pelleted cells were dissolved in PBS containing 1% free fatty acid-free BSA (Sigma) and 2mM EDTA and stained for neutral lipids with 1 mg/mL Bodipy (Sigma) for 20 minutes at RT. Cells were washed with PBS once and measured by flow cytometry using a FC500 Flow Cytometer (Beckman Coulter).

Cytokine measurements

Levels of IL-6 and IL-1ra were measured in 24-hour cell culture supernatants of BMDMs co-cultured with adipocytes by using murine DuoSet ELISA Development kits according to the guidelines of the manufacturer (R&D Systems).

Plasma metabolites

Triglycerides were determined using an enzymatic kit (Roche Molecular Biochemicals). Non-esterified fatty acids were measured with a NEFA-C kit (Wako Diagnostics). Glucose was measured enzymatically using a glucose liquicolor assay kit (Human GmbH). Plasma insulin concentrations were obtained by ultra-sensitive mouse insulin ELISA (Crystal Chem Inc.). Leptin and adiponectin were determined by ELISA (R&D Systems).

Immunohistochemistry

Parrafin-embedded sections of epididymal or inguinal adipose tissue were stained with an F4/80 antibody (AbD Serotec) and counterstained with haematoxylin. Macrophages were visualized with 3,3-diaminobenzidene (DAB, Vector Laboratory).

Western blot

Bone marrow-derived macrophages or adipose tissue were lysed in RIPA buffer (Pierce Thermoscientific) enriched with protease and phosphatase inhibitors (Roche). Total protein was determined using a BCA protein assay (Pierce Thermoscientific) and for each sample 15 µg of protein was loaded on a 4-15% Mini-PROTEAN TGX Precast gel (Bio-Rad). Proteins were

separated by SDS gel electrophoresis and transferred to a PVDF membrane (Bio-Rad) using the Transblot Turbo System (Bio-Rad). Primary antibodies for Cleaved caspase-3 (Bioke), poly ADP ribose polymerase (PARP) (Cell Signalling), and b-tubulin (Sigma) were used at a ratio of 1:1000 and incubated overnight at 4 ^{o}C. All incubations were in Tris-buffered saline with 0.1% Tween-20 (TBS-T), containing 5% dry milk. HRP-conjugated goat-anti-rabbit secondary Ab (Sigma) was used 1:5000 in TBS-T with 5% dry milk. Blots were visualized using the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad).

RNA isolation and qPCR

Total RNA was isolated using TRIzol (Life Technologies), either via phenol/chloroform extraction (BMDMs), or with the RNeasy microkit (Qiagen) (epididymal or inguinal adipose tissue of BMS-treated or Cntrl mice). Reverse transcription was executed using the iScript cDNA synthesis kit (Bio-rad). Real-time quantitative PCR was carried out with a SensiMix SYBR kit (Bioline) in a CFX384 system (Bio-rad). Relative expression levels were calculated after normalization against the housekeeping gene *36B4* using starting quantities. Primer sequences can be found in **Supplementary Table 4**.

Microarray analysis and interpretation

RNA from BMDMs co-cultured with 100 mg epididymal adipose tissue explant for 3 days was purified with an RNAeasy Minikit (Qiagen). After verification of RNA quality with the RNA 6000 Nano assay on a Agilent 2100 Bioanalyzer (Agilent Technologies), 100 ng per sample) was labelled with the Whole-Transcript Sense Target Assay (Affymetrix, Santa Clara, CA, USA; P/N 900652) and hybridized to whole-genome Affymetrix Mouse Gene 1.1 ST arrays (Affymetrix). Quality control and data analysis pipeline have been described in detail (24). Briefly, normalized expression estimates of probe sets were computed by the robust multiarray analysis (RMA) algorithm (25, 26) as implemented in the Bioconductor library *AffyPLM*. Probe sets were redefined according to Dai et al. (27) based on annotations provided by the Entrez Gene database, which resulted in the profiling of 21,187 unique genes (custom CDF v20). Differentially expressed probe sets (genes) were identified by using linear models (library *limma*) and an intensity-based moderated t-statistic (28, 29). Adipose tissue macrophages of obese versus lean mice were obtained as previously described (Chapter 4). Transcriptomes were analysed similarly as those of BMDMs.

Changes in gene expression were related to biologically meaningful changes using gene set enrichment analysis (GSEA) (30), or Ingenuity Pathway Analysis (IPA) (Qiagen). For each comparison, genes were ranked on their t-value that was calculated by the moderated t-test. Statistical significance of GSEA results was determined using 1000 permutations. The library *clusterProfiler* was used for visualization and interpretation of the GSEA results (31). Probe sets that satisfied the criterion of $p<0.01$ were included for Ingenuity Pathway Analysis.

Statistical analysis

When comparing two groups a student's t-test was used for statistical analysis. When comparing more than two groups, a one-way ANOVA or two-way ANOVA with Bonferroni post-hoc test was used as specified at each figure. Statistical analyses were done with Graphpad Prism 5 (GraphPad Software).

RESULTS

Enhanced expression of the efferocytic machinery in adipose tissue macrophages during obesity

Macrophages in obese adipose tissue cluster around dying adipocytes in crown-like structures (6, 7). To investigate whether pathways involved in adipocyte clearance are regulated in adipose tissue macrophages (ATMs) during obesity, we compared transcriptomes of ATMs from obese versus lean mice by microarray and subsequent Ingenuity Pathway Analysis (IPA). Interestingly, clusters of genes related to efferocytosis including 'Phagocytosis', 'Endocytosis' and 'Engulfment of cells' were predicted to be activated in ATMs from obese versus lean mice (**Figure 1A**). A role for efferocytosis of adipocytes and subsequent requirement for lipid processing by macrophages was further corroborated by predicted activation of 'Metabolism of membrane lipid derivatives' and 'Transport of lipid' in ATMs from obese versus lean mice (**Figure 1B,C**).

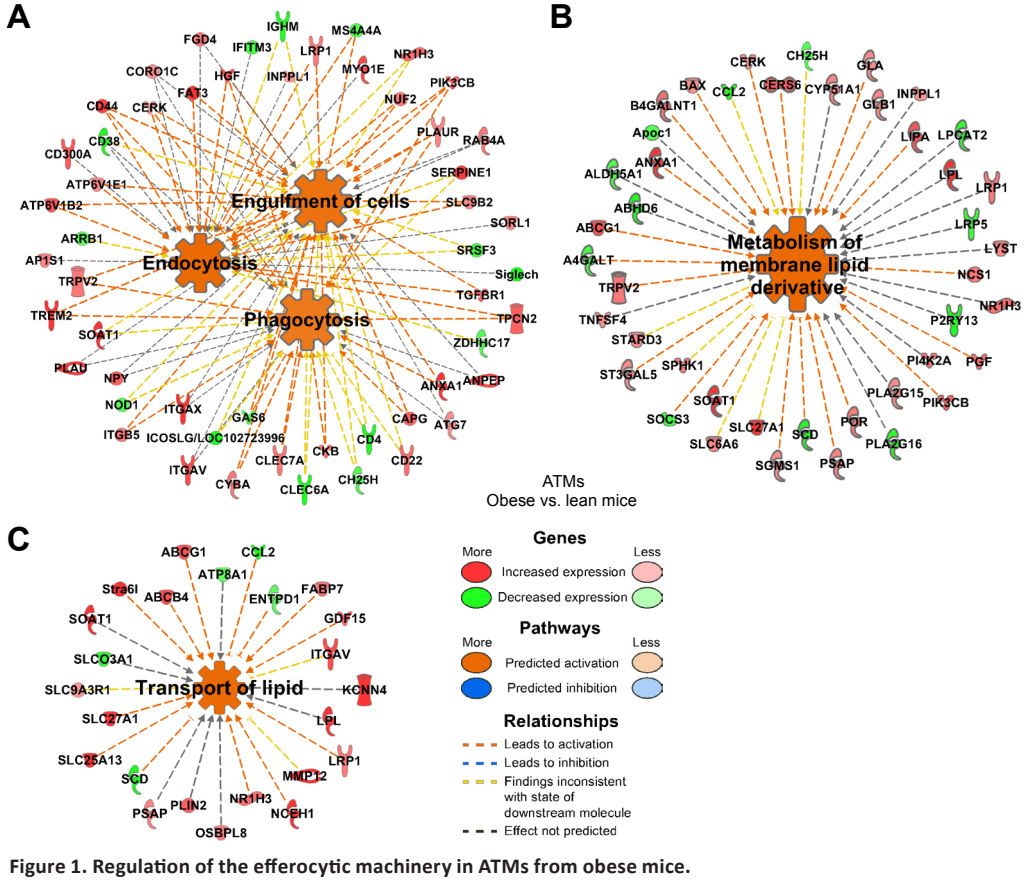

Figure 1. Regulation of the efferocytic machinery in ATMs from obese mice.
Ingenuity Pathway Analysis on the transcriptomes of adipose tissue macrophages (ATMs) isolated from mice on a high-fat diet (obese) or low-fat diet (lean). Predicted activation of pathways involved in efferocytosis (**A**,**B**) and lipid processing (**B**,**C**) in ATMs from obese versus lean mice.

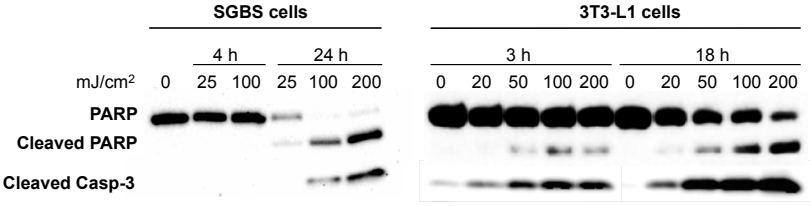

Figure 2. Cell death in UV-irradiated adipocytes.
Presence of cleaved Caspase-3 and Poly ADP ribose polymerase (PARP) in human (Simpson-Golabi-Behmel Syndrome (SGBS) cells) or murine (3T3-L1 cells) adipocytes at two time points after exposure to several intensities of UV irradiation to induce cell death.

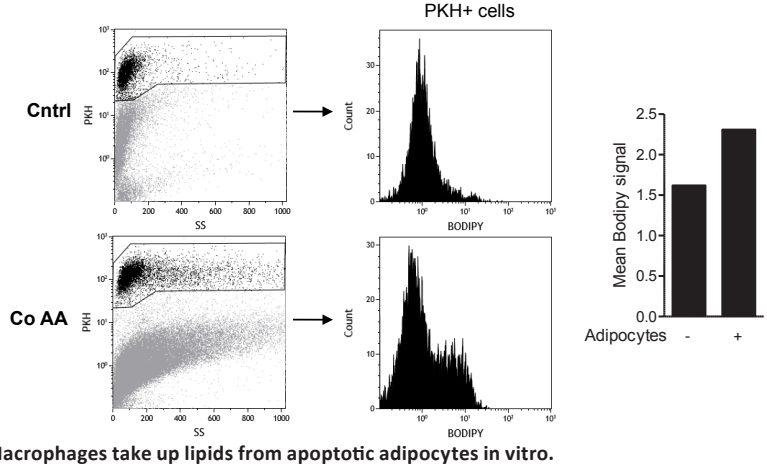

Figure 3. Macrophages take up lipids from apoptotic adipocytes in vitro.
Representative picture of PKH-stained BMDMs either exposed to apoptotic adipocytes (Co AA) or not (Cntrl) and subsequently stained with Bodipy. Selection of macrophages as PKH+ cells (left); mean Bodipy signal in the PKH+ fraction presented as density plot (middle) or as bar graph (right). Measurements done with flow cytometry. SS: side scatter.

Efferocytosis of adipocytes by macrophages

To study efferocytosis of adipocytes by macrophages, we used a co-culture system in which plated bone marrow-derived macrophages (BMDMs) were exposed to dead adipocytes. Controversy exists about the type of adipocyte cell death occurring in obese adipose tissue (32). In our experiments, cell death was induced by irradiation with 25 mJ/cm^2 (apoptotic) or 100 mJ/cm^2 (severe apoptotic) UV light. Dying adipocytes were left in regular culture medium for 18-24 hours, when Caspase 3 and PARP cleavage became apparent (**Figure 2**), before feeding them to macrophages. Uptake of lipids from dead adipocytes was quantified with flow cytometry, by measuring the mean Bodipy signal (lipids) in PKH-positive cells (BMDMs). The higher mean Bodipy signal intensity and increased granularity (side scatter (SS) signal) found in the BMDMs co-cultured with apoptotic adipocytes for 4 hours compared to BMDMs alone is suggestive for macrophages to take up lipids from apoptotic adipocytes (**Figure 3**). To examine transcriptional responses upon efferocytosis, we compared gene expression levels in BMDMs co-cultured with apoptotic or severe apoptotic adipocytes with those in BMDMs co-cultured with live adipocytes. Interestingly, various genes involved in lipid

uptake and processing; including uptake (*Lpl, Cd36*), breakdown (*Lipa, Atp6V0d2*), storage (*Plin2*), and export (*Abcg1*), were higher expressed in macrophages exposed to apoptotic versus live adipocytes (**Figure 4A**). Expression of these genes appeared dose-dependently induced by dead adipocytes (**Figure 4A**), and was only induced upon prolonged (24 hours) exposure to adipocytes with hardly any regulation of these genes found after a 6-hour co-culture, pointing to enhanced uptake of adipocyte-derived lipids over time (**Supplementary Figure 1**). Of note, no regulation of genes classically involved in efferocytosis; the receptor *Mertk,* bridging molecule *Gas6* and adapter protein *Elmo1* (**Figure 4B**), or genes involved in glycolysis (**Figure 4C**) was observed, probably reflecting unique macrophage responses adapted to handling of lipid-filled dead adipocytes.

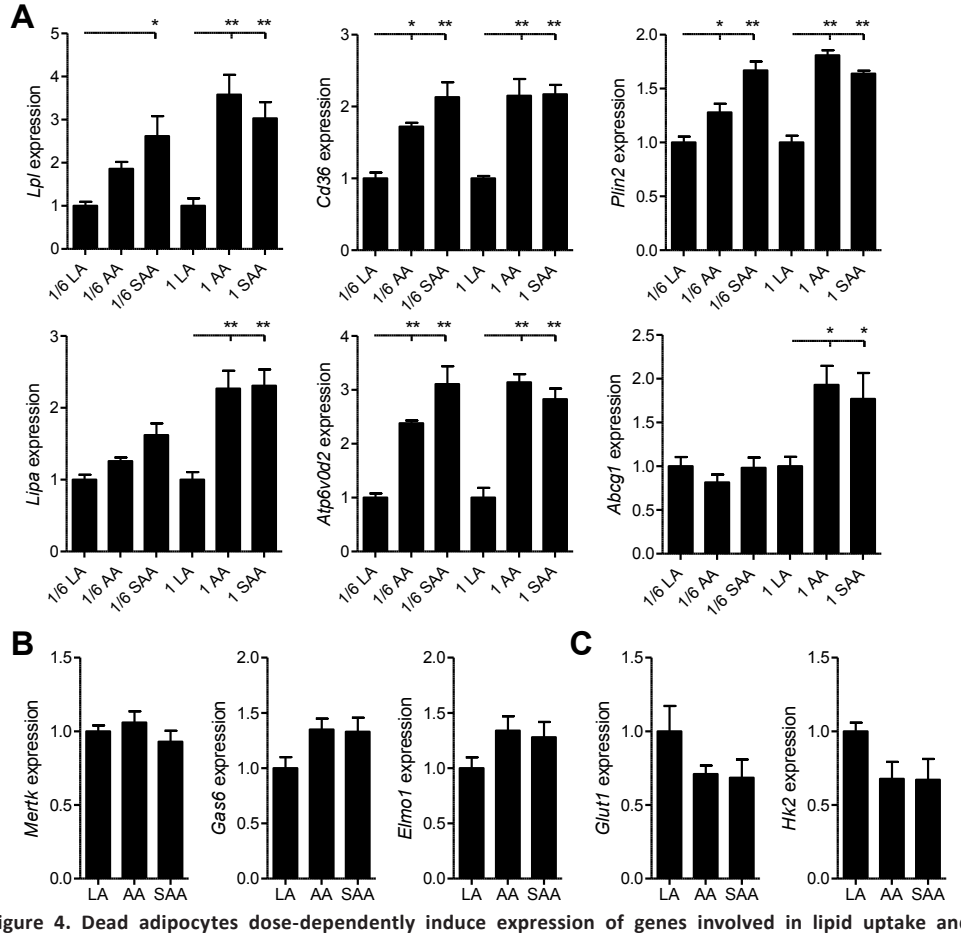

Figure 4. Dead adipocytes dose-dependently induce expression of genes involved in lipid uptake and processing in macrophages.

Relative expression of various genes involved in lipid uptake or processing (**A**), classical phagocytosis (**B**), or glycolysis (**C**) in BMDMs exposed to two concentrations of either live adipocytes (LA), apoptotic adipocytes (AA), or severe apoptotic adipocytes (SAA) (1x or 1/6x of the amount of macrophages) for 24 hours. Co-cultures with live adipocytes were set to 1. The difference in gene expression in macrophages co-cultured with AA vs. LA or SAA vs. LA was tested for significance using an one-way ANOVA with Bonferroni post-hoc test. Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$.

Macrophages in an obese adipose tissue environment react pro-inflammatory on dead adipocytes

We next aimed to uncover whether differences in clearance of adipocytes and/or subsequent processing exist between macrophages in an obese or lean adipose tissue environment. Therefore, we pre-exposed macrophages to 100 mg of lean (LeAT) or obese (ObAT) adipose tissue for 3 days to generate an ATM-alike phenotype *in vitro*, before co-culturing them with apoptotic adipocytes (*experimental setup* **Figure 5A**). To our surprise, only in one out of three individual flow cytometry experiments a significant yet small increase in the amount of neutral lipids taken up from apoptotic adipocytes was found in BMDMs pre-exposed to ObAT compared to LeAT (**Figure 5B**), pointing to relatively equal lipid uptake from apoptotic adipocytes by macrophages in a lean or obese adipose tissue environment.

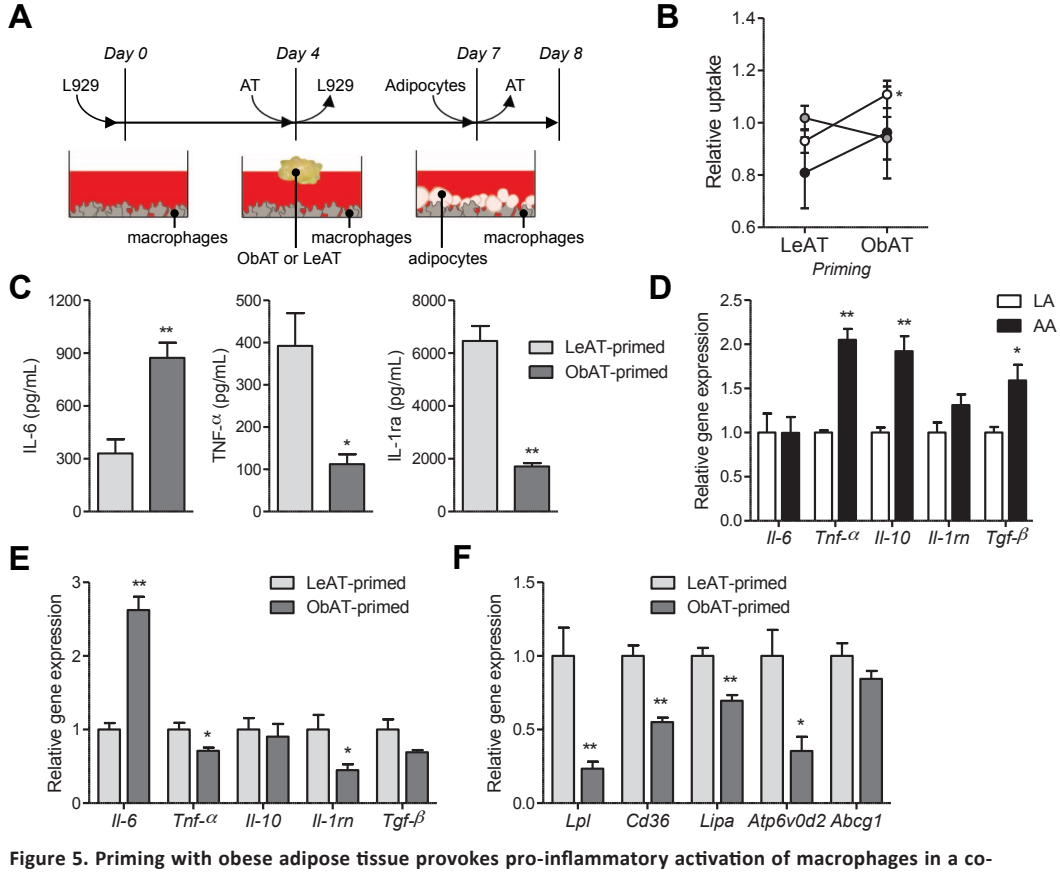

Figure 5. Priming with obese adipose tissue provokes pro-inflammatory activation of macrophages in a co-culture with apoptotic adipocytes.
A Experimental set-up for studying efferocytosis of adipocytes by adipose tissue-primed macrophages in vitro. Medium was refreshed at day 4 and 7. **B** Mean Bodipy signal in PKH-stained BMDMs primed by adipose tissue explants and then co-cultured with apoptotic adipocytes for 4 hours. Three independent flow cytometry experiments are shown. **C** Cytokines measured in the supernatant of BMDMs co-cultured with apoptotic adipocytes for 24 hours, after being primed with lean or obese adipose tissue (LeAT or ObAT) for 3 days. **D** Relative gene expression of anti- (*Il-10, Il-1rn, Tgf-β*) and pro- (*Il-6, Tnf-α*) inflammatory cytokines in BMDMs exposed to live (LA) or apoptotic adipocytes (AA) for 24 hours. Expression in macrophages co-cultured with LA was set to 1.

E Relative gene expression of anti- (*Il-10, Il-1rn, Tgf-β*) and pro- (*Il-6, Tnf-α*) inflammatory cytokines in BMDMs exposed to AA for 24 hours after being primed with LeAT or ObAT for 3 days. Expression in macrophages primed with LeAT was set to 1. **F** Relative expression of genes involved in lipid processing in BMDMs primed with LeAT or ObAT for 3 days and subsequently exposed to AA for 24 hours. Expression in macrophages primed with LeAT was set to 1. Significance was calculated using student's t-tests. Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$.

Rather than differing in quantity, efferocytosis of adipocytes in an obese or lean adipose tissue environment may elicit different inflammatory responses. To study this, IL-6, IL-1ra, TNF-α and IL-10 were measured in supernatants of BMDMs co-cultured with apoptotic adipocytes for 24 hours, after 3 days priming with LeAT or ObAT (*experimental set-up* **Figure 5A**). Whereas BMDMs primed with ObAT secreted more IL-6 during a co-culture with apoptotic adipocytes, they secreted less TNF-α and IL-1ra in response to apoptotic adipocytes than BMDMs primed with LeAT did. No IL-10 could be detected in the supernatants (**Figure 5C**). Strong inflammatory responses upon exposure to apoptotic adipocytes were specific for adipose tissue-primed macrophages, since none of the cytokines could be detected in the supernatant of control macrophages (held in 5% L929) co-cultured with apoptotic adipocytes (data not shown). Interestingly, however, on mRNA level control BMDMs displayed no regulation of *Il-6* or *Il-1rn*, but did increase the expression of *Tnf-α, Il-10* and *Tgf-β* in response to apoptotic adipocytes (**Figure 5D**). In contrast, gene expression levels in BMDMs primed with ObAT versus LeAT were in line with what we found on cytokine level, displaying enhanced *Il-6* yet reduced *Il-1rn* and *Tnf-α* expression upon efferocytosis of adipocytes (**Figure 5E**). Thus, Inflammatory activation of macrophages primed with obese versus lean adipose tissue did not resemble the inflammatory phenotype of control macrophages, and may be indicative of a disrupted response on apoptotic adipocytes. In line with this hypothesis, genes involved in lipid metabolism that were strongly induced in control BMDMs exposed to apoptotic versus live adipocytes (**Figure 4A**) were lower expressed in BMDMs primed with ObAT versus LeAT (**Figure 5F**).

In obese adipose tissue, adipocyte number and size may overwhelm macrophages (32). Interestingly, however, we have found pro-inflammatory responses of macrophages primed by obese versus lean adipose tissue in a co-culture with equal numbers of apoptotic adipocytes, suggestive of distinct regulation in macrophages confronted with cell death in an obese versus lean adipose tissue environment irrespective of the dead adipocyte load. To study this, we looked for differentially regulated gene sets in BMDMs exposed to UV-irradiated (dying) ObAT versus LeAT tissue (100 mJ/cm^2 UV-C light) (**Figure 6A**) for 3 days. By applying gene set enrichment analysis (GSEA) on complete transcriptomes, we found enrichment of gene sets representing ER stress and oxidative stress in macrophages exposed to dying ObAT versus dying LeAT (**Figure 6B**). This result was corroborated by the identification of reactive oxygen species as upstream regulator in macrophages exposed to dying ObAT versus dying LeAT using IPA (activation z-score 2.528, $p<0.01$). Interestingly, ER stress and oxidative stress appeared to be specifically enhanced in macrophages exposed to dying versus live obese but not lean adipose tissue (**Figure 6C**). Hence, cell death in an obese adipose tissue environment

activates ER stress and oxidative stress in macrophages, which may fuel their inflammatory responses (33). Direct contact with apoptotic adipocytes, however, did not induce oxidative stress or ER stress markers in the ObAT-primed BMDMs (data not shown). It is therefore unlikely that oxidative stress or ER stress underlies the inflammatory response on apoptotic adipocytes found in BMDMs primed with ObAT versus LeAT.

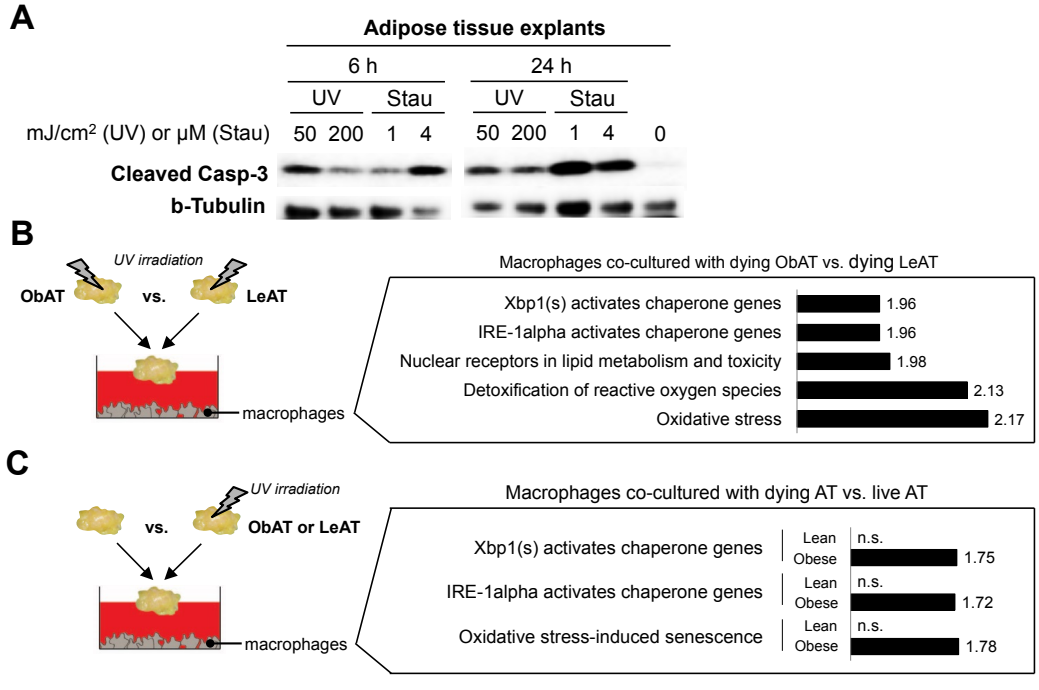

Figure 6. Cell death in obese adipose tissue explants promotes ER stress and oxidative stress in BMDMs.
A Presence of cleaved Caspase-3 and b-Tubulin (loading control) in adipose tissue explant at two time points after irradiation with UV or treatment with Staurosporine (Stau). Enrichment of gene sets related to ER stress and oxidative stress in BMDMs exposed to UV-irradiated ObAT compared to BMDMs exposed to UV-irradiated LeAT for 3 days (**B**), and BMDMs exposed to UV-irradiated AT (lean or obese) compared to BMDMs exposed to non-treated AT (lean or obese) for 3 days (**C**). Gene Set Enrichment Analysis was done on the complete BMDM transcriptomes.

Interferon signalling is impaired in macrophages in an obese adipose tissue environment

Next to activation of oxidative and ER stress, strong depletion of both type I and type II interferon (IFN) signalling was found in macrophages exposed to dying ObAT versus dying LeAT (**Figure 7A**, *experimental set-up* as in **Figure 6B**), which was confirmed by IPA reporting a reduction in interferons as upstream regulators (activation z-score -5.605, $p<0.01$). The IFN gene sets were only depleted in BMDMs exposed to dying versus live ObAT (**Figure 7B,** *experimental set-up* as in **Figure 6C**), suggestive of disruption of IFN signalling in macrophages in an obese adipose tissue environment specifically. Interestingly, IFN signalling appeared to have in vivo relevance as well, evident by depletion of IFN signalling in ATMs from obese versus lean mice (**Figure 7C**). An overview of significantly enriched or depleted gene sets in

the different datasets of BMDMs exposed to adipose tissue can be found in **Supplementary Table 1-3**.

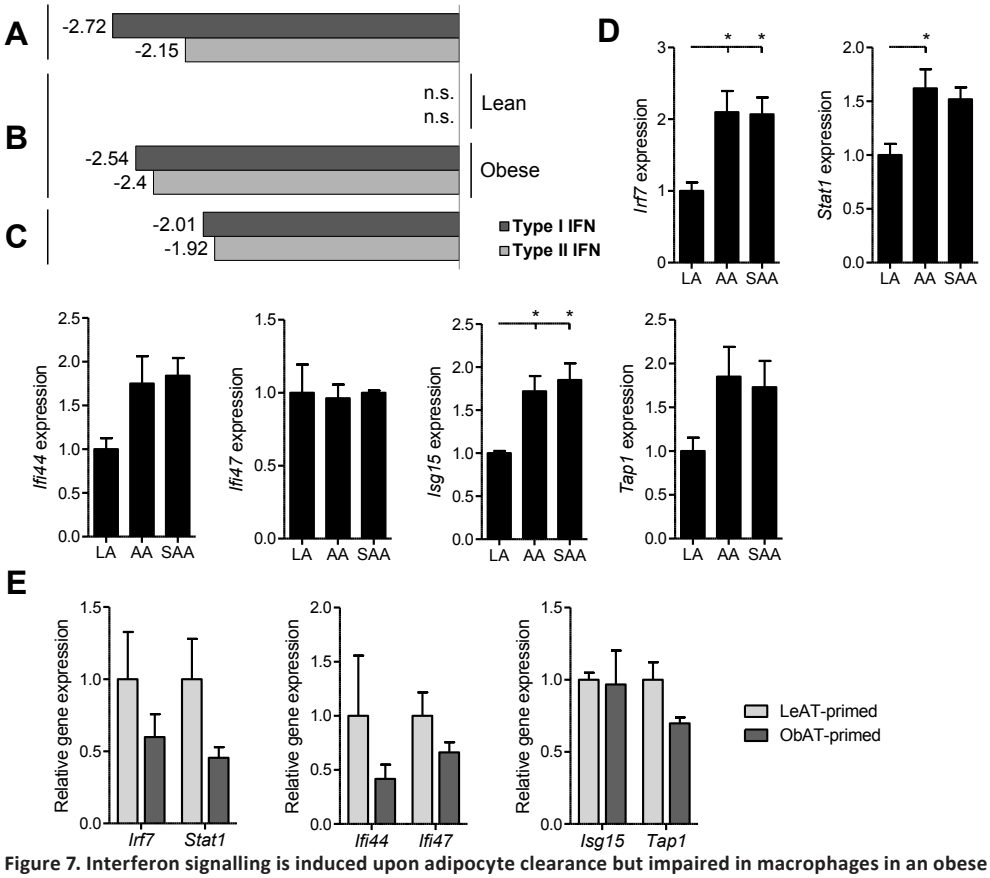

Figure 7. Interferon signalling is induced upon adipocyte clearance but impaired in macrophages in an obese adipose tissue environment.

Interferon (IFN) signalling in BMDMs exposed to UV-irradiated obese (ObAT) versus UV-irradiated lean (LeAT) adipose tissue for 3 days (**A**), in BMDMs exposed to UV-irradiated versus non-treated ObAT (obese) or UV-irradiated versus non-treated LeAT (lean) for 3 days (**B**), or in adipose tissue macrophages isolated form obese versus lean mice (**C**); identified by Gene Set Enrichment Analysis using complete transcriptomes as input. **D** Expression of IFN-related genes in BMDMs co-cultured with live adipocytes (LA), apoptotic adipocytes (AA), or severe apoptotic adipocytes (SAA) for 24 hours. Expression in macrophages co-cultured with LA was set to 1. **E** Expression of IFN-related genes in BMDMs co-cultured with AA for 24 hours after being primed with LeAT or ObAT. Expression in macrophages primed with LeAT was set to 1. The difference in gene expression in macrophages co-cultured with AA vs. LA or SAA vs. LA was tested for significance using an one-way ANOVA with Bonferroni post-hoc test. Differences between ObAT- versus LeAT-primed macrophages were tested for significance using student's t-tests. Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$.

Enhanced interferon signalling in macrophages relates to immunologically silent responses toward apoptotic adipocytes in vitro

Interferon signalling has been shown involved in the regulation of efferocytic capacity of macrophages (34). We therefore reasoned that IFN signalling might be essential for efficient efferocytosis of adipocytes by macrophages and that reduced IFN signalling in macrophages in an obese adipose tissue environment may underlie disrupted processing of apoptotic

adipocytes and/or inflammatory responses. In line with this reasoning genes part of IFN signalling pathways, namely *Irf7, Stat1, Ifi44, Ifi47, Isg15* and *Tap1*, were or tended to be higher expressed in BMDMs co-cultured with apoptotic versus live adipocytes (**Figure 7D**). Interestingly, these genes were lower or not differentially expressed in response to apoptotic adipocytes by BMDMs primed with ObAT versus LeAT (**Figure 7E**). Of note, no clear difference between regulation of Type I-specific (*Ifi-44, Isg15*) versus Type II-specific (*Ifi47, Tap1*) (35) genes was found. Hence, more work is needed to conclude on the relative involvement of the two IFN signalling pathways.

To decipher whether activated IFN signalling is indeed of importance during efferocytosis of adipocytes, macrophages were pre-treated with the type I interferon IFN-γ (10 ng/mL) before co-culturing them with apoptotic adipocytes for 6 or 24 hours. However, no clear difference in expression of the efferocytosis marker genes involved in lipid uptake and processing was found between IFN-γ pre-treated and control macrophages (**Supplementary Figure 2A**), nor in the expression of cytokines (**Supplementary Figure 2B**). Importantly, however, we used IFN-γ pre-treatment as tool for enhancing IFN signalling, yet found many genes involved in both Type I and Type II IFN responses to be reduced upon the pre-treatment (**Supplementary Figure 2C**). Because *Ifn-α,-β*, and *-γ*, nor their receptors *Ifnar* and *Ifngr* were regulated in macrophages exposed to dead adipocytes, IFNs itself are likely not responsible for enhanced IFN signalling in macrophages clearing dead adipocytes.

Relevance of efferocytosis in an obese adipose tissue environment

To examine the relevance of the phagocytic machinery in obese adipose tissue, when adipocyte cell death becomes clearly apparent, we interfered with efferocytosis in mice fed a high-fat diet (HFD) for 10 weeks. To do so, HFD-fed mice were given a daily oral gavage with either vehicle (Cntrl, n=6) or 25 mg BMS-777607 (BMS, n=6) per kg bodyweight for two weeks while remaining on a HFD (*experimental set-up* **Figure 8A**). BMS is an inhibitor of the Mer receptor tyrosine kinase (MerTK) (36), which is involved in phagocytosis and has been shown to affect efferocytosis in fatty tissues upon oral delivery (37). Both total bodyweight and weight of two main adipose tissue depots, inguinal (iWAT) and gonadal (gWAT) adipose tissue, did not change upon BMS treatment (**Figure 8B**), nor did the weight of any other tissue (data not shown). In both iWAT and gWAT no gross histological differences could be observed between BMS-treated and control mice (**Figure 8C**). Interestingly, however, the expression of several cell death markers was increased in iWAT of mice exposed to BMS (**Figure 8D**). Although increased cell death was not apparent in gWAT (**Figure 8E**), microarray analysis and subsequent GSEA of gWAT of BMS-treated mice revealed strong depletion of IFN signalling (**Figure 8F**). Given our finding of reduced IFN signalling in macrophages exposed to cell death in an obese adipose tissue environment (**Figure 7**), depleted IFN signalling in gWAT of BMS-treated animals may be suggestive of impaired efferocytosis. To examine whether BMS treatment affected the inflammatory state of adipose tissue, we measured

the expression of some macrophage markers (*F4/80, Cd206, Cd11c*). In general, there was a trend toward lower macrophage marker expression in the adipose tissue of mice treated with BMS, although only a reduction in F4/80 expression reached significance in iWAT (**Figure 8G**) but not gWAT (**Figure 8H**). We found no differences in plasma glucose, insulin, triglycerides, non-esterfied fatty acids (NEFA), adiponectin, or leptin (**Supplementary Figure 3**). Hence, although BMS-treated animals show signs of impaired efferocytosis in adipose tissue, this did not yet translate into adipose tissue inflammation or gross metabolic disturbances.

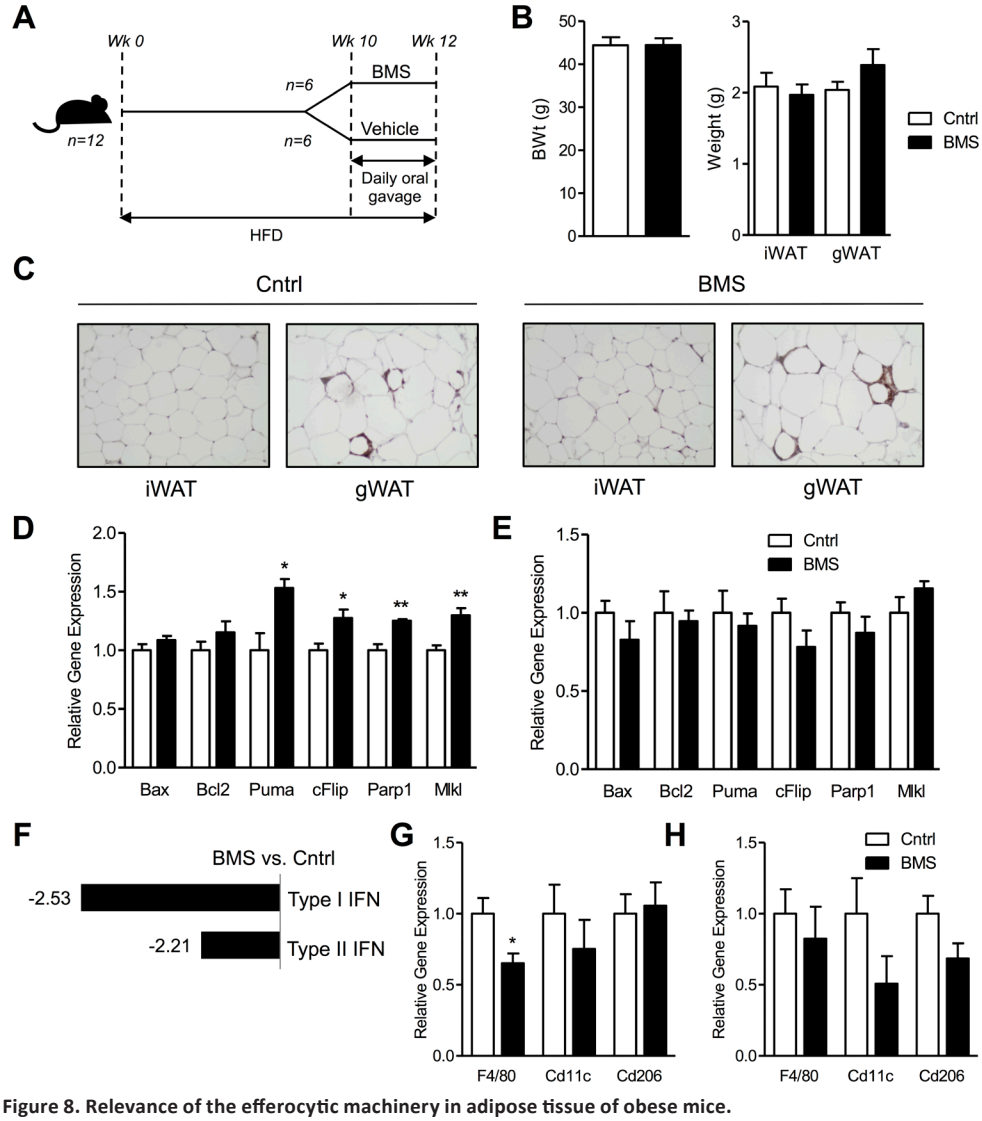

Figure 8. Relevance of the efferocytic machinery in adipose tissue of obese mice.
A Experimental design to examine the role for efferocytosis in adipose tissue of obese mice. In short, mice on a high-fat diet (HFD) for 10 weeks were given a daily oral gavage with either vehicle (Cntrl) or BMS-777607 (BMS) for two weeks (to inhibit the Mer receptor tyrosine kinase that is involved in phagocytosis) while remaining on a HFD. **B** Bodyweight (BWt), and weight of inguinal (iWAT) and gonadal (gWAT) adipose tissue of BMS-treated (BMS) and control (Cntrl) mice. **C** Representative imagines of iWAT and gWAT stained for F4/80 in BMS-treated and Cntrl mice. Relative expression of several cell death markers in iWAT (**D**) and gWAT (**E**) of BMS-treated and Cntrl mice. **F** Depleted Type I and Type II IFN gene sets in gWAT of BMS-treated compared to Cntrl mice, identified by Gene Set Enrichment Analysis using complete transcriptomes as input. Relative expression of macrophage markers in iWAT (**G**) and gWAT (**H**) of BMS-treated and Cntrl mice. Expression in Cntrl mice was set to 1. Differences in gene expression between BMS-treated and Cntrl animals were tested for significance using student's t-tests. Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

Adipocyte death, clearance, and de novo adipogenesis are crucial for adipose tissue functioning and, ultimately, whole body homeostasis (32). Persistent appearance of crown-like structures (CLS) defined by pro-inflammatory macrophages around dead adipocytes in obese adipose tissue points to ineffective adipocyte removal (6, 9, 16, 18). Here, we show that the efferocytic machinery is activated during obesity and that its inhibition increases markers of cell death in obese adipose tissue. In vitro studies, however, point to disrupted macrophage responses toward apoptotic adipocytes in an obese adipose tissue environment. Interestingly, interferon (IFN) signalling appeared to be involved in effective efferocytosis of dead adipocytes, yet was impaired in ATMs part of obese adipose tissue, suggestive of a link between reduced IFN signalling and ATM functioning during obesity.

In lean conditions, adipocyte cell death induced by weight loss or prolonged lipolysis initiates rapid formation of CLS and clearance of dead adipocytes in an anti-inflammatory fashion (13, 14, 38). Recently, clearance of adipocytes by macrophages in CLS has been demonstrated in vitro (11, 12, 16), as well as in obese adipose tissue (12). We observed lipid uptake from dead adipocytes by macrophages primed by obese or lean adipose tissue and found enhanced regulation of the efferocytic machinery in ATMs isolated from obese mice, confirming a role for efferocytosis in the adipose tissue, especially during obesity. Macrophages have been shown to start clearing adipocytes in CLS within an hour (12). Indeed, we found uptake of lipids from adipocytes by macrophages after a 4-hour co-culture. However, most likely complete adipocyte clearance is relatively slow compared to clearance of smaller apoptotic target cells (39), as suggested by a previous study (11) and corroborated by our in vitro transcriptional data revealing little regulation in macrophages after a 6-hour, but profound regulation of genes involved in lipid uptake and processing after a 24-hour co-culture with dead adipocytes.

Classically, efferocytosis is an immunologically silent process, driving the release of anti-inflammatory cytokines (3, 40, 41). Macrophages part of CLS in obese adipose tissue, however, display pro-inflammatory characteristics (9, 16, 18). Incomplete uptake of foreign bodies, sometimes referred to as ‘frustrated phagocytosis’, fuels inflammatory macrophage responses via the release of noxious content (3, 40, 42) and is most often seen when macrophages are confronted with a target much bigger than themselves (42). As such, one could think of a state of ‘frustrated efferocytosis’ in obese adipose tissue, in which adipocyte size is of particular relevance. On the other hand, one may speculate that the increased apoptotic adipocyte cargo in obese adipose tissue overwhelms macrophages. Naturally, processing dead cell material comes with high levels of metabolic stress. In case of adipocytes, macrophages need to deal with extremely high levels of lipids. Not surprisingly, we found enhanced expression of various genes involved in lipid processing in macrophages

co-cultured with apoptotic adipocytes. Importantly, L929-derived (Cntrl) macrophages responded to apoptotic adipocytes in a primarily immunologically silent manner. First, we could not detect any cytokines in the supernatants of control macrophages co-cultured with apoptotic adipocytes. Moreover, on gene expression level a rather pro-efferocytic inflammatory profile was detected, with increased expression of *Il-10* and *Tgf-β*; both well-known to be produced by macrophages during effective, immunologically silent clearance of dead cells (40, 41). Unexpectedly, however, *Tnf-α* was upregulated as well. Although TNF-α is generally considered to interfere with efferocytosis (43), its induction in macrophages co-cultured with dead adipocytes might hold relevance during adipocyte clearance specifically. This is, however, highly speculative and warrants further investigation. Intriguingly, compared to macrophages primed with lean adipose tissue, macrophages primed with obese adipose produced more IL-6, yet less TNF-a and IL-1ra in response to apoptotic adipocytes. This is the opposite of what we observed in control macrophages, suggestive of a disrupted response toward dead adipocytes by macrophages primed with obese versus lean adipose tissue. Although lipid uptake from apoptotic adipocytes was found to be equal in flow cytometry measurements, our finding of reduced expression of genes involved in lipid processing in macrophages primed with obese versus lean adipose tissue corroborates the hypothesis of disrupted responses toward adipocytes by obese adipose-primed macrophages. However, based on our results we are unable to draw any conclusions regarding causal relationships between lipid processing and inflammatory activation upon adipocyte clearance by macrophages.

Interestingly, our data show that priming of macrophages in an obese adipose tissue environment affects future responses toward dead adipocytes, suggestive of obesity-specific rewiring of macrophage pathways involved in efferocytosis of adipocytes. Interferon signalling appeared strongly depleted in macrophages in an obese versus lean adipose tissue environment. Because various IFN-related genes were upregulated during efferocytosis of adipocytes by control macrophages, we hypothesize that IFN signalling is important for effective clearance of dead adipocytes. However, several questions remain to be answered. For example, what drives IFN-related gene expression in macrophages co-cultured with apoptotic adipocytes? Is IFN signalling in macrophages functionally important during adipocyte clearance? If so, are Type I and Type II IFN signalling equally activated and do they both contribute to cellular clearance? Lastly, why is IFN-signalling downregulated in macrophages in an obese versus lean adipose tissue environment?

Initiation of Type I IFN signalling, among which IFN-α and IFN-β, upon recognition of exogenous DNA by innate immune cells is well-described and, together with the Type II interferon IFN-γ, mediates antiviral responses (44, 45). As such, DNA from apoptotic adipocytes engulfed by macrophages may drive expression of IFN-related genes. Although it is not unthinkable that IFN signalling has an exogenous origin, IFN-γ itself was not detectable in the supernatant of adipose tissue, nor in BMDM co-cultured with adipocytes or adipose

tissue.

Interestingly, IFNs may directly affect efferocytosis by macrophages; IFN-α has been shown to enhance phagocytosis of latex beads (34), and IFN-γ stimulates the uptake of apoptotic cells or pathogens (46-48). Not only the uptake itself, but also macrophage responses upon uptake may be affected by IFN signalling. For example, several anti-inflammatory properties have been attributed to Type I IFNs (49), and T-cell-derived IFN-γ has been shown to be involved in immunosuppressive responses of dendritic cells upon efferocytosis (50). Intriguingly, IFN signalling downstream the Type I IFN receptor IFNAR has been shown to enhance expression of the family of TAM receptor tyrosine kinases including Mer tyrosine kinase (MerTK), which, on their turn, reduce inflammatory cytokine production via IFN signalling involving STAT1 and SOCS1 and -3 (51). Hence, IFN signalling may on the one hand affect efferocytosis itself via regulation of its receptors and on the other hand shape inflammatory activation upon efferocytosis of apoptotic cells. As such, impaired IFN signalling in the obese state may be responsible for pro-inflammatory macrophage responses upon efferocytosis of apoptotic adipocytes. Noteworthy, IFNs also reprogram cellular metabolism, including fatty acid oxidation, and affect lipid uptake and processing (52), which on their turn regulate IFN signalling (53).

Hence, many relations between IFN signalling and lipid metabolism, as well as efficient, immunologically silent clearance of dead cells exist and may play a role in adipocyte clearance. Alternatively, given the crucial role for IFNs during macrophage development and functioning (54, 55), reduced IFN signalling might be an a-specific consequence of impaired macrophage functioning. Interestingly, administration of IFN-τ, a Type I IFN, during the development of diet-induced obesity reduced the presence of macrophages displaying classical inflammatory markers in the adipose tissue and could alleviate the development of insulin resistance in mice (56). On the other hand, reducing Type I IFN signalling in adipocytes by adiponectin-driven deletion of *Ifnar1* increased weight gain and impaired glucose tolerance in mice on a high-fat diet (57). Considering the tight interactions between adipocytes and macrophages, one could speculate that impaired IFN signalling and production by ATMs affects adipose tissue functioning via reducing IFN signalling in adipocytes. Future studies are warranted to elucidate the relevance of IFN signalling in macrophages and adipocytes part of obese adipose tissue. Moreover, the role of IFN signalling during efferocytosis, i.e. uptake and processing of adipocytes, and during the shaping of inflammatory responses upon efferocytosis remains to be elucidated, as well as the relevance for Type I versus Type II IFN signalling in ATMs.

Functional impairment of MerTK has been shown to fuel atherosclerosis in humans and mice (58, 59). MerTK recognizes phosphatidylserine on membranes of apoptotic cells by binding to bridging molecules like GAS6 and has been shown critically involved in efferocytosis and anti-inflammatory responses in macrophages (60, 61). Interestingly, despite a relatively low turnover rate of adipocytes – estimated to range from 10%-100% per year in lean individuals yet currently unknown for obese individuals (32) – MerTK inhibition by BMS-777607 (BMS) in

obese mice increased cell death markers in the inguinal adipose tissue (iWAT), and reduced IFN-signalling in gonadal adipose tissue (gWAT), both pointing to impaired clearance of dead adipocytes. Of note, as described above IFN signalling and TAM receptors functioning among which MerTK have shown to be intertwined (51), and inhibition of MerTK in BMS-treated mice might have directly affected IFN signalling.

Differences in adiposity and adipocyte turnover, and immune cell populations including macrophages in iWAT versus gWAT are well-characterized (62), and may explain the distinct transcriptional responses upon BMS-treatment in the different adipose tissue depots. For example, high rates of adipocyte cell death in gWAT (7) may have overruled small changes in adipocyte cell death markers upon treatment with BMS. Alternatively, variation in the inflammatory trait of macrophages that directly relates to their efferocytic capacity and MerTK expression (7, 11, 61) may have contributed to depot-specific differences. Methodologically, oral administration of BMS may have unequally reached the two adipose tissue depots as well.

Although we did observe changes in the adipose tissue transcriptome hinting toward reduced efferocytosis upon BMS treatment, inhibition of MerTK did not affect adipose tissue inflammation or plasma parameters in obese mice. Absence of strong phenotypical changes may relate to a fundamental role for efferocytosis in obese adipose tissue that would facilitate compensatory pathways to ensure adipocyte clearance in BMS-treated mice. Alternatively, classical efferocytosis might hold little relevance in the adipose tissue. Electron microscopy of adipose tissue from obese mice has shown extrusion of lipid droplets from dying adipocytes, taken up by macrophages nearby in CLS (11). Moreover, macrophages have been found to degrade lipids in extracellular acidic compartments at sites of contact with dying adipocytes in CLS (12). Both observations are clearly different from classical efferocytosis of dying cells and appear to depend on lipid catabolism rather than the classical phagocytic machinery. Our in vitro and in vivo observation of enhanced expression of genes involved in lipid processing but not those involved in classical efferocytosis is in line with these studies pointing to unique regulation for adipocyte clearance by ATMs, and may advocate against an important role for MerTK.

In conclusion, we reveal activation of the efferocytic machinery involving lipid processing in ATMs during obesity. In vitro we found disrupted responses toward apoptotic adipocytes when macrophages were primed with obese adipose tissue. Interestingly, this observation is suggestive of changes in the efferocytic response of ATMs irrespective of lipid load, since macrophages primed with obese or lean adipose tissue were exposed to equal numbers of adipocytes. In vivo, however, obesity-driven changes in adipocyte cargo, for example via increased adipocyte number and size (63, 64) or alterations in composition of lipids stored (65-67), may further impede adipocyte clearance by macrophages and thereby further contribute to inflammatory activation of ATMs. Future studies aimed at unravelling regulation of efferocytosis in obese versus lean adipose tissue including the IFN signalling

route are warranted and may provide new targets to improve macrophage functioning and abate adipose tissue inflammation during obesity.

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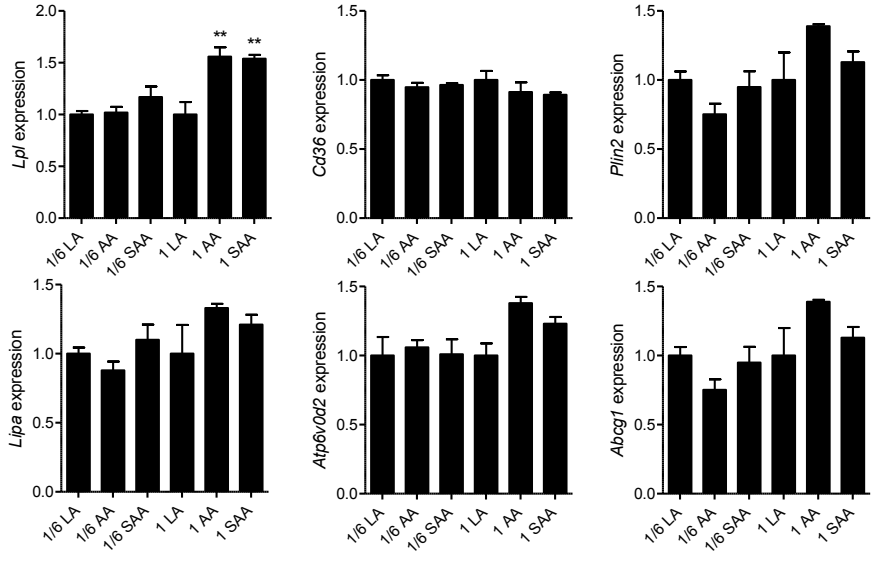
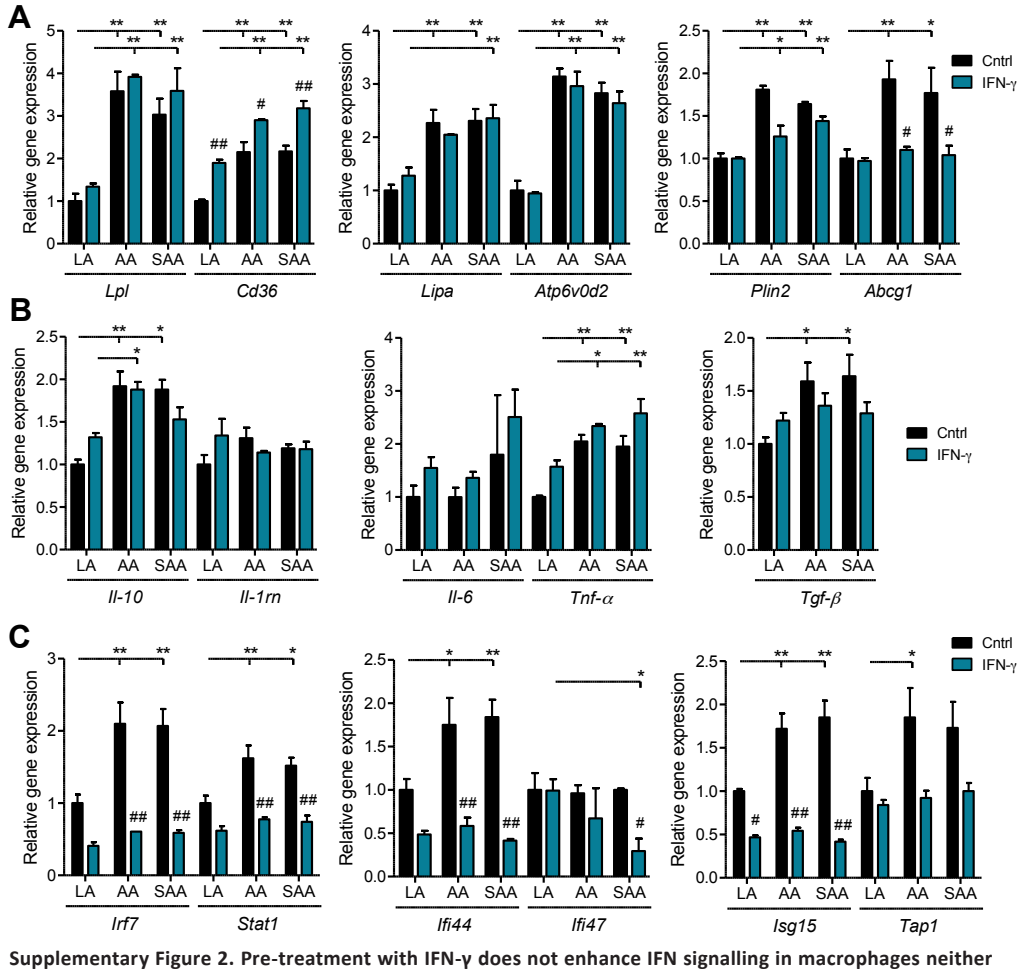
SUPPLEMENTARY FIGURES AND TABLES



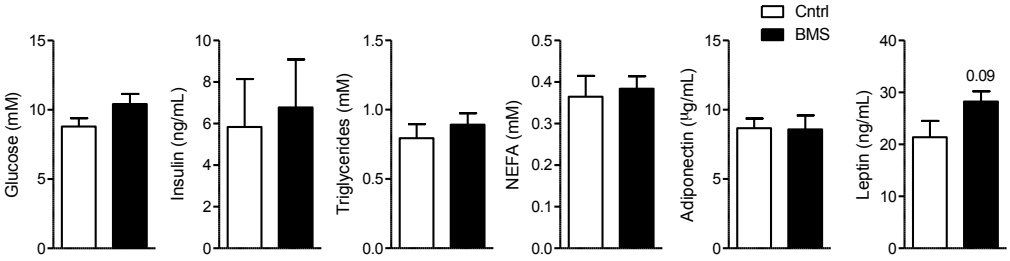
Supplementary Figure 1. No regulation of genes involved in lipid uptake and processing yet after a 6-hour co-culture with dead adipocytes.

Relative expression of several genes involved in lipid uptake and processing in BMDMs exposed to two concentrations of either live adipocytes (LA), apoptotic adipocytes (AA), or severe apoptotic adipocytes (SAA) (1x or 1/6x of the amount of macrophages) for 6 hours. Expression in macrophages co-cultured with LA was set to 1. Significance was calculated using one-way ANOVA with Bonferroni post-hoc tests (AA vs. LA and SAA vs. LA). Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$.



Supplementary Figure 2. Pre-treatment with IFN-γ does not enhance IFN signalling in macrophages neither affects expression of genes involved in adipocyte clearance.

Relative expression of genes involved in lipid processing (**A**), genes encoding cytokines (**B**), or genes involved in IFN signalling (**C**) measured in BMDMs co-cultured with live adipocytes (LA), apoptotic adipocytes (AA), or severe apoptotic adipocytes (SAA) for 24 hours. The BMDMs were either pre-treated with 10 ng/mL IFN-γ for 24 h (IFN-γ) or not (Cntrl). Expression in Cntrl BMDMs co-cultured with LA was set to 1. Significant differences in gene expression were calculated using two-way ANOVA with Bonferroni post-hoc test comparing pre-treatment (IFN-γ vs. cntrl) and response upon cell death (AA vs. LA and SAA vs. LA). Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$ (AA vs. LA or SAA vs. LA), or # $p < 0.05$, ## $p < 0.01$ (IFN-γ vs. Cntrl).



Supplementary Figure 3. Plasma parameters upon BMS-treatment in obese mice.
A Levels of glucose, insulin, triglycerides, non-esterfied fatty acids (NEFA), adiponectin and leptin measured in plasma of mice given BMS-777607 (BMS) or vehicle (Cntrl). Significance was calculated with student's *t-tests*. Data are shown as mean + SEM. * $p < 0.05$, ** $p < 0.01$.

Supplementary Table 1.
A List of significantly (p<0.01) enriched gene sets identified by Gene Set Enrichment Analysis using the complete transcriptome of BMDMs exposed to UV-irradiated obese adipose tissue (ObAT) versus BMDMs exposed to UV-irradiated lean adipose tissue for 3 days. Enriched gene sets in BMDMs exposed to UV-irradiated ObAT with a normalized enrichment score (NES)-score higher than 1.7 were included in the list. All significantly depleted gene sets in BMDMs exposed to UV-irradiated ObAT were included.

	Gene Set	NES
1	COLLAGEN.BIOSYNTHESIS.AND.MODIFYING.ENZYMES	2,6
2	EXTRACELLULAR.MATRIX.ORGANIZATION	2,58
3	COLLAGEN.FORMATION	2,57
4	ASSEMBLY.OF.COLLAGEN.FIBRILS.AND.OTHER.MULTIMERIC.STRUCTURES	2,5
5	INTEGRIN.CELL.SURFACE.INTERACTIONS	2,47
6	NRF2_TARGETS	2,44
7	KEGG_ECM.RECEPTOR.INTERACTION	2,38
8	KEGG_GLUTATHIONE.METABOLISM	2,3
9	WP2310.PODNET.PROTEIN.PROTEIN.INTERACTIONS.IN.THE.PODOCYTE	2,18
10	KEGG_FOCAL.ADHESION	2,17
11	WP412.OXIDATIVE.STRESS	2,17
12	WP85.FOCAL.ADHESION	2,16
13	ECM.PROTEOGLYCANS	2,15
14	DETOXIFICATION.OF.REACTIVE.OXYGEN.SPECIES	2,13
15	KEGG_PROTEOGLYCANS.IN.CANCER	2,12
16	SEMA4D.IN.SEMAPHORIN.SIGNALING	2,1
17	AMINO.ACID.TRANSPORT.ACROSS.THE.PLASMA.MEMBRANE	2,1
18	PPARA_TARGETS	2,1
19	EUKARYOTIC.TRANSLATION.ELONGATION	2,09
20	WP662.AMINO.ACID.METABOLISM	2,08
21	NON.INTEGRIN.MEMBRANE.ECM.INTERACTIONS	2,08
22	CAP.DEPENDENT.TRANSLATION.INITIATION	2,06
23	COMMON.PATHWAY	2,05
24	PHASE.II.CONJUGATION	2,04
25	TRANSLATION	2,04
26	EUKARYOTIC.TRANSLATION.INITIATION	2,04
27	KEGG_CHEMICAL.CARCINOGENESIS	2,04
28	BIOC_EDG1PATHWAY	2,03
29	PLATELET.DEGRANULATION	2,02
30	BIOC_INTRINSICPATHWAY	2,01
31	ELASTIC.FIBRE.FORMATION	2
32	NONSENSE.MEDIATED.DECAY.NMD.INDEPENDENT.OF.THE.EXON.JUNCTION.COMPLEX.EJC.	2
33	KEGG_TIGHT.JUNCTION	2
34	GTP.HYDROLYSIS.AND.JOINING.OF.THE.60S.RIBOSOMAL.SUBUNIT	2
35	WP164.GLUTATHIONE.METABOLISM	1,99
36	WP431.NUCLEAR.RECEPTORS.IN.LIPID.METABOLISM.AND.TOXICITY	1,98
37	SEMA4D.INDUCED.CELL.MIGRATION.AND.GROWTH.CONE.COLLAPSE	1,98
38	X3.UTR.MEDIATED.TRANSLATIONAL.REGULATION	1,97
39	KEGG_PROTEIN.DIGESTION.AND.ABSORPTION	1,97
40	WP318.EICOSANOID.SYNTHESIS	1,97
41	DEGRADATION.OF.THE.EXTRACELLULAR.MATRIX	1,96
42	IRE1ALPHA.ACTIVATES.CHAPERONES	1,96
43	L13A.MEDIATED.TRANSLATIONAL.SILENCING.OF.CERULOPLASMIN.EXPRESSION	1,96
44	XBP1.S.ACTIVATES.CHAPERONE.GENES	1,96
45	KEGG_DRUG.METABOLISM.CYTOCHROME.P450	1,96
46	RESPONSE.TO.ELEVATED.PLATELET.CYTOSOLIC.CA2.	1,96
47	EUKARYOTIC.TRANSLATION.TERMINATION	1,95
48	KEGG_ARRHYTHMOGENIC.RIGHT.VENTRICULAR.CARDIOMYOPATHY.ARVC.	1,95
49	PEPTIDE.CHAIN.ELONGATION	1,95
50	SIGNAL.TRANSDUCTION.BY.L1	1,94
51	WP1273.FOLIC.ACID.NETWORK	1,93
52	GLUTATHIONE.CONJUGATION	1,93
53	BIOLOGICAL.OXIDATIONS	1,93
54	SCAVENGING.BY.CLASS.A.RECEPTORS	1,93
55	KEGG_METABOLISM.OF.XENOBIOTICS.BY.CYTOCHROME.P450	1,92
56	COLLAGEN.DEGRADATION	1,91
57	BINDING.AND.UPTAKE.OF.LIGANDS.BY.SCAVENGER.RECEPTORS	1,9
58	KEGG_ADHERENS.JUNCTION	1,9
59	ACTIVATION.OF.THE.MRNA.UPON.BINDING.OF.THE.CAP.BINDING.COMPLEX.AND.EIFS.AND.SUBSEQUENT.BINDING.TO.43S	1,9
60	SYNDECAN.INTERACTIONS	1,89
61	WP2841.FOCAL.ADHESION.PI3K.AKT.MTOR.SIGNALING.PATHWAY	1,89
62	FORMATION.OF.A.POOL.OF.FREE.40S.SUBUNITS	1,89

63	RIBOSOMAL.SCANNING.AND.START.CODON.RECOGNITION	1,88
64	REGULATION.OF.LIPID.METABOLISM.BY.PEROXISOME.PROLIFERATOR.ACTIVATED.RECEPTOR.ALPHA.PPARALPHA.	1,87
65	KEGG_HIPPO.SIGNALING.PATHWAY	1,87
66	PLCG1.EVENTS.IN.ERBB2.SIGNALING	1,87
67	CHONDROITIN.SULFATE.BIOSYNTHESIS	1,87
68	O.GLYCOSYLATION.OF.TSR.DOMAIN.CONTAINING.PROTEINS	1,87
69	CELL.JUNCTION.ORGANIZATION	1,86
70	VIRAL.MRNA.TRANSLATION	1,86
71	PPARA.ACTIVATES.GENE.EXPRESSION	1,85
72	SMOOTH.MUSCLE.CONTRACTION	1,85
73	FORMATION.OF.TUBULIN.FOLDING.INTERMEDIATES.BY.CCT.TRIC	1,84
74	TRANSLATION.INITIATION.COMPLEX.FORMATION	1,84
75	KEGG_HYPERTROPHIC.CARDIOMYOPATHY.HCM.	1,84
76	PLC.GAMMA1.SIGNALLING	1,83
77	KEGG_GAP.JUNCTION	1,83
78	KEGG_RAP1.SIGNALING.PATHWAY	1,83
79	ABC.FAMILY.PROTEINS.MEDIATED.TRANSPORT	1,83
80	FORMATION.OF.THE.TERNARY.COMPLEX.AND.SUBSEQUENTLY.THE.43S.COMPLEX	1,83
81	PHOSPHOLIPASE.C.MEDIATED.CASCADE	1,83
82	WP339.ESC.PLURIPOTENCY.PATHWAYS	1,81
83	YAP1.AND.WWTR1.TAZ.STIMULATED.GENE.EXPRESSION	1,81
84	EGFR.INTERACTS.WITH.PHOSPHOLIPASE.C.GAMMA	1,8
85	SIGNALING.BY.PDGF	1,8
86	BIOC_P53HYPOXIAPATHWAY	1,79
87	SMAD2.3.PHOSPHORYLATION.MOTIF.MUTANTS.IN.CANCER	1,79
88	KEGG_ALANINE.ASPARTATE.AND.GLUTAMATE.METABOLISM	1,79
89	NCAM1.INTERACTIONS	1,79
90	WP202.HYPERTROPHY.MODEL	1,79
91	CELL.EXTRACELLULAR.MATRIX.INTERACTIONS	1,79
92	CELL.SURFACE.INTERACTIONS.AT.THE.VASCULAR.WALL	1,77
93	SIGNALING.BY.HIPPO	1,77
94	LAMININ.INTERACTIONS	1,77
95	FATTY.ACID.TRIACYLGLYCEROL.AND.KETONE.BODY.METABOLISM	1,76
96	KEGG_LEUKOCYTE.TRANSENDOTHELIAL.MIGRATION	1,76
97	GLYCOLYSIS	1,76
98	SIGNALING.BY.TGF.BETA.RECEPTOR.COMPLEX	1,76
99	TGFBR2.KINASE.DOMAIN.MUTANTS.IN.CANCER	1,76
100	COOPERATION.OF.PREFOLDIN.AND.TRIC.CCT.IN.ACTIN.AND.TUBULIN.FOLDING	1,76
101	SRP.DEPENDENT.COTRANSLATIONAL.PROTEIN.TARGETING.TO.MEMBRANE	1,76
102	LOSS.OF.FUNCTION.OF.TGFBR1.IN.CANCER	1,76
103	SIGNALING.BY.FGFR	1,75
104	PLC.BETA.MEDIATED.EVENTS	1,75
105	LOSS.OF.FUNCTION.OF.SMAD4.IN.CANCER	1,75
106	SIGNALING.BY.TGF.BETA.RECEPTOR.COMPLEX.IN.CANCER	1,75
107	TGFBR1.KD.MUTANTS.IN.CANCER	1,75
108	LOSS.OF.FUNCTION.OF.SMAD2.3.IN.CANCER	1,75
109	KEGG_PENTOSE.PHOSPHATE.PATHWAY	1,75
110	PREFOLDIN.MEDIATED.TRANSFER.OF.SUBSTRATE.TO.CCT.TRIC	1,75
111	WP488.ALPHA6.BETA4.INTEGRIN.SIGNALING.PATHWAY	1,74
112	ARACHIDONIC.ACID.METABOLISM	1,74
113	NONSENSE.MEDIATED.DECAY.NMD.ENHANCED.BY.THE.EXON.JUNCTION.COMPLEX.EJC.	1,74
114	SMAD4.MH2.DOMAIN.MUTANTS.IN.CANCER	1,74
115	G.PROTEIN.MEDIATED.EVENTS	1,74
116	KEGG_PI3K.AKT.SIGNALING.PATHWAY	1,74
117	LOSS.OF.FUNCTION.OF.TGFBR2.IN.CANCER	1,74
118	TGFBR2.MSI.FRAMESHIFT.MUTANTS.IN.CANCER	1,74
119	KEGG_BIOSYNTHESIS.OF.UNSATURATED.FATTY.ACIDS	1,74
120	LIPID.DIGESTION.MOBILIZATION.AND.TRANSPORT	1,73
121	DAG.AND.IP3.SIGNALING	1,73
122	CA2.PATHWAY	1,73
123	NONSENSE.MEDIATED.DECAY.NMD.	1,73
124	ACTIVATION.OF.MATRIX.METALLOPROTEINASES	1,73
125	SIGNALING.BY.ERBB2	1,72
126	TGFBR1.LBD.MUTANTS.IN.CANCER	1,72
127	SMAD2.3.MH2.DOMAIN.MUTANTS.IN.CANCER	1,72
128	DOWNSTREAM.SIGNALING.OF.ACTIVATED.FGFR	1,71
129	WP79.TRYPTOPHAN.METABOLISM	1,71
130	WP1251.METAPATHWAY.BIOTRANSFORMATION	1,7
131	LIPOPROTEIN.METABOLISM	1,7

Supplementary Table 1.
B List of significantly (p<0.01) depleted gene sets identified by Gene Set Enrichment Analysis using the complete transcriptome of BMDMs exposed to UV-irradiated obese adipose tissue (ObAT) versus BMDMs exposed to UV-irradiated lean adipose tissue for 3 days. Enriched gene sets in BMDMs exposed to UV-irradiated ObAT with a normalized enrichment score (NES)-score higher than 1.7 were included in the list. All significantly depleted gene sets in BMDMs exposed to UV-irradiated ObAT were included.

	Gene Set	NES
1	MITOTIC.PROMETAPHASE	-2,77
2	RESOLUTION.OF.SISTER.CHROMATID.COHESION	-2,75
3	INTERFERON.ALPHA.BETA.SIGNALING	-2,72
4	KEGG_DNA.REPLICATION	-2,53
5	WP150.DNA.REPLICATION	-2,52
6	MITOTIC.M.M.G1.PHASES	-2,4
7	SEPARATION.OF.SISTER.CHROMATIDS	-2,37
8	MITOTIC.METAPHASE.AND.ANAPHASE	-2,36
9	MITOTIC.ANAPHASE	-2,36
10	G2.M.CHECKPOINTS	-2,34
11	NUCLEOSOME.ASSEMBLY	-2,34
12	CELL.CYCLE.MITOTIC	-2,33
13	DEPOSITION.OF.NEW.CENPA.CONTAINING.NUCLEOSOMES.AT.THE.CENTROMERE	-2,31
14	ACTIVATION.OF.THE.PRE.REPLICATIVE.COMPLEX	-2,29
15	INTERFERON.SIGNALING	-2,28
16	CELL.CYCLE	-2,28
17	CHROMOSOME.MAINTENANCE	-2,25
18	M.PHASE	-2,2
19	KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS	-2,18
20	KEGG_FANCONI.ANEMIA.PATHWAY	-2,18
21	DNA.STRAND.ELONGATION	-2,17
22	ACTIVATION.OF.ATR.IN.RESPONSE.TO.REPLICATION.STRESS	-2,17
23	MEIOTIC.SYNAPSIS	-2,15
24	INTERFERON.GAMMA.SIGNALING	-2,15
25	MEIOSIS	-2,13
26	MITOTIC.G1.G1.S.PHASES	-2,13
27	KEGG_HOMOLOGOUS.RECOMBINATION	-2,11
28	THE.ROLE.OF.NEF.IN.HIV.1.REPLICATION.AND.DISEASE.PATHOGENESIS	-2,1
29	DNA.REPLICATION	-2,1
30	BIOC_IL7PATHWAY	-2,08
31	E2F.MEDIATED.REGULATION.OF.DNA.REPLICATION	-2,06
32	S.PHASE	-2,05
33	KEGG_MISMATCH.REPAIR	-2,04
34	KEGG_MEASLES	-2,04
35	WP571.FAS.PATHWAY.AND.STRESS.INDUCTION.OF.HSP.REGULATION	-2,03
36	STING.MEDIATED.INDUCTION.OF.HOST.IMMUNE.RESPONSES	-2,02
37	WP413.G1.TO.S.CELL.CYCLE.CONTROL	-2,02
38	CONDENSATION.OF.PROPHASE.CHROMOSOMES	-2,01
39	WP450.IL.2.SIGNALING.PATHWAY	-2
40	MEIOTIC.RECOMBINATION	-2
41	DNA.DAMAGE.TELOMERE.STRESS.INDUCED.SENESCENCE	-1,99
42	G0.AND.EARLY.G1	-1,99
43	WP190.CELL.CYCLE	-1,99
44	CYTOKINE.SIGNALING.IN.IMMUNE.SYSTEM	-1,98
45	G1.S.TRANSITION	-1,98
46	G1.S.SPECIFIC.TRANSCRIPTION	-1,98
47	KEGG_CELL.CYCLE	-1,97
48	KEGG_INFLUENZA.A	-1,97
49	WP1253.TYPE.II.INTERFERON.SIGNALING.IFNG.	-1,97
50	TELOMERE.MAINTENANCE	-1,96
51	KEGG_ALLOGRAFT.REJECTION	-1,96
52	SYNTHESIS.OF.DNA	-1,96
53	IRF3.MEDIATED.INDUCTION.OF.TYPE.I.IFN	-1,94
54	M.G1.TRANSITION	-1,91
55	DNA.REPLICATION.PRE.INITIATION	-1,9
56	KEGG_PRIMARY.IMMUNODEFICIENCY	-1,9
57	PRC2.METHYLATES.HISTONES.AND.DNA	-1,89
58	CELL.CYCLE.CHECKPOINTS	-1,88
59	NEF.MEDIATES.DOWN.MODULATION.OF.CELL.SURFACE.RECEPTORS.BY.RECRUITING.THEM.TO.CLATHRIN.ADAPTERS	-1,88
60	FANCONI.ANEMIA.PATHWAY	-1,87
61	WP2087.MIRNA.REGULATION.OF.DNA.DAMAGE.RESPONSE	-1,86
62	BIOC_ATRBRCAPATHWAY	-1,86

63	KEGG_VIRAL.CARCINOGENESIS	-1,85
64	WP1271.TOLL.LIKE.RECEPTOR.SIGNALING.PATHWAY	-1,85
65	KEGG_HERPES.SIMPLEX.INFECTION	-1,83
66	NEF.MEDIATED.DOWNREGULATION.OF.MHC.CLASS.I.COMPLEX.CELL.SURFACE.EXPRESSION	-1,83
67	KEGG_ANTIGEN.PROCESSING.AND.PRESENTATION	-1,82
68	TELOMERE.C.STRAND.LAGGING.STRAND.SYNTHESIS	-1,82
69	ENDOSOMAL.VACUOLAR.PATHWAY	-1,82
70	PACKAGING.OF.TELOMERE.ENDS	-1,81
71	RNA.POLYMERASE.I.PROMOTER.OPENING	-1,81
72	KEGG_GRAFT.VERSUS.HOST.DISEASE	-1,81
73	KEGG_SYNAPTIC.VESICLE.CYCLE	-1,81
74	SIGNALING.BY.RHO.GTPASES	-1,8
75	BIOC_G1PATHWAY	-1,8
76	LYSOSOME.VESICLE.BIOGENESIS	-1,79
77	RHO.GTPASE.CYCLE	-1,79
78	KEGG_AUTOIMMUNE.THYROID.DISEASE	-1,79
79	KEGG_TOLL.LIKE.RECEPTOR.SIGNALING.PATHWAY	-1,79
80	NEGATIVE.REGULATORS.OF.RIG.I.MDA5.SIGNALING	-1,79
81	DNA.METHYLATION	-1,76
82	KEGG_VIRAL.MYOCARDITIS	-1,75
83	BIOC_TNFR1PATHWAY	-1,75
84	KINESINS	-1,74
85	BIOC_IL12PATHWAY	-1,73
86	CLATHRIN.DERIVED.VESICLE.BUDDING	-1,72
87	KEGG_GLYCOSAMINOGLYCAN.DEGRADATION	-1,71
88	TRANS.GOLGI.NETWORK.VESICLE.BUDDING	-1,7
89	TRAF6.MEDIATED.IRF7.ACTIVATION	-1,7
90	BIOC_CELLCYCLEPATHWAY	-1,7
91	INSULIN.RECEPTOR.RECYCLING	-1,7
92	KEGG_LYSOSOME	-1,67
93	RIG.I.MDA5.MEDIATED.INDUCTION.OF.IFN.ALPHA.BETA.PATHWAYS	-1,67
94	KEGG_TYPE.I.DIABETES.MELLITUS	-1,67
95	EXTENSION.OF.TELOMERES	-1,67
96	KEGG_CYTOSOLIC.DNA.SENSING.PATHWAY	-1,65
97	KEGG_OXIDATIVE.PHOSPHORYLATION	-1,65
98	KERATAN.SULFATE.KERATIN.METABOLISM	-1,65
99	WP297.IL.7.SIGNALING.PATHWAY	-1,63
100	KEGG_NATURAL.KILLER.CELL.MEDIATED.CYTOTOXICITY	-1,62
101	KEGG_B.CELL.RECEPTOR.SIGNALING.PATHWAY	-1,62
102	KEGG_APOPTOSIS	-1,61
103	KEGG_BASE.EXCISION.REPAIR	-1,61
104	KEGG_T.CELL.RECEPTOR.SIGNALING.PATHWAY	-1,6
105	KEGG_HTLV.I.INFECTION	-1,59
106	HATS.ACETYLATE.HISTONES	-1,59
107	REGULATION.OF.DNA.REPLICATION	-1,59
108	SENESCENCE.ASSOCIATED.SECRETORY.PHENOTYPE.SASP.	-1,59
109	KEGG_HEPATITIS.B	-1,59
110	CYTOSOLIC.SENSORS.OF.PATHOGEN.ASSOCIATED.DNA	-1,58
111	KEGG_OOCYTE.MEIOSIS	-1,57
112	REMOVAL.OF.LICENSING.FACTORS.FROM.ORIGINS	-1,57
113	WP274.B.CELL.RECEPTOR.SIGNALING.PATHWAY	-1,54
114	WP480.T.CELL.RECEPTOR.SIGNALING.PATHWAY	-1,53
115	WP1248.OXIDATIVE.PHOSPHORYLATION	-1,53
116	RESPIRATORY.ELECTRON.TRANSPORT	-1,53
117	KEGG_JAK.STAT.SIGNALING.PATHWAY	-1,52
118	CELLULAR.SENESCENCE	-1,5
119	KEGG_HEMATOPOIETIC.CELL.LINEAGE	-1,5
120	HOST.INTERACTIONS.OF.HIV.FACTORS	-1,49
121	WP295.ELECTRON.TRANSPORT.CHAIN	-1,49
122	KEGG_ALZHEIMER.S.DISEASE	-1,48
123	KEGG_TUBERCULOSIS	-1,47
124	DNA.REPAIR	-1,47
125	KEGG_RIG.I.LIKE.RECEPTOR.SIGNALING.PATHWAY	-1,46
126	ION.CHANNEL.TRANSPORT	-1,42
127	KEGG_TRANSCRIPTIONAL.MISREGULATION.IN.CANCER	-1,39
128	WP2292.CHEMOKINE.SIGNALING.PATHWAY	-1,36

Supplementary Table 2.
A List of significantly (p<0.01) enriched gene sets identified by Gene Set Enrichment Analysis using the complete transcriptome of BMDMs exposed to UV-irradiated lean adipose tissue (LeAT) versus BMDMs exposed to non-treated LeAT for 3 days. Enriched gene sets in BMDMs exposed to UV-irradiated LeAT with a normalized enrichment score (NES)-score higher than 1.7 were included in the list. All significantly depleted gene sets in BMDMs exposed to UV-irradiated LeAT were included.

	Gene Set	NES
1	KEGG_ADHERENS.JUNCTION	2,31
2	KEGG_HIPPO.SIGNALING.PATHWAY	2,18
3	WP150.DNA.REPLICATION	2,17
4	WP413.G1.TO.S.CELL.CYCLE.CONTROL	2,17
5	KEGG_TGF.BETA.SIGNALING.PATHWAY	2,17
6	DNA.STRAND.ELONGATION	2,13
7	METABOLISM.OF.NUCLEOTIDES	2,13
8	EXTENSION.OF.TELOMERES	2,13
9	MISMATCH.REPAIR	2,1
10	FORMATION.OF.TUBULIN.FOLDING.INTERMEDIATES.BY.CCT.TRIC	2,07
11	CYTOSOLIC.TRNA.AMINOACYLATION	2,06
12	ACTIVATION.OF.THE.PRE.REPLICATIVE.COMPLEX	2,06
13	METABOLISM.OF.NON.CODING.RNA	2,05
14	SNRNP.ASSEMBLY	2,05
15	MITOTIC.G1.G1.S.PHASES	2,05
16	SIGNALING.BY.HIPPO	2,05
17	WP2310.PODNET.PROTEIN.PROTEIN.INTERACTIONS.IN.THE.PODOCYTE	2,04
18	KEGG_GAP.JUNCTION	2,04
19	PYRIMIDINE.METABOLISM	2,04
20	KEGG_TIGHT.JUNCTION	2,03
21	WP113.TGF.BETA.SIGNALING.PATHWAY	2,03
22	CHONDROITIN.SULFATE.BIOSYNTHESIS	2,03
23	KEGG_PYRIMIDINE.METABOLISM	2,02
24	BASIGIN.INTERACTIONS	2,02
25	KEGG_RIBOSOME.BIOGENESIS.IN.EUKARYOTES	2,01
26	KEGG_DNA.REPLICATION	2
27	DOUBLE.STRAND.BREAK.REPAIR	2
28	SIGNALING.BY.BMP	1,99
29	WP512.ID.SIGNALING.PATHWAY	1,98
30	TRANSCRIPTION.COUPLED.NER.TC.NER.	1,97
31	WP1270.ENDOCHONDRAL.OSSIFICATION	1,97
32	KEGG_AXON.GUIDANCE	1,96
33	PREFOLDIN.MEDIATED.TRANSFER.OF.SUBSTRATE.TO.CCT.TRIC	1,96
34	KEGG_SPLICEOSOME	1,96
35	MRNA.SPLICING.MINOR.PATHWAY	1,95
36	GAP.FILLING.DNA.REPAIR.SYNTHESIS.AND.LIGATION.IN.TC.NER	1,95
37	KEGG_RAP1.SIGNALING.PATHWAY	1,95
38	KEGG_MISMATCH.REPAIR	1,95
39	MITOCHONDRIAL.PROTEIN.IMPORT	1,94
40	COOPERATION.OF.PREFOLDIN.AND.TRIC.CCT.IN.ACTIN.AND.TUBULIN.FOLDING	1,94
41	KEGG_RNA.TRANSPORT	1,93
42	INTERACTIONS.OF.VPR.WITH.HOST.CELLULAR.PROTEINS	1,93
43	GAP.FILLING.DNA.REPAIR.SYNTHESIS.AND.LIGATION.IN.GG.NER	1,92
44	G1.PHASE	1,91
45	CHOLESTEROL.BIOSYNTHESIS	1,9
46	CYCLIN.D.ASSOCIATED.EVENTS.IN.G1	1,9
47	WP190.CELL.CYCLE	1,89
48	PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA	1,89
49	CELL.EXTRACELLULAR.MATRIX.INTERACTIONS	1,89
50	NON.INTEGRIN.MEMBRANE.ECM.INTERACTIONS	1,88
51	VPR.MEDIATED.NUCLEAR.IMPORT.OF.PICS	1,88
52	TELOMERE.C.STRAND.LAGGING.STRAND.SYNTHESIS	1,87
53	YAP1.AND.WWTR1.TAZ.STIMULATED.GENE.EXPRESSION	1,87
54	BIOC_NOS1PATHWAY	1,87
55	ACTIVATION.OF.ATR.IN.RESPONSE.TO.REPLICATION.STRESS	1,87
56	HOMOLOGOUS.RECOMBINATION.REPAIR	1,86
57	KEGG_BIOSYNTHESIS.OF.UNSATURATED.FATTY.ACIDS	1,86
58	HIV.LIFE.CYCLE	1,85
59	EXTRACELLULAR.MATRIX.ORGANIZATION	1,85
60	KEGG_ARRHYTHMOGENIC.RIGHT.VENTRICULAR.CARDIOMYOPATHY.ARVC.	1,85
61	INTERACTIONS.OF.REV.WITH.HOST.CELLULAR.PROTEINS	1,85
62	LAGGING.STRAND.SYNTHESIS	1,85

63	KEGG_STEROID.BIOSYNTHESIS	1,83
64	CELL.JUNCTION.ORGANIZATION	1,82
65	G2.M.CHECKPOINTS	1,82
66	G1.S.TRANSITION	1,82
67	HOMOLOGOUS.RECOMBINATION.REPAIR.OF.REPLICATION.INDEPENDENT.DOUBLE.STRAND.BREAKS	1,82
68	KSRP.DESTABILIZES.MRNA	1,82
69	E2F.MEDIATED.REGULATION.OF.DNA.REPLICATION	1,82
70	SEMAPHORIN.INTERACTIONS	1,81
71	SYNTHESIS.AND.INTERCONVERSION.OF.NUCLEOTIDE.DI.AND.TRIPHOSPHATES	1,81
72	DARPP.32.EVENTS	1,81
73	RNA.POLYMERASE.I.TRANSCRIPTION.TERMINATION	1,81
74	NUCLEAR.IMPORT.OF.REV.PROTEIN	1,8
75	WP1983.SPLICING.FACTOR.NOVA.REGULATED.SYNPATIC.PROTEINS	1,8
76	CHONDROITIN.SULFATE.DERMATAN.SULFATE.METABOLISM	1,8
77	SYNDECAN.INTERACTIONS	1,8
78	BIOC_CELLCYCLEPATHWAY	1,8
79	SIGNAL.TRANSDUCTION.BY.L1	1,79
80	DNA.REPAIR	1,78
81	KEGG_CELL.CYCLE	1,78
82	REGULATION.OF.CHOLESTEROL.BIOSYNTHESIS.BY.SREBP.SREBF.	1,78
83	WP1268.DIURNALLY.REGULATED.GENES.WITH.CIRCADIAN.ORTHOLOGS	1,78
84	TRNA.AMINOACYLATION	1,77
85	RNA.POLYMERASE.III.TRANSCRIPTION	1,77
86	NUCLEOTIDE.EXCISION.REPAIR	1,76
87	RNA.POLYMERASE.III.ABORTIVE.AND.RETRACTIVE.INITIATION	1,76
88	MRNA.SPLICING.MAJOR.PATHWAY	1,76
89	RNA.POLYMERASE.I.TRANSCRIPTION.INITIATION	1,76
90	CELL.CELL.COMMUNICATION	1,75
91	WP523.REGULATION.OF.ACTIN.CYTOSKELETON	1,75
92	BIOC_GHPATHWAY	1,75
93	S.PHASE	1,75
94	ACTIVATION.OF.GENE.EXPRESSION.BY.SREBF.SREBP.	1,75
95	CELL.CELL.JUNCTION.ORGANIZATION	1,75
96	ADRENALINE.NORADRENALINE.INHIBITS.INSULIN.SECRETION	1,75
97	KEGG_FOCAL.ADHESION	1,74
98	SMAD4.MH2.DOMAIN.MUTANTS.IN.CANCER	1,74
99	MRNA.SPLICING	1,74
100	LOSS.OF.FUNCTION.OF.SMAD4.IN.CANCER	1,74
101	TGFBR2.KINASE.DOMAIN.MUTANTS.IN.CANCER	1,74
102	TRANSPORT.OF.MATURE.TRANSCRIPT.TO.CYTOPLASM	1,74
103	TGFBR1.LBD.MUTANTS.IN.CANCER	1,74
104	SIGNALING.BY.TGF.BETA.RECEPTOR.COMPLEX	1,73
105	LOSS.OF.FUNCTION.OF.TGFBR2.IN.CANCER	1,73
106	LOSS.OF.FUNCTION.OF.SMAD2.3.IN.CANCER	1,73
107	SIGNALING.BY.TGF.BETA.RECEPTOR.COMPLEX.IN.CANCER	1,73
108	SEMA4D.IN.SEMAPHORIN.SIGNALING	1,73
109	REV.MEDIATED.NUCLEAR.EXPORT.OF.HIV.RNA	1,73
110	WP2185.PURINE.METABOLISM	1,72
111	LOSS.OF.FUNCTION.OF.TGFBR1.IN.CANCER	1,72
112	SMAD2.3.PHOSPHORYLATION.MOTIF.MUTANTS.IN.CANCER	1,72
113	ABORTIVE.ELONGATION.OF.HIV.1.TRANSCRIPT.IN.THE.ABSENCE.OF.TAT	1,72
114	A.TETRASACCHARIDE.LINKER.SEQUENCE.IS.REQUIRED.FOR.GAG.SYNTHESIS	1,72
115	TGFBR2.MSI.FRAMESHIFT.MUTANTS.IN.CANCER	1,71
116	OPIOID.SIGNALLING	1,71
117	KEGG_SIGNALING.PATHWAYS.REGULATING.PLURIPOTENCY.OF.STEM.CELLS	1,71
118	VIRAL.MESSENGER.RNA.SYNTHESIS	1,71
119	BASE.EXCISION.REPAIR	1,71
120	RNA.POLYMERASE.I.PROMOTER.ESCAPE	1,71
121	TRANSPORT.OF.THE.SLBP.DEPENDANT.MATURE.MRNA	1,71
122	WP258.TGF.BETA.RECEPTOR.SIGNALING.PATHWAY	1,7
123	SMAD2.3.MH2.DOMAIN.MUTANTS.IN.CANCER	1,7
124	KEGG_NUCLEOTIDE.EXCISION.REPAIR	1,7
125	WP509.NUCLEAR.RECEPTORS	1,7

Supplementary Table 2.
B List of significantly (p<0.01) depleted gene sets identified by Gene Set Enrichment Analysis using the complete transcriptome of BMDMs exposed to UV-irradiated lean adipose tissue (LeAT) versus BMDMs exposed to non-treated LeAT for 3 days. Enriched gene sets in BMDMs exposed to UV-irradiated LeAT with a normalized enrichment score (NES)-score higher than 1.7 were included in the list. All significantly depleted gene sets in BMDMs exposed to UV-irradiated LeAT were included.

	Gene Set	NES
1	KEGG_SYNAPTIC.VESICLE.CYCLE	-1,99
2	KEGG_OTHER.GLYCAN.DEGRADATION	-1,93
3	BIOC_DCPATHWAY	-1,88
4	KEGG_LYSOSOME	-1,88
5	HDMS.DEMETHYLATE.HISTONES	-1,82
6	KEGG_STAPHYLOCOCCUS.AUREUS.INFECTION	-1,8
7	IMMUNOREGULATORY.INTERACTIONS.BETWEEN.A.LYMPHOID.AND.A.NON.LYMPHOID.CELL	-1,73
8	KEGG_FRUCTOSE.AND.MANNOSE.METABOLISM	-1,72
9	TOLL.LIKE.RECEPTORS.CASCADES	-1,6
10	TOLL.LIKE.RECEPTOR.4.TLR4.CASCADE	-1,52
11	PEROXISOMAL.LIPID.METABOLISM	-1,79
12	KEGG_NF.KAPPA.B.SIGNALING.PATHWAY	-1,56
13	WP1271.TOLL.LIKE.RECEPTOR.SIGNALING.PATHWAY	-1,54
14	KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS	-1,5
15	WP480.T.CELL.RECEPTOR.SIGNALING.PATHWAY	-1,49
16	INSULIN.RECEPTOR.RECYCLING	-1,78
17	KEGG_SNARE.INTERACTIONS.IN.VESICULAR.TRANSPORT	-1,7
18	LATENT.INFECTION.OF.HOMO.SAPIENS.WITH.MYCOBACTERIUM.TUBERCULOSIS	-1,67
19	RIG.I.MDA5.MEDIATED.INDUCTION.OF.IFN.ALPHA.BETA.PATHWAYS	-1,49
20	PHAGOSOMAL.MATURATION.EARLY.ENDOSOMAL.STAGE.	-1,64

Supplementary Table 3.
A List of significantly (p<0.01) enriched gene sets identified by Gene Set Enrichment Analysis using the complete transcriptome of BMDMs exposed to UV-irradiated obese adipose tissue (ObAT) versus BMDMs exposed to non-treated ObAT for 3 days. Enriched gene sets in BMDMs exposed to UV-irradiated ObAT with a normalized enrichment score (NES)-score higher than 2 were included in the list. All significantly depleted gene sets in BMDMs exposed to UV-irradiated ObAT were included.

	Gene Set	NES
1	CELL.CYCLE	2,96
2	CELL.CYCLE.MITOTIC	2,96
3	MITOTIC.G1.G1.S.PHASES	2,89
4	KEGG_RIBOSOME.BIOGENESIS.IN.EUKARYOTES	2,82
5	MITOTIC.M.M.G1.PHASES	2,82
6	KEGG_RNA.TRANSPORT	2,75
7	G1.S.TRANSITION	2,72
8	WP413.G1.TO.S.CELL.CYCLE.CONTROL	2,71
9	TRANSPORT.OF.MATURE.MRNA.DERIVED.FROM.AN.INTRON.CONTAINING.TRANSCRIPT	2,7
10	M.G1.TRANSITION	2,68
11	MITOTIC.PROMETAPHASE	2,68
12	DNA.REPLICATION.PRE.INITIATION	2,67
13	CELL.CYCLE.CHECKPOINTS	2,67
14	S.PHASE	2,65
15	MITOTIC.ANAPHASE	2,65
16	G2.M.CHECKPOINTS	2,65
17	MITOTIC.METAPHASE.AND.ANAPHASE	2,65
18	INTERACTIONS.OF.REV.WITH.HOST.CELLULAR.PROTEINS	2,64
19	RESOLUTION.OF.SISTER.CHROMATID.COHESION	2,64
20	DNA.REPLICATION	2,64
21	SEPARATION.OF.SISTER.CHROMATIDS	2,63
22	PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA	2,62
23	TRANSPORT.OF.MATURE.TRANSCRIPT.TO.CYTOPLASM	2,61
24	M.PHASE	2,59
25	SNRNP.ASSEMBLY	2,59
26	ACTIVATION.OF.ATR.IN.RESPONSE.TO.REPLICATION.STRESS	2,57
27	WP190.CELL.CYCLE	2,57
28	NUCLEAR.IMPORT.OF.REV.PROTEIN	2,56
29	METABOLISM.OF.NON.CODING.RNA	2,56
30	KEGG_SPLICEOSOME	2,55
31	METABOLISM.OF.RNA	2,55
32	ASSEMBLY.OF.THE.PRE.REPLICATIVE.COMPLEX	2,55
33	REV.MEDIATED.NUCLEAR.EXPORT.OF.HIV.RNA	2,54
34	SYNTHESIS.OF.DNA	2,51
35	MITOTIC.G2.G2.M.PHASES	2,5
36	NEP.NS2.INTERACTS.WITH.THE.CELLULAR.EXPORT.MACHINERY	2,47
37	EXPORT.OF.VIRAL.RIBONUCLEOPROTEINS.FROM.NUCLEUS	2,47
38	ACTIVATION.OF.THE.PRE.REPLICATIVE.COMPLEX	2,47
39	G2.M.TRANSITION	2,47
40	TRANSPORT.OF.RIBONUCLEOPROTEINS.INTO.THE.HOST.NUCLEUS	2,45
41	TRNA.AMINOACYLATION	2,45
42	WP150.DNA.REPLICATION	2,45
43	RECRUITMENT.OF.MITOTIC.CENTROSOME.PROTEINS.AND.COMPLEXES	2,45
44	VPR.MEDIATED.NUCLEAR.IMPORT.OF.PICS	2,44
45	TRANSPORT.OF.MATURE.MRNAS.DERIVED.FROM.INTRONLESS.TRANSCRIPTS	2,44
46	INTERACTIONS.OF.VPR.WITH.HOST.CELLULAR.PROTEINS	2,44
47	TRANSPORT.OF.THE.SLBP.DEPENDANT.MATURE.MRNA	2,43
48	TRANSPORT.OF.MATURE.MRNA.DERIVED.FROM.AN.INTRONLESS.TRANSCRIPT	2,43
49	TRANSPORT.OF.THE.SLBP.INDEPENDENT.MATURE.MRNA	2,43
50	LATE.PHASE.OF.HIV.LIFE.CYCLE	2,42
51	CHROMOSOME.MAINTENANCE	2,42
52	CYTOSOLIC.TRNA.AMINOACYLATION	2,41
53	G1.S.DNA.DAMAGE.CHECKPOINTS	2,41
54	REGULATION.OF.DNA.REPLICATION	2,4
55	MRNA.SPLICING.MAJOR.PATHWAY	2,4
56	REGULATION.OF.GLUCOKINASE.BY.GLUCOKINASE.REGULATORY.PROTEIN	2,39
57	CENTROSOME.MATURATION	2,38
58	COOPERATION.OF.PREFOLDIN.AND.TRIC.CCT.IN.ACTIN.AND.TUBULIN.FOLDING	2,38
59	PREFOLDIN.MEDIATED.TRANSFER.OF.SUBSTRATE.TO.CCT.TRIC	2,38
60	KEGG_AMINOACYL.TRNA.BIOSYNTHESIS	2,38
61	KEGG_DNA.REPLICATION	2,38
62	DNA.STRAND.ELONGATION	2,37

63	LOSS.OF.NLP.FROM.MITOTIC.CENTROSOMES	2,37
64	LOSS.OF.PROTEINS.REQUIRED.FOR.INTERPHASE.MICROTUBULE.ORGANIZATION.FROM.THE.CENTROSOME	2,36
65	GLUCOSE.TRANSPORT	2,36
66	MRNA.SPLICING	2,36
67	KEGG_CELL.CYCLE	2,36
68	REGULATION.OF.MRNA.STABILITY.BY.PROTEINS.THAT.BIND.AU.RICH.ELEMENTS	2,35
69	FORMATION.OF.TUBULIN.FOLDING.INTERMEDIATES.BY.CCT.TRIC	2,35
70	HIV.LIFE.CYCLE	2,35
71	REMOVAL.OF.LICENSING.FACTORS.FROM.ORIGINS	2,34
72	NUCLEAR.PORE.COMPLEX.NPC.DISASSEMBLY	2,34
73	MITOCHONDRIAL.TRANSLATION.TERMINATION	2,33
74	MITOCHONDRIAL.TRANSLATION.ELONGATION	2,33
75	MITOCHONDRIAL.TRANSLATION.INITIATION	2,31
76	HEXOSE.TRANSPORT	2,31
77	MITOCHONDRIAL.TRANSLATION	2,31
78	ORC1.REMOVAL.FROM.CHROMATIN	2,3
79	SWITCHING.OF.ORIGINS.TO.A.POST.REPLICATIVE.STATE	2,3
80	EXTENSION.OF.TELOMERES	2,3
81	METABOLISM.OF.MRNA	2,3
82	INFLUENZA.LIFE.CYCLE	2,3
83	P53.DEPENDENT.G1.S.DNA.DAMAGE.CHECKPOINT	2,29
84	P53.DEPENDENT.G1.DNA.DAMAGE.RESPONSE	2,29
85	INFLUENZA.INFECTION	2,28
86	CDT1.ASSOCIATION.WITH.THE.CDC6.ORC.ORIGIN.COMPLEX	2,27
87	METABOLISM.OF.NUCLEOTIDES	2,27
88	NUCLEAR.ENVELOPE.BREAKDOWN	2,26
89	TRANSLATION	2,26
90	STABILIZATION.OF.P53	2,24
91	VIRAL.MESSENGER.RNA.SYNTHESIS	2,23
92	UBIQUITIN.MEDIATED.DEGRADATION.OF.PHOSPHORYLATED.CDC25A	2,23
93	INFLUENZA.VIRAL.RNA.TRANSCRIPTION.AND.REPLICATION	2,22
94	WP157.GLYCOLYSIS.AND.GLUCONEOGENESIS	2,22
95	UBIQUITIN.DEPENDENT.DEGRADATION.OF.CYCLIN.D1	2,22
96	P53.INDEPENDENT.DNA.DAMAGE.RESPONSE	2,21
97	CHAPERONIN.MEDIATED.PROTEIN.FOLDING	2,21
98	REGULATION.OF.PLK1.ACTIVITY.AT.G2.M.TRANSITION	2,21
99	MITOTIC.PROPHASE	2,2
100	CYCLIN.E.ASSOCIATED.EVENTS.DURING.G1.S.TRANSITION	2,2
101	G1.PHASE	2,2
102	P53.INDEPENDENT.G1.S.DNA.DAMAGE.CHECKPOINT	2,19
103	REGULATION.OF.MITOTIC.CELL.CYCLE	2,19
104	APC.C.MEDIATED.DEGRADATION.OF.CELL.CYCLE.PROTEINS	2,18
105	TELOMERE.MAINTENANCE	2,18
106	CYCLIN.D.ASSOCIATED.EVENTS.IN.G1	2,18
107	REGULATION.OF.ORNITHINE.DECARBOXYLASE.ODC.	2,17
108	ACTIVATION.OF.APC.C.AND.APC.C.CDC20.MEDIATED.DEGRADATION.OF.MITOTIC.PROTEINS	2,17
109	POST.ELONGATION.PROCESSING.OF.INTRON.CONTAINING.PRE.MRNA	2,16
110	MRNA.3.END.PROCESSING	2,16
111	UBIQUITIN.DEPENDENT.DEGRADATION.OF.CYCLIN.D	2,16
112	TELOMERE.C.STRAND.LAGGING.STRAND.SYNTHESIS	2,16
113	DNA.REPAIR	2,15
114	TRANSCRIPTION	2,15
115	CDK.MEDIATED.PHOSPHORYLATION.AND.REMOVAL.OF.CDC6	2,15
116	RNA.POLYMERASE.II.TRANSCRIPTION	2,15
117	REGULATION.OF.APC.C.ACTIVATORS.BETWEEN.G1.S.AND.EARLY.ANAPHASE	2,14
118	E2F.MEDIATED.REGULATION.OF.DNA.REPLICATION	2,14
119	REGULATORY.RNA.PATHWAYS	2,14
120	WP310.MRNA.PROCESSING	2,14
121	KSRP.DESTABILIZES.MRNA	2,14
122	BASIGIN.INTERACTIONS	2,13
123	CYCLIN.A.B1.ASSOCIATED.EVENTS.DURING.G2.M.TRANSITION	2,13
124	AUTODEGRADATION.OF.THE.E3.UBIQUITIN.LIGASE.COP1	2,13
125	CYCLIN.A.CDK2.ASSOCIATED.EVENTS.AT.S.PHASE.ENTRY	2,13
126	APC.C.CDH1.MEDIATED.DEGRADATION.OF.CDC20.AND.OTHER.APC.C.CDH1.TARGETED.PROTEINS.IN.LATE.MITOSIS.EARLY.G1	2,13
127	HIV.INFECTION	2,12
128	BIOC_G2PATHWAY	2,12
129	DEGRADATION.OF.DVL	2,11
130	TRANSCRIPTIONAL.REGULATION.BY.SMALL.RNAS	2,11
131	GAP.FILLING.DNA.REPAIR.SYNTHESIS.AND.LIGATION.IN.GG.NER	2,1
132	BIOC_ATRBRCAPATHWAY	2,09
133	REGULATION.OF.ACTIVATED.PAK.2P34.BY.PROTEASOME.MEDIATED.DEGRADATION	2,09

134	TRISTETRAPROLIN.TTP.DESTABILIZES.MRNA	2,09
135	APC.C.CDC20.MEDIATED.DEGRADATION.OF.MITOTIC.PROTEINS	2,09
136	KEGG_PYRIMIDINE.METABOLISM	2,08
137	KEGG_MRNA.SURVEILLANCE.PATHWAY	2,08
138	REGULATION.OF.APOPTOSIS	2,07
139	GAP.FILLING.DNA.REPAIR.SYNTHESIS.AND.LIGATION.IN.TC.NER	2,07
140	APOPTOSIS	2,07
141	KEGG_GLYCOLYSIS.GLUCONEOGENESIS	2,07
142	CAP.DEPENDENT.TRANSLATION.INITIATION	2,07
143	CDC20.PHOSPHO.APC.C.MEDIATED.DEGRADATION.OF.CYCLIN.A	2,07
144	GLI3.IS.PROCESSED.TO.GLI3R.BY.THE.PROTEASOME	2,06
145	SCF.SKP2.MEDIATED.DEGRADATION.OF.P27.P21	2,06
146	G0.AND.EARLY.G1	2,06
147	VIF.MEDIATED.DEGRADATION.OF.APOBEC3G	2,06
148	G1.S.SPECIFIC.TRANSCRIPTION	2,06
149	KEGG_NUCLEOTIDE.EXCISION.REPAIR	2,05
150	HH.LIGAND.BIOGENESIS.DISEASE	2,05
151	LAGGING.STRAND.SYNTHESIS	2,05
152	ORGANELLE.BIOGENESIS.AND.MAINTENANCE	2,05
153	GLOBAL.GENOMIC.NER.GG.NER.	2,05
154	SCF.BETA.TRCP.MEDIATED.DEGRADATION.OF.EMI1	2,05
155	APC.C.CDC20.MEDIATED.DEGRADATION.OF.SECURIN	2,04
156	MRNA.SPLICING.MINOR.PATHWAY	2,04
157	EUKARYOTIC.TRANSLATION.INITIATION	2,04
158	PROTEIN.FOLDING	2,04
159	AUF1.HNRNP.D0.DESTABILIZES.MRNA	2,04
160	MITOCHONDRIAL.TRNA.AMINOACYLATION	2,02
161	PROCESSIVE.SYNTHESIS.ON.THE.LAGGING.STRAND	2,02
162	DEGRADATION.OF.GLI2.BY.THE.PROTEASOME	2,01
163	KEGG_PENTOSE.PHOSPHATE.PATHWAY	2,01
164	NRF2_TARGETS	2,01
165	TRANSCRIPTION.COUPLED.NER.TC.NER.	2,01
166	RNA.POLYMERASE.II.TRANSCRIPTION.TERMINATION	2
167	PURINE.METABOLISM	2
168	AUTODEGRADATION.OF.CDH1.BY.CDH1.APC.C	2
169	WP567.EUKARYOTIC.TRANSCRIPTION.INITIATION	2
170	ASYMMETRIC.LOCALIZATION.OF.PCP.PROTEINS	2
171	KEGG_MISMATCH.REPAIR	2

Supplementary Table 3.
B List of significantly (p<0.01) depleted gene sets identified by Gene Set Enrichment Analysis using the complete transcriptome of BMDMs exposed to UV-irradiated obese adipose tissue (ObAT) versus BMDMs exposed to non-treated ObAT for 3 days. Enriched gene sets in BMDMs exposed to UV-irradiated ObAT with a normalized enrichment score (NES)-score higher than 2 were included in the list. All significantly depleted gene sets in BMDMs exposed to UV-irradiated ObAT were included.

	Gene Set	NES
1	INTERFERON.ALPHA.BETA.SIGNALING	-2,54
2	INTERFERON.GAMMA.SIGNALING	-2,4
3	KEGG_ALLOGRAFT.REJECTION	-2,28
4	SMOOTH.MUSCLE.CONTRACTION	-2,27
5	TRAF6.MEDIATED.IRF7.ACTIVATION	-2,22
6	KEGG_AUTOIMMUNE.THYROID.DISEASE	-2,16
7	KEGG_STAPHYLOCOCCUS.AUREUS.INFECTION	-2,13
8	MUSCLE.CONTRACTION	-2,02
9	KEGG_GRAFT.VERSUS.HOST.DISEASE	-2,01
10	BIOC_NKTPATHWAY	-1,96
11	WP1253.TYPE.II.INTERFERON.SIGNALING.IFNG.	-1,93
12	THE.ROLE.OF.NEF.IN.HIV.1.REPLICATION.AND.DISEASE.PATHOGENESIS	-1,9
13	KEGG_TYPE.I.DIABETES.MELLITUS	-1,88
14	BIOC_ALKPATHWAY	-1,84
15	KEGG_INFLAMMATORY.BOWEL.DISEASE.IBD.	-1,82
16	NEGATIVE.REGULATORS.OF.RIG.I.MDA5.SIGNALING	-1,79
17	SIGNALING.BY.RHO.GTPASES	-1,78
18	RHO.GTPASE.CYCLE	-1,78
19	INTERFERON.SIGNALING	-1,78
20	KEGG_MEASLES	-1,77
21	RIG.I.MDA5.MEDIATED.INDUCTION.OF.IFN.ALPHA.BETA.PATHWAYS	-1,76
22	KEGG_ANTIGEN.PROCESSING.AND.PRESENTATION	-1,75
23	CYTOKINE.SIGNALING.IN.IMMUNE.SYSTEM	-1,75
24	KEGG_TOXOPLASMOSIS	-1,7
25	KEGG_TUBERCULOSIS	-1,68
26	WP1271.TOLL.LIKE.RECEPTOR.SIGNALING.PATHWAY	-1,67
27	KEGG_INFLUENZA.A	-1,67
28	PEPTIDE.LIGAND.BINDING.RECEPTORS	-1,6
29	GPCR.LIGAND.BINDING	-1,59
30	WP1397.ODORANT.GPCRS	-1,57
31	KEGG_OSTEOCLAST.DIFFERENTIATION	-1,57
32	KEGG_HERPES.SIMPLEX.INFECTION	-1,57
33	KEGG_CYTOKINE.CYTOKINE.RECEPTOR.INTERACTION	-1,56
34	KEGG_LYSOSOME	-1,54
35	WP447.ADIPOGENESIS.GENES	-1,53
36	CLASS.A.1.RHODOPSIN.LIKE.RECEPTORS.	-1,49
37	ANTIGEN.PRESENTATION.FOLDING.ASSEMBLY.AND.PEPTIDE.LOADING.OF.CLASS.I.MHC	-1,86
38	BIOC_CTLA4PATHWAY	-1,85
39	REGULATION.OF.IFNA.SIGNALING	-1,84
40	STING.MEDIATED.INDUCTION.OF.HOST.IMMUNE.RESPONSES	-1,8
41	KEGG_TOLL.LIKE.RECEPTOR.SIGNALING.PATHWAY	-1,7
42	KEGG_VIRAL.MYOCARDITIS	-1,68
43	KEGG_SYSTEMIC.LUPUS.ERYTHEMATOSUS	-1,66
44	KEGG_LEISHMANIASIS	-1,6
45	KEGG_PROTEIN.DIGESTION.AND.ABSORPTION	-1,51
46	DEFENSINS	-1,85
47	NEF.MEDIATES.DOWN.MODULATION.OF.CELL.SURFACE.RECEPTORS.BY.RECRUITING.THEM.TO.CLATHRIN.ADAPTERS	-1,8
48	CD28.DEPENDENT.PI3K.AKT.SIGNALING	-1,78
49	NEF.MEDIATED.DOWNREGULATION.OF.MHC.CLASS.I.COMPLEX.CELL.SURFACE.EXPRESSION	-1,78
50	ELASTIC.FIBRE.FORMATION	-1,65
51	G.ALPHA.12.13.SIGNALLING.EVENTS	-1,65
52	WP41.GPCRS.OTHER	-1,46
53	BIOC_TOB1PATHWAY	-1,79
54	REGULATION.OF.FZD.BY.UBIQUITINATION	-1,73
55	GROWTH.HORMONE.RECEPTOR.SIGNALING	-1,67
56	KEGG_HEPATITIS.C	-1,54
57	WP458.INFLAMMATORY.RESPONSE.PATHWAY	-1,71
58	O.GLYCOSYLATION.OF.TSR.DOMAIN.CONTAINING.PROTEINS	-1,71
59	MOLECULES.ASSOCIATED.WITH.ELASTIC.FIBRES	-1,68
60	ENDOSOMAL.VACUOLAR.PATHWAY	-1,79
61	KEGG_RAS.SIGNALING.PATHWAY	-1,33

Supplementary Table 4.
Sequences of primers used for examining mRNA levels using RT-qPCR.

Gene	3' primer	5' primer
Abcg1	CCTACCACAACCCAGCAGACT	CGAGGTCTCTCTTATAGTCAG
Atp6v0d2	AGCCAGCCTAACTCAGC	GCTTCTTCCTCATCTCCGTGTC
Bax	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
Bcl2	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATGC
Cd11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTCA
Cd206	GCTTCCGTCACCCTGTATG	GTGTGTCATTCTTACACTCCCAT
Cd36	TCCAGCCAATGCCTTTGC	TGGAGATTACTTTTCAGTGCAGAA
cFlip	GCTCCAGAATGGGCGAAGTAA	ACGGATGTGCGGAGGTAAAAA
Elmo1	AGCTCCATGAACGAATACAGTCA	CACCATCTGAGTGAGAAGTGAGA
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Gas6	CCGCGCCTACCAAGTCTTC	CGGGGTCGTTCTCGAACAC
Glut1	GGTCACCATCTTGGAGCTGTTC	ACCTGCCTTCTCGAAGATGCT
Hk2	CGTGTCCCTACCTTTGGGTT	CCAGGTCAAACTCCTCTCGC
Ifi44	AACTGACTGCTCGCAATAATGT	GTAACACAGCAATGCCTCTTGT
Ifi47	AGCAGATGAATCCGCTGATGT	CGTGGAAATTGGGTGTCCC
Il-10	CTGGACAACATACTGCTAACCG	GGGCATCACTTCTACCAGGTAA
Il-1rn	AAATCTGCTGGGGACCCTAC	TGAGCTGGTTGTTTCTCAGG
Il-6	AGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Irf7	CCCCAGCCGGTGATCTTTC	CACAGTGACGGTCCTCGAAG
Isg15	GGTGTCCGTGACTAACTCCAT	TGGAAAGGGTAAGACCGTCCT
Lipa	TGTTCGTTTTCACCATTGGGA	CGCATGATTATCTCGGTCACA
Lpl	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG
Mertk	TGCGTTTAATCACACCATTGGA	TGCCCCGAGCAATTCCTTTC
Mlkl	AATTGTACTCTGGGAAATTGCCA	TCTCCAAGATTCCGTCCACAG
Parp1	GGCAGCCTGATGTTGAGGT	GCGTACTCCGCTAAAAAGTCAC
Plin2	GCCTCTCAACTGGCTGGTAG	ACAGCAAAAGGGGTCATCTG
Puma	AGCAGCACTTAGAGTCGCC	CCTGGGTAAGGGGAGGAGT
Stat1	TCACAGTGGTTCGAGCTTCAG	GCAAACGAGACATCATAGGCA
Tap1	CCAACGTGTGCGAGTCCATTA	AGAGTGAGGTACGGTGACCC
Tgf-β	CTTCAATACGTCAGACATTCGGG	GTAACGCCAGGAATTGTTGCTA
Tnf-α	CAACCTCCTCTCTGCCGTCAA	TGACTCCAAAGTAGACCTGCCC
36B4	ATGGGTACAAGCGCGTCCTG	GCCTTGACCTTTTCAGTAAG

7

A role for TLR10 in obesity and adipose tissue morphology

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ABSTRACT

Toll like receptors (TLRs) are expressed in adipose tissue and promote adipose tissue inflammation during obesity. Recently, anti-inflammatory properties have been attributed to TLR10 in myeloid cells, the only member of the TLR family with inhibitory activity. In order to assess whether TLR10-induced inhibition of inflammation may be protective during the development of obesity and metabolic abnormalities we used transgenic human *TLR10* mice (hTLR10tg) and wild type (WT) controls on a C57B6J background. HFD-feeding enhanced ***TLR10*** expression in the adipose tissue, and HFD-fed hTLR10tg mice displayed reduced adipocyte size, adipose tissue weight, and a trend toward lower plasma insulin levels compared to WT mice.

In humans, obese individuals with polymorphisms in the *TLR10* gene displayed reduced macrophage infiltration in the adipose tissue accompanied by a trend to lower leptin levels and higher adiponectin levels in plasma. In healthy individuals with the same polymorphisms in the *TLR10* gene we did not observe any difference in plasma concentrations of leptin and adiponectin.

We conclude that TLR10 impacts adipose tissue morphology in obesity. Larger studies in humans are warranted to assess its potential value as therapeutic target in metabolic syndrome and type 2 diabetes.

INTRODUCTION

The prevalence of obesity is rising worldwide promoting the development of the metabolic syndrome and closely related metabolic disturbances such as insulin resistance and type 2 diabetes [1]. Adipose tissue from obese individuals is characterized by an increased presence of pro-inflammatory macrophages [2]. Various animal studies have shown similar outcomes and subsequently demonstrated that the pro-inflammatory macrophage phenotype contributes to the development of inflammation that interferes with insulin signaling pathways. Ultimately, this pro-inflammatory trait leads to the development of insulin resistance during obesity [1, 3].

Toll-like receptors (TLRs) have been shown to contribute to the development of obesity-induced inflammation [4]. As innate immunity pattern recognition receptors (PRR), TLRs sense pathogen-components to induce an inflammatory response. Moreover, recently endogenous activators of TLRs have been identified, among which free fatty acids [5]. In the adipose tissue TLRs are expressed on both macrophages and adipocytes and were described to be involved in the development of metabolic abnormalities including insulin resistance [4, 6].

Numerous in vitro studies have shown that TLR2 and TLR4 activation promotes insulin resistance by inducing activation of JNK, p38 and IKK kinases that inhibit the phosphorylation of the insulin receptor substrate thus disturbing insulin signaling [4, 6-8]. In support of these results, several studies in murine experimental models revealed that absence of TLR2 or TLR4 protects against the development of obesity-induced insulin resistance [9]. In obese individuals, high levels of free fatty acids are thought to either directly or indirectly drive the TLR4-signaling cascade in adipose tissue resulting in pro-inflammatory cytokine secretion and subsequent deterioration in insulin signalling pathways [10].

Recently, TLR10, a member of the TLR family receiving little attention so far, has been described to be the only TLR with anti-inflammatory properties in human myeloid cells. Blocking TLR10 in monocytes was proven to increase pro-inflammatory cytokine production upon stimulation with various stimuli [11]. Moreover, single nucleotide polymorphisms (SNPs) in the *TLR10* gene correlated with altered levels of cytokine production [11]. Various mechanisms of action may explain the anti-inflammatory effects of TLR10. First, TLR10 can form dimmers with other TLRs such as TLR1, TLR2 and TLR6, blocking their signaling upon ligand binding. Second, TLR10 has been shown to promote the production of the anti-inflammatory cytokine IL-1Ra [11]. Moreover, a recent study showed that TLR10 has the capacity to block signals from other TLRs by suppressing both MyD88- and TRIF-dependent signaling [12].

In order to investigate whether TLR10 has anti-inflammatory properties in adipose tissue and to decipher the role of this receptor during the development of obesity and associated metabolic abnormalities, we used various approaches involving both animal and human studies. Firstly, we investigated the impact of a high-fat diet (HFD) versus low-fat diet (LFD) in *TLR10* transgenic mice. Secondly, we evaluate metabolic effects of polymorphisms in the *TLR10* gene in two human cohorts including both obese individuals and healthy controls.

Our results revealed upregulation of h*TLR10* in adipose tissue of mice during HFD-feeding. Moreover, despite similar weight gain upon LFD- or HFD-feeding, hTLR10tg mice had reduced adipose tissue weight, smaller adipocytes, and a trend towards less crown-like structures (CLSs) and lower circulating insulin levels than their wild type controls. In obese individuals carrying SNPs in the *TLR10* gene we observed reduced macrophage numbers in the adipose tissue and a trend to lower leptin plasma levels and higher adiponectin plasma levels whereas no differences in adipokine levels were observed in healthy controls carrying the same SNPs in *TLR10*.

MATERIALS AND METHODS

Human cohorts

302 obese individuals (IN-CONTROL study of the Cardiovascular research Netherlands Project) and more than 500 individuals (500 Functional Genomics Project) from the Human Functional Genomics project (HGFP) were genotyped as described before [13]. Of all individuals of the IN-CONTROL study and a total of 427 individuals of the 500 Functional Genomics Project, BMI and adipokines levels (leptin and adiponectin) could be measured and compared between individuals carrying a SNP and individuals with wild type (WT) alleles of the *TLR10* gene. Leptin and adiponectin concentrations in plasma were measured by ELISA according to manufacturer's protocol (R&D, The Netherlands).

In addition, subcutaneous adipose tissue was obtained from 265 out of 302 obese individuals by performing needle biopsy after informed consent was obtained. The tissue was formalin fixed and paraffin-embedded and cross-sections (5μm) were stained by using the DAB (3'3 diaminobenzidine) method and anti-human CD68 antibody (Bio Rad, Veenendaal, The Netherands). Adipocyte size and the number of CLSs could be determined for 265 individuals. The HGFP was approved by the Ethical Committee of Radboud University Medical Center, Nijmegen (no. 42561.091.12). Experiments were conducted according to the principles expressed in the Declaration of Helsinki. Samples of venous blood were drawn after informed consent was obtained. For more information see http://www.humanfunctionalgenomics.org/site/.

Animal study

Because mice lack a functional *TLR10* gene, a constitutive human *TLR10* knock-in mouse was generated by Taconic Artemis using targeted transgenesis. Using recombination-mediated cassette exchange (RMCE), a CAG promoter cassette, the human *TLR10* open reading frame (ORF), and the hGH polyadenylation signal and an additional polyadenylation signal, were inserted into the ROSA26 locus. This obtained RMCE vector was transfected into the TaconicArtemis-C57BL/6 ES cell line. Positive Neomycin selection was used to select recombinant clones. Positively selected blastocytes were transferred into embryos through which chimeric mice were attained. Using a Caliper LabChip GX device, sample analysis has been performed. Animals were fully back-crossed to a C57/Black6 background before they were used for experiments. Genotyping of the mice was performed using PCR and 1.5% agarose gel electrophoresis with the following primers: wild type forward: 5' CTCTTCCCTCGTGATCTGCAACTCC; wild type reverse: 5' CATGTCTTTAATCTACCTCGATGG; *TLR10* cond-forward: 5' GACAGCAGAGGGTGATGCTC; *TLR10* cond-reverse: 5' CTTCCTCACAGATAGGCATGG; positive control forward: 5' GAGACTCTGGCTACTCATCC; positive control reverse: 5' CCTTCAGCAAGAGCTGGGGAC. The PCR conditions were: 95 °C for 5min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, followed by 72 °C for 10 min.

Human *TLR10* transgenic (hTLR10tg) and C57BL/6j (WT) male mice aged between 12-16 weeks were given a low-fat diet (LFD) or a high-fat diet (HFD) with 10%, respectively 45%, of energy derived from fat [14] for 16 weeks (n=9 for the HFD-fed mice, n=10 for the LFD-fed mice). Bodyweight and food intake was measured weekly. Glycaemic control was assessed by an oral glucose tolerance test (oGTT). Mice were fasted overnight after which glucose was administered (2g/kg) followed by measurements of circulating glucose at 20, 40, 60, 90 and 120 minutes after glucose administration. Blood glucose concentration was measured with an Accu-chek glucosemeter (Roche Diagnostics, Almere, The Netherlands). The study was approved by the animal experimental committee in Nijmegen, The Netherlands, and all experiments were performed according to approved guidelines (Legislation for the protection of animals used for scientific purposes - Environment - European Commission).

Plasma measurements

Triglycerides (TG) were determined using commercially available enzymatic kits from Roche Molecular Biochemicals (Indianapolis, IN). Free fatty acids (FFAs) were measured using the NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, The Netherlands). Plasma insulin concentrations were measured by ELISA according to the manufacturer's instructions (ultra sensitive mouse insulin ELISA kit, Crystal Chem Inc., IL, USA). Glucose (Liquicolor, Human GmbH, Wiesbaden, Germany) was measured enzymatically following manufacturer's protocols. Leptin, adiponectin and IL-1Ra were determined by ELISA according to manufacturer's protocol (R&D Systems, The Netherlands). Serum amyloid A (SAA) was measured with an ELISA assay (Tridelta Development Ltd., Maynooth, Ireland).

Liver, pancreas and adipose tissue histology

Liver and gonadal adipose tissue (gWAT) were formalin-fixed and paraffin-embedded, and cross-sections (5 µm) were stained by the DAB (3'3 diaminobenzidine) method using an anti-F4/80 antibody (AbD Serotec). Inflammation was assessed by determining the number of crown-like structures (CLSs) in four non-overlapping fields (at 20x magnification) using software as previously described [15].

Pancreatic tissue was stained with the DAB (3'3 diaminobenzidine) method using an anti-insulin antibody from Santa Cruz biotechnology. The Langerhans islets surface was determined using the software Image J (https://imagej.net/Welcome).

RNA isolation and gene expression analysis

After dissection, tissues were snap frozen using liquid nitrogen and stored at -80 before further processing. RNA was isolated using TRIzol reagent (Life Technologies Europe BV, Bleiswijk, The Netherlands) and quantity was measured with a Nano Drop Spectrophotometer (Nanodrop technologies, Montchanin, DE, USA).

For gene expression analysis, cDNA was generated using reverse transcription with

iScript (Biorad) following manufacturer's instructions. Gene expression analysis was done by quantitative PCR using SYBR green-based quantification (Applied Biosystems, Foster City, CA, USA). Primers were selected from the Harvard Primer Bank and are available upon request. *36b4* was used as housekeeping gene.

Statistical analysis

Data are represented as mean + SEM. Graphs were made and statistical analysis was done with Graphpad Prism 5.03 (La Jolla, USA). To examine the effects of both diet and genotype in our murine model we used a two-way ANOVA analysis with Bonferroni posthoc testing. For human data, the relation between two variables was tested with correlation studies and differences between two groups were tested with an unpaired Mann-Whitney t-test. The difference between allele frequencies was calculated using a chi-square test. A p-value <0.05 was considered significant.

RESULTS

Animal study

To learn more about the role of TLR10 during diet-induced obesity, wild type (WT) and human *TLR10* transgenic (hTLR10tg) mice were fed a low-fat diet (LFD) or high-fat diet (HFD) for 16 weeks. As shown in **Figure 1A**, WT and hTLR10tg HFD-fed animals displayed a similar increase in bodyweight compared to animals receiving the LFD ($p<0.0001$). Noticeably, food intake was similar between groups as well (**Figure 1B**). The HFD induced an increase in brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) in both WT and hTLR10tg mice and an increase in liver weight only in transgenic mice. Interestingly, the transgenic mice had lower white adipose tissue mass with significantly less iWAT and gonadal white adipose tissue (gWAT) than WT controls (**Figure 1C**).

Next, we measured several circulating parameters related to metabolism in both LFD- and HFD-fed animals. As shown in **Figure 1D** and **1E**, plasma triglyceride (TG) and non-esterified fatty acid (FFA) levels were similar across genotypes and significantly higher in mice on a HFD. To learn more about the potential role of TLR10 on obesity-induced inflammation, circulating concentrations of IL-1 receptor antagonist (IL-1Ra) and serum amyloid a (SAA) were measured. IL-1Ra, a marker of inflammation [16] and produced by the adipose tissue [17], was similarly increased by HFD-feeding in both genotypes (**Figure 1F**). SAA levels, an acute phase protein produced by the liver and adipose tissue [18], neither were not significantly different between genotypes (**Figure 1G**).

We subsequently zoomed in on potential effects of TLR10 in adipose tissue by measuring circulating concentrations of two adipokines. Whereas leptin was enhanced by HFD-feeding in both genotypes (**Figure 1H**) no significant differences were found between adiponectin levels upon a HFD (**Figure 1I**). Interestingly, there was a trend to lower leptin levels in the hTLR10tg mice versus WT mice on a HFD (**Figure 1H**).

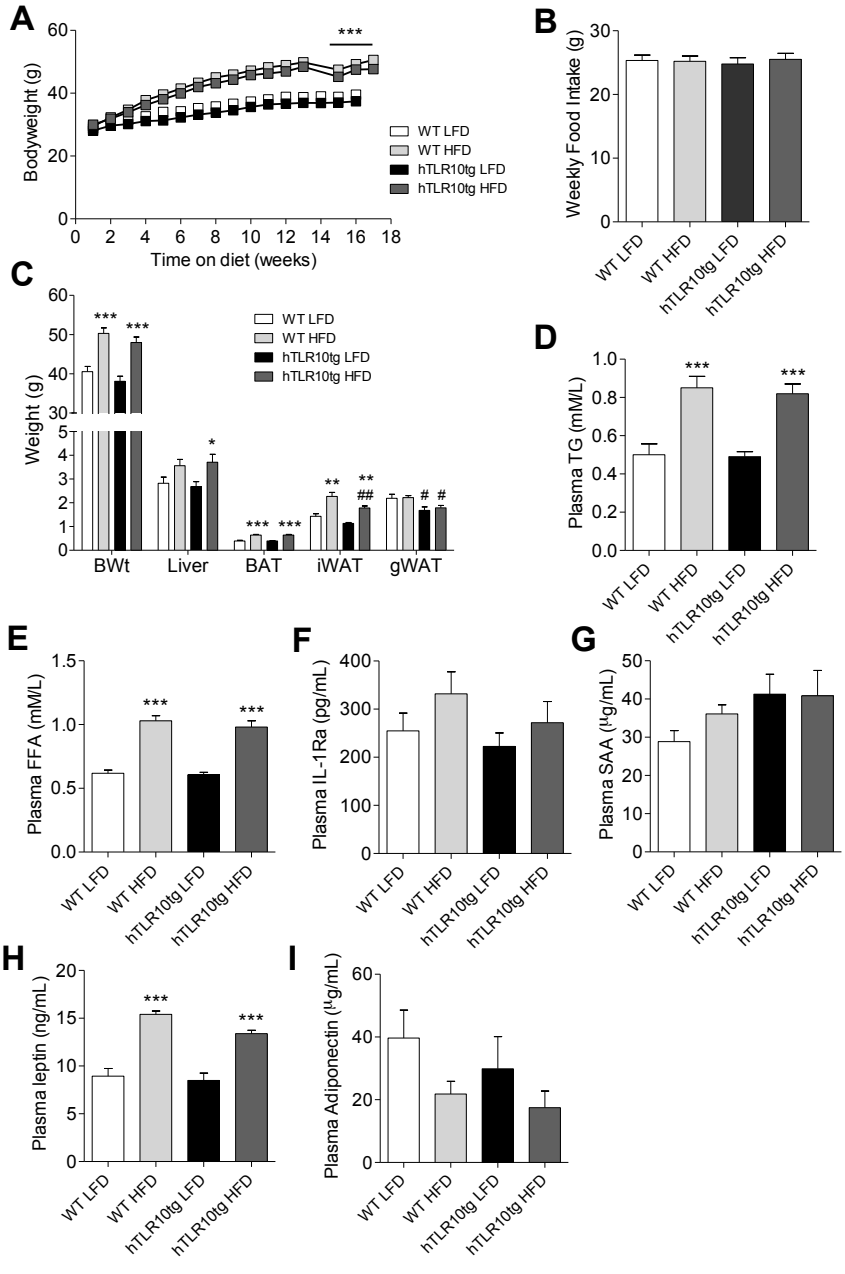

Figure 1. Bodyweight, organ weight and plasma parameters in WT and hTLR10tg mice on a low- or high-fat diet

Bodyweight (BWt) (**A**,**C**) and food intake (**B**) of wild type (WT) and transgenic mice (hTLR10tg) on a high-fat diet (HFD) or a low-fat diet (LFD) for 16 weeks. (**C**) Weight of the liver, interscapular brown adipose tissue (BAT), inguinal white adipose tissue (iWAT) and gonadal white adipose tissue (gWAT) at the end of a 16 week LFD or HFD intervention. Plasma triglycerides (TG) (**D**), non-esterified fatty acids (FFA) (**E**), the inflammatory markers interleukin-1 receptor antagonist (IL-1Ra) (**F**) and serum amyloid A (SAA) (**G**), and the adipokines leptin (**H**) and adiponectin (**I**) in WT and transgenic mice on a LFD or HFD for 16 weeks. All data are expressed as mean + SEM; *p<0.05, **p<0.001, ***p<0.0001 effect of the diet; #p<0.05, ##p<0.001 effect of the genotype.

TLR10-dependent effects on HFD-induced adipose tissue inflammation

During the development of obesity, several robust morphological changes occur in the adipose tissue: adipocytes increase in volume to accommodate fat mass expansion and macrophages are invading the adipose tissue and form so-called crown-like structures (CLSs) when surrounding dying adipocytes [19].

Interestingly, we observed up-regulation of h*TLR10* expression in gWAT of HFD-fed hTLR10tg mice compared to transgenic animals on a LFD (**Figure 2A**), suggestive of activation of the TLR10 pathway in the adipose tissue during obesity. Zooming in on adipose tissue morphology, we observed that hTLR10tg mice had smaller adipocytes than WT animals ($p<0.05$) (**Figure 2B**). In addition, there was a trend to less macrophages surrounding dead adipocytes in so-called CLSs in the transgenic group (**Figure 2C**). Besides numbers, phenotypical changes of macrophages are important in determining adipose tissue inflammation. Therefore, we measured expression levels of macrophage marker genes in gWAT. As shown in **Figure 2D** expression levels of macrophage markers *F4/80*, *Cd68, Cd11c, Cd11b, Cd206* and *Arg1* and the pro-inflammatory receptor *Tlr2* were significantly increased by the HFD intervention in hTLR10 transgenic mice. Interestingly, gene expression levels of *Cd206,* a marker for anti-inflammatory macrophages [20], was significantly enhanced in HFD-fed hTLR10tg mice compared to WT controls on the same diet. Of note, we checked the expression levels of T-cell marker *Cd4*, but we did not observe any difference between the four mice groups (data not shown). Next, we measured expression levels of downstream effector genes related to inflammation. As shown in **Figure 2E**, the HFD intervention caused an increased expression of the chemokine *Mcp-1* in both WT and transgenic mice. In hTLR10tg mice, expression levels of *Tnfα*, but also the anti-inflammatory cytokines *Il-10* and *Il-1rn* tended to be higher than in WT mice upon a HFD (**Figure 2E**).

Besides the adipose tissue, the liver importantly contributes to metabolic abnormalities during obesity. HFD-feeding induced steatosis in both genotypes and hepatic gene expression changes were similar between WT and hTRL10tg animals (**Figure 2F** and **2G**), suggesting that the liver is largely unaffected by h*TLR10* expression in mice on a HFD.

A disturbance in glycaemic control is a common consequence in the development of obesity. Inflammation of the adipose tissue may partly be setting off detrimental changes in glycaemic control [21]. To evaluate the role of TLR10 in this process, both genotypes were exposed to an oral glucose tolerance test (oGTT). As shown in **Figure 3A** and **3B** there were no differences in glucose tolerance between hTLR10tg and WT mice, although in the LFD group higher glucose levels were measured in transgenic animals 60 minutes after glucose injection. Interestingly, measurements of plasma insulin revealed a tendency to lower levels in animals with *TLR10* expression, suggestive of improved insulin sensitivity in the hTLR10tg mice and a potential protective role for TLR10 against the development of insulin resistance (**Figure 3C**). The differences in plasma insulin levels appeared to be unrelated to β-cell size (**Figure 3D** and **3E**).

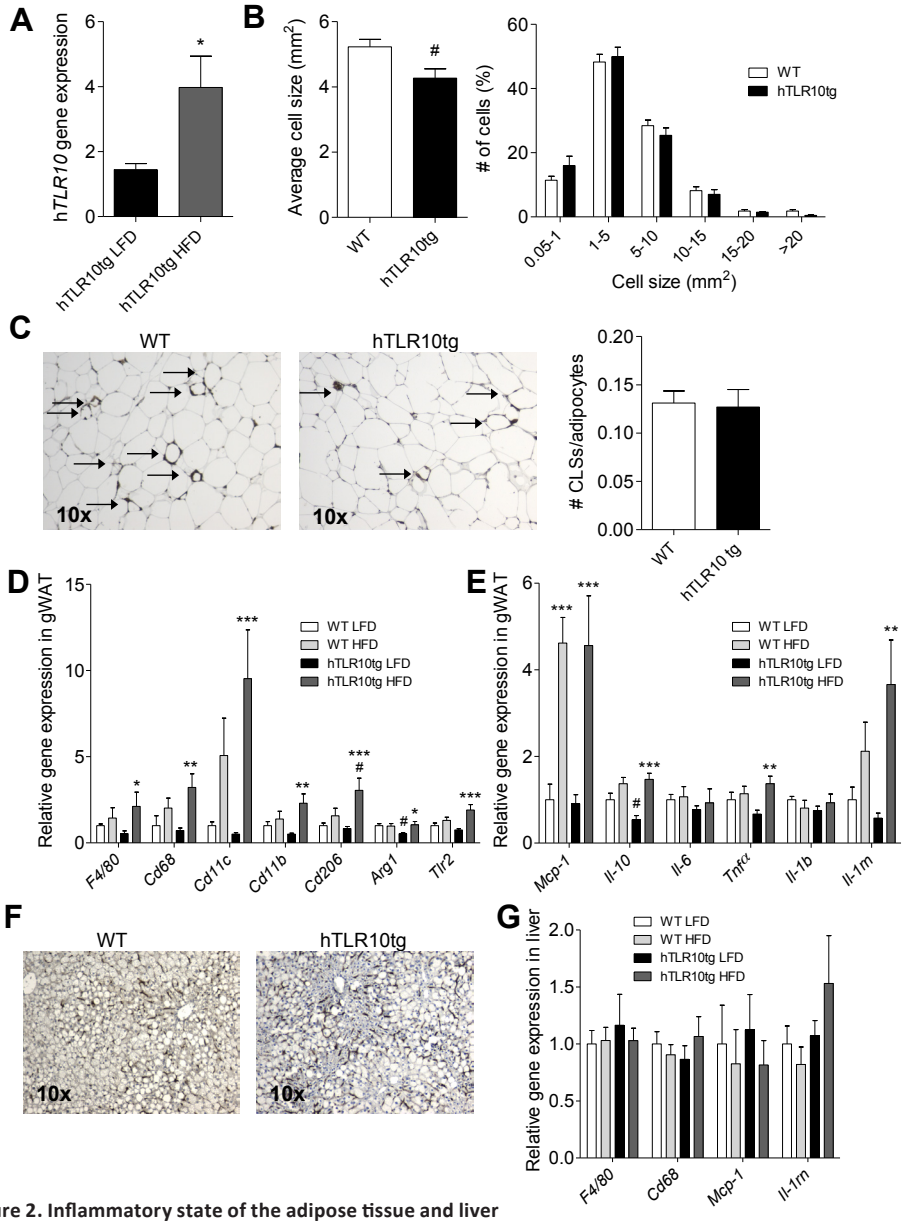

Figure 2. Inflammatory state of the adipose tissue and liver

(**A**) Human *TLR10* expression in gonadal adipose tissue (gWAT) of transgenic (hTLR10tg) mice after a low-fat diet (LFD) or high-fat diet (HFD) intervention for 16 weeks. (**B**) Adipocyte size in HFD-fed hTLR10tg vs wild type (WT) mice after 16 weeks of diet intervention. (**C**) Representative images of gWAT stained for F4/80 in wild type (WT) and hTLR10tg mice after 16 weeks of HFD intervention at a 10x magnification with arrows indicating the presence of crown-like structures (CLSs), and average number of CLSs in the HFD groups. (**D**) Relative gene expression of *F4/80, Cd68, Cd11b, Cd11c, Cd206, Arg-1* and *Tlr2* of WT and hTLR10tg mice on a LFD or HFD. (**E**) Relative gene expression levels of the chemokine *Mcp-1* and several inflammatory cytokines in gWAT of WT and hTLR10tg mice. (**F**) Representative image of the liver stained for F4/80 in the HFD groups at a 10x magnification. (**G**) Relative mRNA levels of several inflammatory markers in the liver of WT and hTLR10tg mice on a LFD or HFD for 16 weeks (**H**). All data are expressed as mean + SEM; *p<0.05, **p<0.001, ***p<0.0001 effect of the diet; #p<0.05 effect of the genotype.

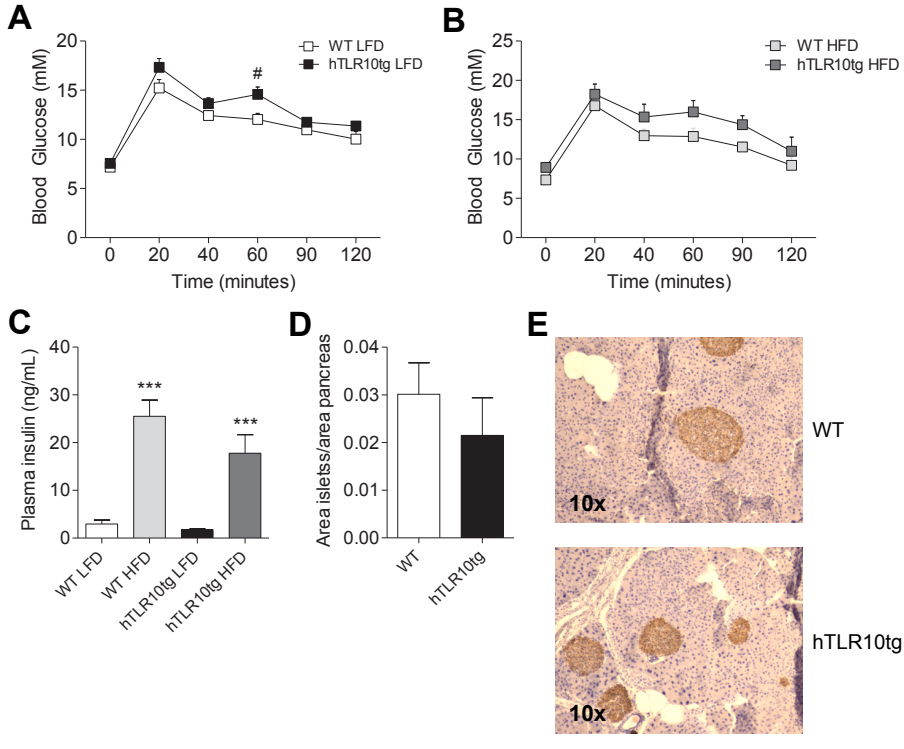

Figure 3. Glucose homeostasis
An oral glucose tolerance test (oGTT) performed at the end of the low-fat diet (LFD) (**A**) or high-fat diet (HFD) (**B**) intervention in wild type (WT) and transgenic (hTLR10tg) mice. (**C**) Plasma levels of insulin. (**D**) Relative presence of insulin islets in the pancreas of WT and hTLR10tg mice determined by calculating average islet surface area over average pancreas surface. (**E**) Pancreas slides stained for β cells. All data are expressed as mean + SEM; ***p<0.0001 effect of the diet; #p<0.05 effect of the genotype.

Human studies

Finally, we set out to validate our findings in humans. To do so, we compared adipocytes and macrophages numbers in the subcutaneous adipose tissue between obese individuals carrying SNPs in the *TLR10* gene versus obese individuals with WT alleles. In addition, BMI and plasma levels of the adipokines adiponectin and leptin were compared between either obese or lean individuals carrying SNPs in *TLR10* and individuals carrying WT alleles.

As expected, BMI positively correlated with plasma leptin levels, and negatively correlated with plasma adiponectin levels in both lean (**Figure 4A**) and obese individuals (**Figure 4B**). Three polymorphisms in *TLR10* associated with altered inflammatory responses were evaluated: rs11096955, rs11096957, rs4129009 [11, 22, 23]. Due to the fact that rs11096955 and rs11096957 are in strong linkage disequilibrium as calculated with LDlink (LDlink | An Interactive Web Tool for Exploring Linkage Disequilibrium in Population Groups), they are carried by the same individuals and data for both SNPs are shown in one graph. No difference in SNP frequencies was observed between obese versus lean subjects (data not shown).

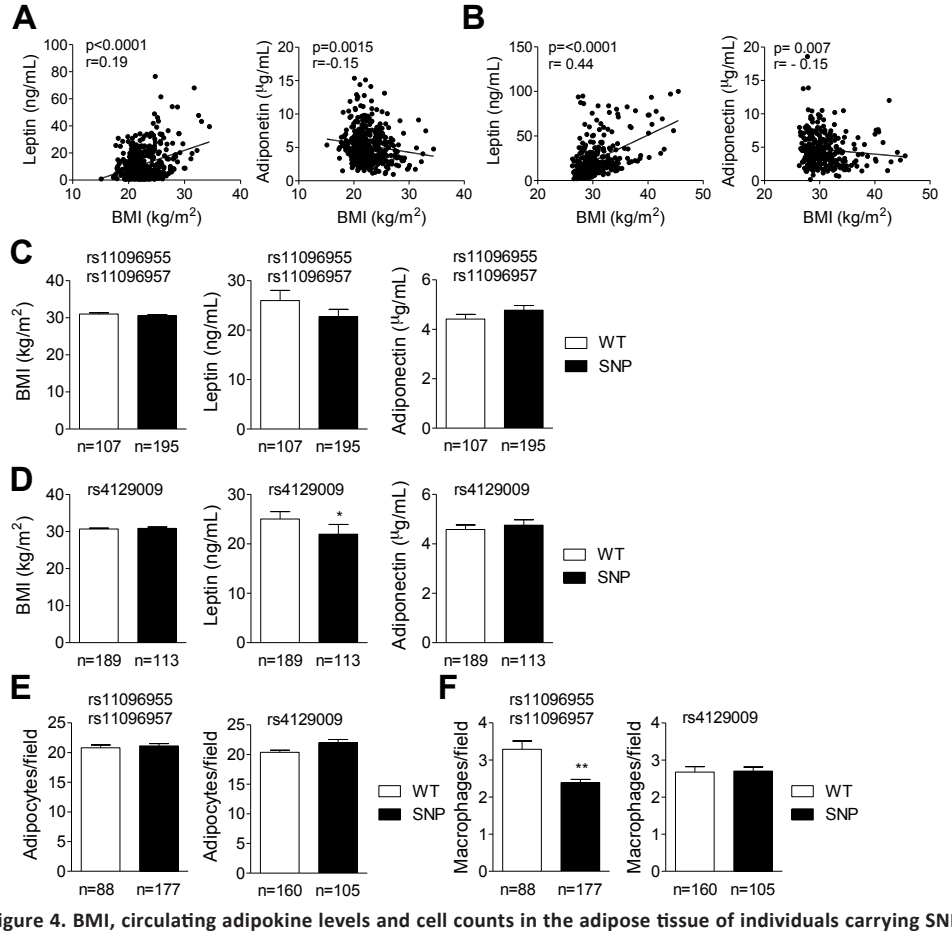

Figure 4. BMI, circulating adipokine levels and cell counts in the adipose tissue of individuals carrying SNPs in the *TLR10* gene

The Body Mass Index (BMI) calculated for 302 obese individuals and 427 healthy controls genotyped for three SNPs in the *TLR10* gene: rs11096955, rs11096957 and rs4129009. BMI plotted against plasma leptin and adiponectin in healthy controls (**A**) and obese individuals (**B**). BMI, plasma leptin and plasma adiponectin levels of obese individuals carrying the rs11096955 or rs11096957 allele (**C**), or the rs4129009 allele (**D**) compared to obese individuals carrying wild type (WT) alleles of *TLR10*. Number of adipocytes (**E**) and macrophages (CD68+ cells) (**F**) counted at a 40x magnification in obese individuals bearing WT alleles, the rs11096955 and/or rs11096957 SNP, or the rs4129009 SNP in *TLR10*. All data are represented as mean + SEM. *p<0.05, **p<0.01.

In both cohorts there was no difference in BMI between individuals carrying the mutant allele and individuals carrying WT alleles (**Figure 4C**, **4D**, and **Supplementary Figure 1**). In contrast, plasma leptin levels were significantly lower in obese individuals carrying the rs4129009 mutant allele (**Figure 4D**) and a similar trend was observed for rs11096955 and rs11096957 (**Figure 4C**). In the same cohort of obese individuals plasma adiponectin levels tended to be higher in individuals carrying either SNP in *TLR10* as compared to individuals bearing WT alleles (**Figure 4C** and **4D**). In lean individuals we did not observe a difference in plasma leptin and adiponectin levels between individuals with a mutation versus those carrying WT alleles (**Supplementary Figure 1**).

In our cohort of obese subjects we went on by assessing adipocytes number and macrophage infiltration in subcutaneous adipose tissue (**Figure 4E** and **4F**). There was no difference in adipocyte counts between individuals carrying the WT allele versus individuals carrying the mutant allele (**Figure 4E**). In contrast, macrophage numbers were significantly lower in individuals bearing the rs11096955 or rs11096957 mutant allele compared to individuals bearing WT alleles, whilst no such difference was observed for rs4129009 (**Figure 4F**).

DISCUSSION

Pattern recognition receptors, including Toll-like receptors (TLRs), not only sense foreign danger signals but can also be activated by endogenous signals including fatty acids [5]. During obesity, TLR signalling is thought to contribute to pro-inflammatory responses and metabolic abnormalities [24]. Interestingly, within the family of TLRs, TLR10 is the only receptor assigned with anti-inflammatory properties [11] and therefore may have the potential to modulate the development of metabolic disturbances during obesity. Using a transgenic mouse model expressing human *TLR10*, we demonstrate a role for TLR10 in adiposity illustrated by reduced adipose tissue weight and adipocyte size in hTLR10tg compared to wild type (WT) mice, despite equal bodyweight gain. In addition, plasma insulin levels tended to be lower in animals bearing h*TLR10*.

Analysing a cohort of obese individuals we observed that polymorphisms in the *TLR10* gene do not affect adipocyte numbers but appear to reduce macrophage numbers in the adipose tissue. Interestingly, individuals bearing the rs4129009 mutation have lower plasma levels for leptin compared to individuals bearing wild type (WT) alleles and a trend towards higher plasma adiponectin levels suggesting a possible effect of TLR10 on adipose tissue homeostasis. Together, these results are suggestive of an implication for TLR10 in adipose tissue remodelling and homeostasis. In lean individuals, polymorphisms in *TLR10* appeared not to affect BMI, plasma leptin or adiponectin, pointing to an interaction between *TLR10* activation and adipose tissue homeostasis specifically in obesity and states characterized by low-grade chronic inflammation.

Earlier work from our group has shown that *TLR10* is expressed in both adipocytes and non-adipocyte cells of human adipose tissue [25]. The function of TLR10 in adipocytes vs. non-adipocyte cells is currently unknown. Based on our in vivo results TLR10 affects both macrophages and adipocytes in the adipose tissue of mice, and cell-specific *TLR10* transgenic models will be valuable for elucidating the role of TLR10 in adipocytes and macrophages specifically during the development of obesity.

The *TLR10* expressed in adipose tissue macrophages may affect adipose tissue inflammation by determining macrophage phenotype. Based on our results, one might speculate that the presence and/or activation of TLR10 in macrophages drives polarization towards a more anti-inflammatory trait. This may partly be explained by the inhibitory effects of TLR10 on other TLRs that are known to promote pro-inflammatory properties of these cells [26]. To decipher what molecular pathways controlled by TLR10 may impact macrophage polarization, more research is needed. Moreover, potential ligand(s) driving TLR10 activation have not been identified yet. In the context of adipose tissue, certain types of saturated fatty acids, shown to activate other TLRs [5], may also have the potential to drive TLR10 stimulation. One approach may therefore be to test the effects of diets differing

in fatty acid composition and to perform in vitro studies with various fatty acids to decipher the contribution of different types of fatty acids on TLR10 activation in macrophages.

Interestingly, our results demonstrate that the presence of TLR10 lowers adipocyte size during HFD-feeding. Similarly, inhibition of TLR2 has been previously shown to reduce adipocyte hypertrophy [27]. Since human TLR10 specifically inhibits TLR2-mediated responses in vivo and in vitro [11], the decrease in adipocyte size in hTLR10tg animals may partly be explained by this mechanism of action. The *Tlr2* gene was significantly up-regulated by the diet intervention in transgenic mice only and tended to be higher in hTLR10tg versus WT mice on a HFD, suggestive of a relation between TLR10 and TLR2. Using a cross-linking antibody for TLR10 in an in vitro system that contains activators of other TLRs that are of relevance during obesity, for example fatty acids, may provide insight into the role for these interactions between TLR10 and pro-inflammatory TLRs like TLR2 in adipocyte hypertrophy, as well as in driving macrophage phenotypes.

On a systemic level, the effects of TLR10 on glucose homeostasis were modest. However, plasma insulin concentrations tended to be lower in animals expressing *TLR10*, suggestive of increased insulin sensitivity. Hence, activating TLR10 during obesity may have some beneficial metabolic effects.

One may argue, however, that over-expressing the human *TLR10* gene into a mouse model also has downsides. Although it allowed us to establish the effects of TLR10 in various tissues during the development of obesity, several important issues still need to be addressed. For example, is the molecular machinery for a fully functional TLR10 pathway present in adipose tissue? Are there unknown endogenous pathways that either promote or interfere with TLR10 signalling in the transgenic animal model? Does the *Tlr10* pseudogene present in in mice [28] interact with the human *TLR10* gene, thereby affecting our results?

Our results in humans demonstrate that obese individuals carrying the rs11096955 or rs11096957 SNP in *TLR10* have significantly reduced macrophage numbers. Moreover, individuals bearing the rs4129009 mutation have significantly lower levels of leptin in plasma compared to individuals bearing the WT allele, and display a trend towards higher plasma adiponectin levels. Similar trends in plasma leptin and adiponectin were observed in individuals bearing either the rs11096955 or rs11096957 allele. Together these results are suggestive of a protective role of *TLR10* SNPs in humans. Interestingly, this interaction between TLR10 activation and adipose tissue homeostasis was specific for obese individuals, suggestive of a role for TLR10 only when a state of low-grade chronic inflammation has developed. Additional measurements assessing adipose tissue inflammation and insulin sensitivity in obese individuals carrying one of the three SNPs in *TLR10* will be needed to evaluate whether the observed reduction in macrophage number affects adipose tissue functioning as well. In addition, the functional consequences of the analysed polymorphisms in TLR10 are not clear. Our data imply that certain SNPs in *TLR10* would result in a gain

of function and subsequently could ameliorate disturbances in adipose tissue homeostasis during obesity. The *TLR10* SNPs described above are the consequence of missense mutations which, among nonsynonymous mutations (mutation that affect protein structure), can have a large palette of functional manifestations. Since it is difficult to separate the effects of our mutations from interactions with the environment and genomic profile of each individual, further studies using mice carrying these SNPs in *TLR10* might help clarifying their functional consequences [29]. Ultimately, cross-linking TLR10 in the adipose tissue of obese individuals could be useful to understand the influence of TLR10 on adipose tissue metabolism and metabolic disturbances during obesity.

In conclusion, although more work is needed, our results demonstrate a role for TLR10 in adipose tissue morphology during obesity.

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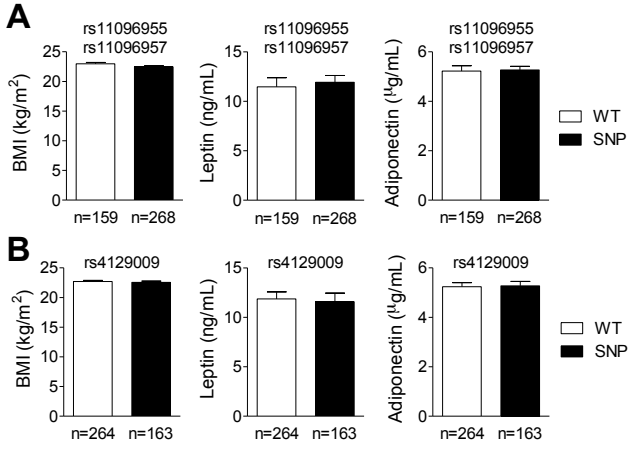
SUPPLEMENTARY FIGURE



Supplementary Figure 1. BMI and circulating adipokine levels of lean individuals carrying SNPs in the *TLR10* gene

BMI, plasma leptin and plasma adiponectin levels of lean individuals carrying the rs11096955 or rs11096957 allele (A), or the rs4129009 allele (B) compared to lean individuals carrying wild type (WT) alleles of *TLR10*.

8

General discussion

Macrophages and their monocyte precursors are challenged with a plethora of stressors on a daily basis. Regardless of the numerous occasions at which they succeed in maintaining or re-establishing homeostasis, macrophages are particularly known for their role in a variety of pathologies that are associated with systemic chronic inflammation. These pathologies include various metabolic diseases, such as Type 2 Diabetes and cardiovascular disease, in which systemic chronic inflammation is an integral part of disease progression and outcome (1). Already in the early '90s, expanding adipose tissue was identified to be an important source of inflammatory cytokines that interfere with insulin sensitivity in obese individuals (2-6). Macrophages were pinpointed as predominant source of these elevated pro-inflammatory cytokines in obese adipose tissue, following from an increase in number and shift toward a pro-inflammatory phenotype during obesity (7-10). Since then, multiple other immune cells have been shown to populate the adipose tissue, including dendritic cells, mast cells, neutrophils, B-cells and T-cells (1, 11). Although most likely alterations in the composition and number of immune cells together contribute to the development of a state of low-grade inflammation in obese adipose tissue (1, 11), macrophages have retained particular interest. On the one hand they build upon their role as phagocytes by clearing dead adipocytes (12, 13), yet also acquire several adipose tissue-tailored functions including lipid buffering (14), and communicate with other immune cells to maintain immune balance in the adipose tissue (15, 16). Consequential to being a central player for adipose tissue homeostasis in the lean state, continuous pro-inflammatory activation of ATMs in the obese state severely affects adipose tissue functioning. As such, ATMs constitute an important link between obesity and metabolic disturbances including insulin resistance and, eventually, Type 2 Diabetes (7, 10, 17).

Due to their pivotal role during the development of adipose tissue inflammation there is a strong urge for understanding mechanisms underlying macrophage activation in obese adipose tissue. Although considerable progress has been made, the underlying causes and phenotypical and functional characteristics of macrophage activation in obese adipose tissue are still far from completely understood. In this thesis, several aspects related to activation of macrophages and their monocyte precursors were evaluated. First, we revealed and clarified specificity in metabolic routes used by human monocytes in response to stimulation with various microbial stimuli (**Chapter 2**). From these insights into the role of intracellular metabolism upon activation of core immunological pathways in macrophage precursors, we went on by examining intracellular metabolism in macrophages residing in the complex adipose tissue environment. We identified adipose tissue macrophages (ATMs) to be uniquely metabolically activated during obesity, and showed that several metabolic routes critically contribute to their inflammatory activation (**Chapter 4**). The ATM origin, phenotype and function, as well as potential factors that may drive the ATM phenotype were reviewed in **Chapter 3**, and the relevance of lipids as driver of the ATM phenotype further investigated in **Chapter 5**. We evaluated clearance of adipocytes by macrophages in an obese

versus lean adipose tissue environment, and showed that immunologically silent responses to dead adipocytes are disrupted in macrophages primed by obese adipose tissue (**Chapter 6**). Lastly, we examined the impact of TLR10 on the adipose tissue in lean and obese mice and humans (**Chapter 7**). The different insights on ATM metabolism and inflammatory activation as obtained in this thesis are presented in **Figure 3**. In this final chapter, the potential of targeting intracellular metabolism for shaping functional output of ATMs and, ultimately, abrogating adipose tissue inflammation, will be evaluated. In this light, the relevance and implications of our findings are discussed and suggestions for future research are made.

Retrieving constructive adipose tissue macrophages during obesity

First, it is important to realize that whatever the cause of the inflammatory activation of macrophages is, the initial purpose was to conquest a stressor – either microbial or non-microbial of origin – that is or could be potentially harmful (18). In case of the adipose tissue, ATMs are known to buffer lipids released during pronounced adipocyte lipolysis or from over-engorged adipocytes, in order to protect other tissues from high and potentially toxic levels of lipids (14, 19). Moreover, they clear dead adipocytes from the adipose tissue that may otherwise release their noxious content into the interstitial fluid (13, 18, 20). In the lean state these actions are effectively executed in an immunologically silent or anti-inflammatory manner. Obese adipose tissue, however, is characterized by a state of chronic inflammation reflective of homeostatic imbalance that may be importantly fuelled by macrophages incapable to effectively execute the above mentioned functions. A future challenge will be to initiate ATM adaptations that support their functioning conform increasing demands of the obese adipose tissue environment. In this way, we may prevent or combat pathological inflammatory activation of macrophages during obesity that contributes to the development of insulin resistance.

Intracellular metabolism translates environmental factors into functional output

The last decade, new insights in the field of 'immunometabolism' have brought forward intracellular metabolism as essential driver of macrophage function including inflammatory cytokine release and phagocytosis (21-23). As such, macrophage metabolism is currently being viewed as potential target for therapeutic interventions aimed at curtailing inflammation in various pathologies that are defined by excessive macrophage activation.

Monocytes and macrophages can be activated by various stimuli or '*stressors*'. These may include pathogens or pathogen-associated molecular patterns (PAMPs) on the one hand, and dead cells or damage-associated molecular patterns (DAMPs) on the other. These *stressors* require functional responses of monocytes or macrophages, for example phagocytosis, that are fuelled by specific metabolic routes (**Figure 1,** *arrow I and III*).

Indeed, metabolic reprogramming of macrophages highly depends on the type of *stressor* that activates them (21). This is clearly illustrated by the diverging degree to which various metabolic routes are used by human monocytes stimulated with different microbial stimuli (**Chapter 2**). We found that both lipopolysaccharide (LPS) and Pam_3CysSK_4 (P3C), as well as whole bacterial lysates, increase glycolysis yet predominantly vary in the activation of oxidative phosphorylation (OXPHOS) after stimulation. In fact, LPS was unique in minimizing mitochondrial spare respiratory capacity (SRC) of human monocytes, disabling monocytes to use their mitochondria for ATP production. Importantly, other environmental *'cues'* like nutrient availability, cytokines, growth factors and oxygen will also significantly shape the metabolic trait of monocytes or macrophages (22) (**Figure 1**, *arrow II*). For example, human monocytes stimulated with LPS that essentially use glucose for ATP generation via glycolysis, have been shown to switch toward OXPHOS as source for ATP production upon glucose deprivation (24). In a tissue environment, a combination of *stressors* and *cues* demands for specific macrophage functions and drives unique metabolic traits. Indeed, the metabolic trait of ATMs – characterized by increased OXPHOS and glycolysis (**Chapter 4**) – is not only driven by *stressors* that are most likely non-pathogenic of origin, including factors derived from stressed or dying adipocytes, but also by cytokines, nutrients and various other local *cues*.

Following this line of reasoning, intracellular metabolism of macrophages is the cumulative outcome of environmental *stressors*, either pathogenic or non-pathogenic of origin, and other environmental *cues*, like nutrient availability or cytokines. Together these *stressors* and *cues* tailor functional macrophage responses in an environment with specific spatial and temporal characteristics and demands. Changing the metabolic routes used by macrophages will affect their functional output (25)(**Chapter 2** & **4**) (**Figure 1**, *arrow III*), and alter the environment, for example via the secretion of lactate (**Figure 1**, *arrow IV*). In addition, execution of function will change the environment (e.g. by removing the stressor) (**Figure 1**, *arrow V*) and demand for new metabolic choices (e.g. introduce the need for metabolic processing of an embodied stressor) (**Figure 1,** *arrow VI*). Hence, the environment, macrophage metabolism, and macrophage function are highly intertwined with intracellular metabolism as central spill translating environmental factors into macrophage function. As a consequence, targeting intracellular metabolism for shaping or directing macrophage function requires full understanding of 1) metabolic routes driving certain macrophage outputs and 2) what and how *stressors* and *cues* affect macrophage metabolism in a given environment.

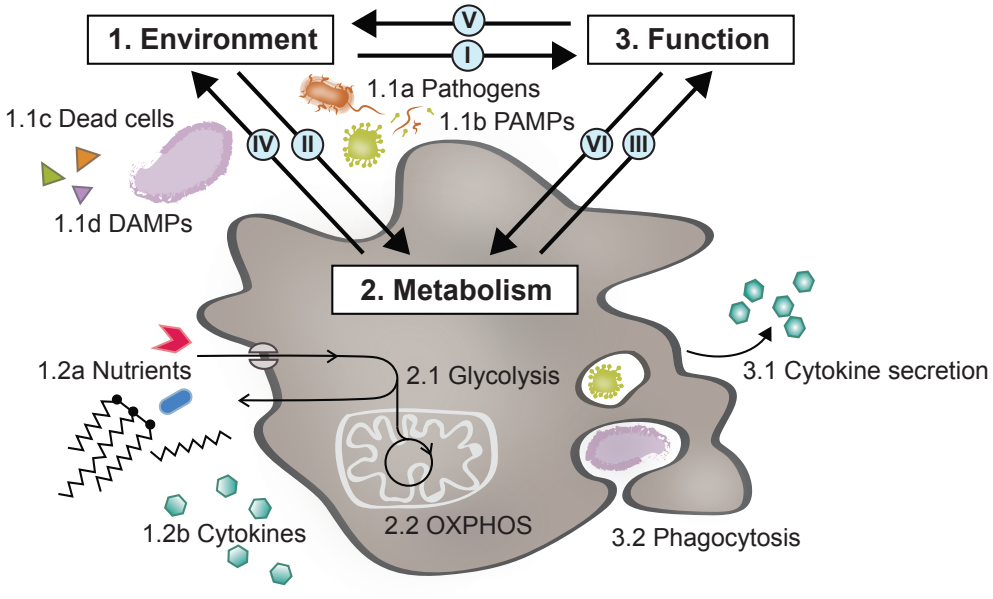


Figure 1. **Representation of interactions between the environment, monocyte or macrophage metabolism, and their function.**
In each different environment, being the circulation or a specific tissue, monocytes or macrophages are confronted with a unique spectrum of stimuli (1) that together shape their phenotype (2+3). These stimuli encompass pathogens and dead cells including their derivatives; danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) (1.1), but also include other molecules present in the environment, such as cytokines and nutrients (1.2). For the sake of clarity, these stimuli will be referred to as '*stressors*' (1.1) and '*cues*' (1.2). *Stressors* demand for a specific action of monocyte or macrophages (function), for example phagocytosis or cytokine production (I), but may also shape intracellular metabolism via binding Toll-like receptors that activate several intracellular signalling pathways affecting metabolic choices (II). *Cues* do not directly demand for a specific function but can shape macrophage functioning, including via reprogramming intracellular metabolism (II) that is needed for monocyte or macrophage function (III). By removing nutrients from the environment, and secreting end-products of intracellular metabolism (IV) on the one hand, and removing a *stressor*, or secreting cytokines (V) on the other hand, monocytes or macrophages change their environment as well. Lastly, monocytes or macrophages adapt their intracellular metabolism upon the execution of a certain function, for example to enable processing of engulfed *stressors* (VI).

Intracellular metabolism to support function: cytokine release and phagocytosis

Knowing what metabolic routes underlie different functional outputs of monocytes or macrophages is undoubtedly essential if one aims to target intracellular metabolism for therapeutic gain. Interestingly, we found that glycolysis importantly contributes to cytokine release in monocytes and macrophages irrespective of the manifested *stressor* (**Chapter 2 & 4**). First, monocytes stimulated with LPS or P3C were both characterized by cytokine release and enhanced glycolysis, and inhibition of glycolysis using 2-deoxyglucose (2-DG) strongly reduced cytokine secretion (**Chapter 2**). Similarly, ATMs from obese adipose tissue and obese adipose tissue-primed macrophages displayed increased glycolysis and higher cytokine secretion as compared to their lean counterparts. Accordingly, competing with glycolysis using 2-DG decreased cytokine release by ATMs (**Chapter 4**). A dominant role for

glycolysis in cytokine release has been proven to generally hold truth in innate immune cells stimulated with various pathogenic stimuli (26-28). However, two things should be taken into account. First, 2-DG but also other inhibitors widely used to block glycolysis or other metabolic routes induce considerable off-target effects, blunting the interpretation of data obtained using these inhibitors. Robust examination of metabolic pathways involved in macrophage functioning is considered a future challenge and will be addressed accordingly later in this discussion. Second, most data including ours are derived from in vitro experiments using medium in which glucose (and glutamine) is prominently available, yet that lacks some other primary nutrients like fatty acids (FAs). As a consequence, macrophages in vivo may use a more complex combination of nutrients hence metabolic pathways upon activation depending on local nutrient availability. In case of the adipose tissue, lipids may compose an important metabolic substrate. It would therefore be worthwhile to combine ex vivo measurements with measurements of metabolites or enzyme activity in snap-frozen ATMs, to verify the in vivo findings of metabolic reprogramming and learn more about underlying pathways leading to the specific metabolic trait of these cells. Nevertheless, GLUT1, the dominant glucose transporter in macrophages, is highly expressed by macrophages in crown-like structures (CLSs) of obese adipose tissue, pointing to a role for glycolysis in activated ATMs in vivo as well (29).

The contribution of OXPHOS to cytokine release appears to particularly rely on the *stressor* involved. Specifically, monocytes stimulated for twenty-four hours with P3C display dependency on OXPHOS for fuelling cytokine secretion, while no such dependency was found in monocytes stimulated with LPS (**Chapter 2**). In ATMs we did not use Rotenone to impair OXPHOS via inhibition of Complex I of the electron transport chain, yet found lower cytokine release upon the inhibition of glucose oxidation and, to a lesser extent, fatty acid oxidation (FAO), especially in ATMs derived from lean adipose tissue (**Chapter 4**). Hence, OXPHOS does – to some extent – contribute to cytokine release in ATMs as well as P3C- but not LPS-stimulated monocytes. In innate immune cells OXPHOS may, however, particularly fuel other functions than cytokine release, for example phagocytosis (30). Indeed, we found OXPHOS to contribute to phagocytosis in P3C-stimulated but not LPS-stimulated monocytes, the latter being characterized by lower mitochondrial capacity and phagocytic capacity to start with (**Chapter 2**). Other studies, however, did find a role for glycolysis during phagocytosis by monocytes or macrophages (31, 32). Although many reasons may explain this discrepancy, it might well be reflective of the different phagocytosis assays used in the experiments, further corroborating stressor-specific remodelling of monocyte and macrophage metabolism. A schematic representation of the contribution of energy metabolism to function in human monocytes stimulated with LPS or P3C is provided in **Figure 2**.

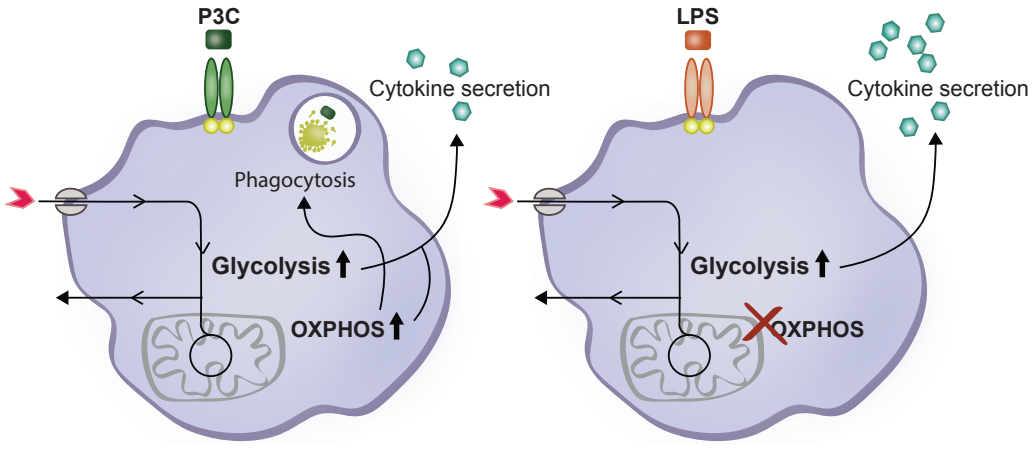


Figure 2 Representation of metabolic regulation in human monocytes stimulated with P3C or LPS, and its relation to their functional output.
Different pathogenic stimuli, including Pam_3CysSK_4 (P3C) and lipopolysaccharide (LPS), uniquely reprogram monocyte metabolism to facilitate functioning. Glycolysis is enhanced both by P3C, that activates Toll-like receptor (TLR)2, and by LPS, that activates TLR4, and fuels cytokine release upon stimulation with either stimulus. In contrast, oxidative phosphorylation (OXPHOS) is enhanced upon P3C-stimulation, but not changed in LPS-stimulated monocytes. Consequentially, OXPHOS contributes to cytokine release in P3C- but not LPS-stimulated monocytes. Moreover, OXPHOS but not glycolysis fuels phagocytosis upon P3C stimulation. In line with reduced mitochondrial capacity upon LPS stimulation, the phagocytic capacity of monocytes stimulated with LPS is low.

In contrast to phagocytosis of bacterial components upon activation of Toll-like receptors (TLRs), phagocytosis in the adipose tissue most likely involves clearance of damaged, dying or dead adipocytes predominantly. In general, uptake of apoptotic cells (efferocytosis) is considered to mechanistically differ from phagocytosis of pathogens. In fact, increasing evidence suggests adipocyte clearance to be different from 'classical' efferocytosis as well (33). Instead of engulfing the complete dying cell and subsequent intracellular breakdown (34), adipocytes are probably far too big for complete uptake by the markedly smaller macrophages. Indeed, macrophages were found to process bits of adipocytes (35), in line with a gradual decrease in size of dead adipocytes part of CLSs (36). Moreover, adipocyte clearance has recently been shown to involve exocytosis of lysosomes into extracellular acidic compartments formed near dying adipocytes, suggestive of extracellular instead of intracellular breakdown of the efferocytic load (13, 20). However, many aspects regarding recognition and processing of dead adipocytes by macrophages remain unclear. An interesting aspect that warrants further attention is whether or not macrophage clearance of dead adipocytes is solely reactive to the release of content due to rupture of the adipocyte membrane, or involves specific recognition of cell death markers like phosphatidylserine (PS) at the cell surface of a dying adipocyte. Although subtle, we found signs of increased cell death in the adipose tissue upon inhibition of receptors of the TAM receptor tyrosine kinase family that recognise PS at the dead cell membrane (37)(**Chapter 6**). This would suggest that the well-controlled efferocytic machinery plays a role during adipocyte clearance as well, and is potentially sensitive for dysregulation during obesity.

Regardless of passive or active recognition of dead adipocytes by ATMs, dead adipocytes are complex cells to deal with. Considering the schematic overview given in **Figure 1**, dead adipocytes may be qualified both as *stressor* and *cue*; requiring the clearance of a corpse as well as high amounts of lipids that demand for effective lipid processing by the macrophage. Indeed, macrophages exposed to dead adipocytes display increased expression of genes involved in lipid uptake and processing including lysosomal acid lipase compared to macrophages exposed to live adipocytes (**Chapter 6**). Of note, no difference in the expression of genes involved in glycolysis was found, suggestive of lipid handling to be a specific requirement for effective adipocyte clearance by macrophages. In vivo, ATMs part of obese adipose tissue are characterized by enhanced lysosomal biogenesis and activity, accompanied by increased expression of several markers of lipid uptake, storage, breakdown and export (19, 20, 38)(**Chapter 5 & 6**). As such, ATMs populating obese adipose tissue have been identified to be metabolically rather than inflammatory activated (38). Interestingly, the metabolically activated phenotype of ATMs has recently been shown to be crucial for adipocyte clearance (20), suggestive of a critical link between intracellular metabolism and efferocytosis of adipocytes by ATMs. We observed increased OXPHOS and glycolysis in macrophages in an obese adipose tissue environment (**Chapter 4**), and it would be interesting to examine whether OXPHOS and/or glycolysis are of importance during adipocyte clearance. To do so, intracellular metabolism should be examined in macrophages that take up or have taken up parts of dead adipocytes. Unfortunately, however, it represents a technical challenge to determine macrophage metabolism during adipocyte clearance by real time energy flux measurements as both macrophages and adipocytes display metabolic activity. Nevertheless, considering the profound size and high lipid content of dead adipocytes one could speculate that both lysosomal lipases and OXPHOS, in particular fatty acid oxidation (FAO), will be crucial for macrophages to effectively process dead adipocytes.

Of note, in addition to cytokine release and adipocyte clearance, ATMs have been shown involved in adipogenesis, angiogenesis, extracellular matrix remodelling and antigen presentation (39). Although these functions were not addressed in this thesis, they should be taken into account when studying the relation between intracellular energy metabolism and ATM functioning. Moreover, although we have and will predominantly focus on catabolic routes, metabolism can also be used for the production of building blocks or signalling molecules (25). The contribution of such anabolic metabolism to ATM functioning forms an interesting field for future studies as well.

The adipose tissue environment as driver of macrophage metabolism and function

The source and nature of lipids in the adipose tissue

Various compounds present in the adipose tissue environment may affect the ATM phenotype. Lipids, however, are generally considered to play a central role. Indeed, already in the lean state, we found genes involved in lipid handling to be a unique feature of ATMs (**Chapter 5**). During obesity, the expression of lipid-related genes in ATMs is further upregulated (**Chapter 5 & 6**). As described above, ATMs may be exposed to lipids via damaged or dying adipocytes that increase in both number and size during obesity (13, 40, 41)(**Chapter 6**). An alternative route of exposure is via adipocyte lipolysis. This has been demonstrated upon weight loss or β-adrenergic stimulation that both increase adipocyte lipolysis and drive lipid-loading by macrophages (14, 42). During obesity, adipocyte lipolysis is enhanced by virtue of increased total fat mass, and may be further increased when adipocytes become resistant to insulin (20, 43, 44). Lastly, ATMs may be exposed to lipids through lipoproteins (45). The relevance of lipoproteins as source of lipids for ATMs is, however, questionable (46), especially in mouse models without any genetic manipulation that ensures the development of dyslipidaemia.

While adipocyte lipolysis will predominantly enhance the concentration of free fatty acids (FFAs) in the direct surroundings of ATMs, stressed adipocytes have been shown to secrete small lipid droplets that may contain both FFAs and triglycerides (TGs) (47). Moreover, extracellular degradation of apoptotic adipocytes (13) results in either complete or partial breakdown of lipid droplets, thereby contributing to the pool of both TGs and FFAs. The relative contribution of FFAs versus TGs as drivers of the ATM phenotype is currently unclear. Although our in vitro model of macrophages exposed to adipose tissue-conditioned medium points to a prominent role for FFAs (**Chapter 5**), reduced lipid loading upon silencing of lipoprotein lipase (LPL) – involved in hydrolysis of extracellular TGs – in ATMs part of obese adipose tissue is suggestive of a role for TGs as well (48). In addition, the mechanism of uptake of TGs and FAs remains to be elucidated. Although LPL-induced lipid-loading of ATMs (48) points to a dominant role for extracellular breakdown of TGs, TGs and FAs itself or as part of lipid droplets may also be taken up via endocytosis or phagocytosis. Hence, the relevance of FFAs and TGs for shaping the ATM phenotype as well as the mechanism of uptake involved needs further study, and may bring to light key pathways involved in driving the ATM phenotype that can potentially be targeted for clinical purposes.

Increased lipid exposure as key driver of the ATM phenotype during obesity

We observed increased OXPHOS and glycolysis in macrophages exposed to a synthetic TG emulsion called Intralipid (**Chapter 5**), similar to metabolic activation found in macrophages exposed to adipose tissue-conditioned medium (**Chapter 5**) and in ATMs part of obese adipose tissue (**Chapter 4**). This finding strongly suggests that lipids are an important driver

of the unique metabolic phenotype of ATMs in obesity. The lipid-laden appearance of ATMs in CLSs part of obese adipose tissue has been directly linked to a pro-inflammatory ATM phenotype, suggestive of a crucial role for lipids in driving inflammatory properties of ATMs as well (12, 49-51). Indeed, lipids are considered to form the basis of inflammation observed in metabolic diseases including atherosclerosis and obesity (52, 53). Via activating TLR signalling (54, 55), generation of bioactive lipid mediators (56), and ER stress (57) either driven by changes in the composition of lipid membranes (58, 59) or reactive oxygen species (ROS) (60), lipids may induce pro-inflammatory macrophage responses. Interestingly, because macrophage glycolysis was strongly induced by lipids (**Chapter 4** & **5**) and appeared to predominantly contribute to inflammatory cytokine release by ATMs isolated from obese adipose tissue (**Chapter 4**), one may speculate glycolysis to be an alternative route through which lipids fuel inflammatory macrophage responses in obese adipose tissue. Such a relation between glycolysis and inflammatory cytokine release did not hold true in bone marrow-derived macrophages (BMDMs) exposed to Intralipid (**Chapter 5**), however, and may require lipotoxicity involving TLR activation, lipid mediators and ER stress as well. Because Intralipid is enriched in unsaturated FAs, it may lack such lipotoxic potential that is associated with saturated FAs predominantly (55, 57, 61).

Macrophages in a lipid-enriched environment as the adipose tissue are expected to develop a mechanism to limit lipotoxicity. The formation of lipid droplets is one way to neutralize otherwise toxic FFAs (62). Limiting lipid uptake may be an alternative approach. Interestingly, we observed strong upregulation of *angiopoietin-like 4* (*Angptl4*) in macrophages in lipid-rich environments including the adipose tissue (**Chapter 5**). As endogenous inhibitor of LPL, ANGPTL4 may protect macrophages from lipid overloading and thus lipotoxicity. Surprisingly, however, ANGPTL4 appeared not to be involved in lipid loading of macrophages in an adipose tissue environment in vitro (**Chapter 5**). This may be explained by a predominant presence of FFAs over TGs in adipose tissue-conditioned medium, thus LPL-independent lipid uptake, or may point toward roles other than inhibiting LPL for ANGPTL4 in macrophages. Intriguingly, despite equal lipid loading, *Angptl4*$^{-/-}$ BMDMs primed with adipose tissue explants displayed enhanced cytokine release upon LPS stimulation compared to wild-type BMDMs. Such increased inflammatory activation of macrophages lacking *Angptl4* was not apparent without adipose tissue-priming, suggestive of the existence of a link between exposure to lipids, ANGPTL4, and inflammatory activation of macrophages. In addition to being a well-known target gene of peroxisome proliferator activated receptors (PPARs), *Angptl4* expression is also regulated via HIF-1α in myeloid cells (63). We observed upregulation of *Hif-1α* and several of its target genes in macrophages in an obese adipose tissue environment (**Chapter 4**). As target of HIF-1α, *Angptl4* might be directly involved in inflammatory responses of macrophages and be part of a negative feedback mechanism limiting pro-inflammatory activation in lipid-enriched environments (**Chapter 4**). To what extent and in what manner ANGPTL4 is involved in lipid uptake and lipotoxicity

including inflammatory activation of macrophages in the obese adipose tissue environment is an interesting question that requires further investigation.

Additional factors potentially involved in shaping the pro-inflammatory ATM phenotype

Intriguingly, macrophages exposed to excessive amounts of lipids in lean adipose tissue do acquire a lipid-laden appearance, yet in contrast to the obese state do not develop pro-inflammatory characteristics (14, 42, 64). In fact, they display an anti-inflammatory phenotype and promote adipose tissue remodelling through adipocyte clearance and adipogenesis (14, 42). It is therefore unlikely that exposure to dead adipocytes or lipids on itself is sufficient to explain functional output and inflammatory characteristics of ATMs in the obese state. Rather, other changes in the adipose tissue environment make macrophages respond differently in obese versus lean adipose tissue.

Lipid species

In addition to an increase in the amount of lipids, the composition of lipids in the adipose tissue has been shown to shift toward a more saturated profile in the obese state (65-68). Since saturated FAs are well known for inducing lipotoxicity, this shift in lipid composition may contribute to the more pro-inflammatory ATM responses observed during obesity. Moreover, saturated FAs may interfere with lipid raft integrity and membrane fluidity that are essential for efferocytosis (59, 61, 69, 70), and may be critical for adipocyte clearance as well. In general, by inducing a pro-inflammatory phenotype, saturated FAs may indirectly interfere with macrophage functions other than cytokine release. Indeed, both stimulation of adipogenesis and effective efferocytosis are associated with an anti-inflammatory macrophage phenotype (42, 71) and may be disrupted upon lipid-induced inflammatory activation of macrophages.

DAMPs and TLR-signalling

In addition to changes in lipid composition, obesity-induced changes in *stressors* or *cues* in the adipose tissue environment may affect macrophage functional output directly, or via shaping their response toward dead adipocytes or lipids. First, stressed or dying adipocytes release DAMPs such as DNA (72) and HMGB1 (73, 74) that may activate ATMs via binding various receptors including TLRs. Indeed, TLR expression and signalling, predominantly that of TLR2 and TLR4, have been shown enhanced in obese adipose. Activation of these receptors was found to contribute to adipose tissue inflammation during obesity and, ultimately, impaired insulin sensitivity (75, 76). Interestingly, signalling downstream of TLRs may not only affect macrophage responses directly, by activation of phagocytosis or cytokine production (77) (**Chapter 2**), but also indirectly, by interfering with lipid handling by macrophages. For example, LPS-induced activation of TLR4 on macrophages in a lipid-rich environment fuels lipotoxicity and cell death by enhancing ceramide production (78) and triggering lysosomal

damage and depletion (79).

Recently, TLR10 has been identified to be the first and so far only anti-inflammatory member of the TLR family. Via dimerization with other TLRs including TLR2, TLR10 can inhibit pro-inflammatory signalling (80). Given that pro-inflammatory activation in obese adipose tissue has been shown to involve TLR activation, we hypothesized that introducing human *TLR10* into the mouse genome would attenuate the development of adipose tissue inflammation during obesity. We did, however, not find profound effects of human *TLR10* on the development of adipose tissue inflammation in mice on a high-fat diet for 16 weeks (**Chapter 7**). Interestingly, however, *TLR10* transgenic mice displayed reduced adipose tissue mass and adipocyte size, pointing toward a role for *TLR10* in adiposity. In humans, obese individuals carrying single nucleotide polymorphisms (SNPs) in *TLR10* had or tended to have lower leptin levels in plasma and reduced macrophage numbers in the adipose tissue. Intriguingly, no such associations were detected in lean individuals, suggestive of a role for TLR10 in adipose tissue specifically in obesity when a state of low-grade inflammation develops. Whether or not these SNPs in *TLR10* affect adipose tissue inflammation and insulin sensitivity, and whether *TLR10* mediates its effects in adipocytes, macrophages, or other immune cells, requires further investigation.

Adipokines and cytokines

Hypertrophic, stressed adipocytes may also be a source of pro-inflammatory adipokines (81-83). Leptin is one of the most well-known adipokines that directly correlates with adipose tissue mass (84) and favours inflammatory activation of macrophages (85). Although we found no changes in intracellular metabolism of macrophages stimulated with leptin in vitro (**Chapter 4**), leptin has been shown to promote lipid body formation in macrophages (86). As a consequence, leptin may well affect lipid handling by macrophages in a lipid-rich environment such as the adipose tissue, yet this requires further study.

In addition to adipokines, immune cell- or adipocyte-derived cytokines may also affect macrophage responses in a lipid-rich environment. For example, IL-4 has recently been shown to enhance clearance of adipocytes, most likely by increasing macrophage capacity for lipid uptake and FAO (41). The concentration of various cytokines in the adipose tissue rises during the development of obesity (87) yet pro-inflammatory cytokines gain relative abundance over anti-inflammatory cytokines at progressed stages of obesity, which may relate to alterations in the abundance and activation state of immune cell populations (1, 11). Such a shift in cytokine abundance may importantly affect macrophage metabolism and function. On the one hand, anti-inflammatory cytokines, such as IL-4 and IL-10, promote OXPHOS in general and FAO specifically (88-91), whereas on the other hand pro-inflammatory cytokines, including IL-6 and TNF-α, have been shown to enhance glycolysis (92, 93). These experiments, however, have been mainly done in vitro and it would therefore be relevant to examine how exactly cytokines present in the obese versus lean adipose tissue reprogram macrophage responses including intracellular metabolism in a lipid-

rich environment. Interestingly, we found strong depletion of interferon (IFN)-signalling pathways in macrophages in obese adipose tissue (**Chapter 6**). Whether impaired IFN signalling by metabolically activated macrophages in obese adipose tissue is the result of reduced presence of external IFNs, reflects reduced initiation of macrophage IFN responses upon external cues other than IFNs (for instance exogenous DNA (94)), or can be considered a general outcome measure of reduced or altered macrophage functioning, remains to be elucidated. In dendritic cells autocrine IFN signalling has been shown to increase FAO (95), yet if and how exactly lower IFN signalling affects ATM metabolism warrants future studies as well. Interestingly, oral administration of IFN-τ, a Type I IFN, during high-fat diet (HFD)-feeding of mice reduced the pro-inflammatory trait of ATMs and alleviated insulin resistance (96). On the other hand, depletion of IFNAR, a key receptor of Type I IFNs, in adipocytes has been shown to increase weight gain and aggravate glucose intolerance in HFD-fed mice (97). Given the numerous interactions between macrophages and adipocytes, decreased IFN signalling and release by ATMs may importantly affect adipocyte functioning in obese adipose tissue. Hence, raising IFN signalling in ATMs during obesity may provide a potential mechanism for improving adipose tissue functioning that deserves further attention.

Nutrients

At all times, nutrient availability will critically determine macrophage metabolism. For example, lactate has been shown to importantly affect macrophage metabolism in a tumour environment (98). Although we found no evidence for lactate on its own driving the ATM metabolic phenotype in vitro (Chapter 4), this does not exclude a role for lactate in shaping metabolic programming of macrophages in a lipid-rich environment as the adipose tissue. The potential of nutrients to interfere with effective lipid processing is clearly illustrated by glutamine that fuels lipotoxicity, i.e. lysosomal damage and cell death, in classically activated macrophages in a lipid-rich environment (99). In contrast to glutamine, glucose appears not to enhance lipid-induced cell death in macrophages (79). However, glucose may importantly compete with FAs for processing as a result of the reciprocal relation between glucose and FAO known as the Randle cycle; in which metabolic intermediates of one pathway inhibit enzymes of the other (100). Because glycolysis itself has been shown crucial for inflammatory cytokine release by ATMs (Chapter 4), increased glucose levels during obesity may, similar to macrophage activation via TLRs or cytokines that stimulate glycolysis, importantly impact on metabolic choices and functional output of ATMs.

Targeting intracellular metabolism to align adipose tissue macrophage functioning with increased environmental demands

Considering the many factors present in obese adipose tissue that all potentially affect the ATM phenotype, various different approaches may be undertaken to reduce the pro-inflammatory trait of ATMs populating obese adipose tissue. Anti-inflammatory approaches have been numerous and, following from the tight connections between immunity and

metabolism, in animal models often positively affect systemic metabolism as well (101). Unfortunately, however, the clinical effect of most anti-inflammatory drugs has been somewhat disappointing so far (102, 103). Importantly, acute, short-lived inflammatory signalling in the adipose tissue is of functional relevance, not only for combatting pathogens, but also for healthy adipose tissue expansion and homeostasis (104). Because processing of lipids will always be a key function of ATMs part of obese adipose tissue, it might be more effective to aim at reducing the burden of lipids in ATMs. The more because lipotoxicity is considered to be an important driver of maladaptive inflammatory activation of ATMs in obesity (12, 49).

Limiting lipid uptake

The most obvious approach for reducing the lipid burden would be to limit lipid uptake. Importantly, however, studies interfering with lipid uptake or handling by ATMs during obesity point to a similar role for lipid buffering during obesity as during fasting; when ATMs abate excessive leakage of potentially toxic FFAs into the circulation (14). First, aggravated lipid buffering capacity of ATMs through selective depletion of *Lpl* results in increased circulating FFAs and reduced glucose tolerance in leptin-deficient o*b/ob* mice without affecting bodyweight or adiposity (48). Second, introducing bone marrow that is depleted from *Fatp1*, an acyl-CoA synthetase facilitating intracellular lipid trafficking and involved in FA uptake; increases weight gain, adiposity and adipose tissue inflammation, and aggravates glucose intolerance in mice fed a HFD (105). Hence, even though lipids may underlie inflammatory activation of ATMs during obesity, lipid buffering by ATMs is of great functional relevance. Therefore, interfering with lipid uptake of ATMs holds limited therapeutic potential. Rather, efforts should be directed at expanding the lipid buffering and handling capacity of ATMs to render them capable of bearing the continuous flow of lipids in obese adipose tissue, without introducing lipotoxicity.

Increasing lipid breakdown

Both storage in lipid droplets (LDs) and oxidation in mitochondria are effective ways to neutralize otherwise toxic FAs (62, 106). Interestingly, macrophages in obese adipose tissue already show enhanced lipid storage as evidenced by their lipid-laden appearance. Moreover, increased lysosomal biogenesis and intracellularly activity in ATMs populating obese adipose tissue are suggestive of a continuous cycle of lipid processing that involves both lipid storage in LDs and subsequent lysosomal degradation of lipids from LDs (19). An intriguing question that remains to be answered, however, is whether lipids taken up by ATMs in obese adipose tissue – either directly or via lipolysis of LDs – are oxidized for ATP production in mitochondria. Interestingly, carnitine palmitoyltransferase 1α (CPT1α), a rate-limiting enzyme for FAO, has been detected in ATMs of obese individuals (107). Our unpublished data hint toward FAO in macrophages in an obese adipose tissue environment as well. However, despite increased exposure to lipids, FAO was not higher in macrophages

in an obese compared to lean adipose tissue environment, illustrated by a profound (~25%) but equal reduction in OXPHOS upon the injection of etomoxir in BMDMs exposed to obese or lean adipose tissue explants. Although just as 2-DG, etomoxir has been shown to have various side-effects (108, 109), one may speculate FAO to be the missing link in ATMs that are increasingly exposed to lipids during the development of obesity.

Importantly, a nutrient overload as present in obesity has been described to lead to 'mitochondrial grid-lock' in a variety of tissues including the muscle, liver and adipose tissue, characterized by impaired fuel switching and a persistent battle for oxidation between glucose, fatty acids, and amino acids (110-113). For ATMs, this may have significant consequences since oxidation of other metabolites present in the obese adipose tissue environment may importantly interfere with FAO. Interestingly, acylcarnitines, the by-products of incomplete oxidation of FAs, have been shown to promote pro-inflammatory cytokine release by macrophages in vitro (114). Although this study provided acylcarnitines extracellularly, one could hypothesize that increased lysosomal breakdown of TGs into FAs without subsequent (complete) oxidation in mitochondria of ATMs increases the concentration of intracellular acylcarnitines to toxic levels, thereby fuelling inflammatory activation. This is highly speculative, however, and studies involving isotope tracers of FAs will be essential for examining the fate of lipids taken up by ATMs and unravelling the degree of (partial) FAO in mitochondria. In vitro, interference with FAO of macrophages in a lipid (palmitate)-rich environment increases their inflammatory signalling, whereas enhancing FAO reduces inflammatory activation of macrophages (106, 107). Accordingly, raising FAO in ATMs part of obese adipose tissue may be effectively enhance their lipid handling capacity and decrease inflammatory activation. Ultimately, efficient lipid processing by ATMs may not only limit intracellular lipotoxicity, but also allow for timely removal and processing of dead adipocytes preventing the deterioration of dead adipocytes and release of DAMPs that contribute to inflammation and auto-immunity (18).

Hence, enhancing mitochondrial capacity and driving metabolic choices toward FAO may promote decisive, functional mitochondria that support the ATM capacity to buffer lipids released upon high levels of adipocyte lipolysis or cell death during obesity. A future challenge will be to identify enzymes or central regulators crucial for enhancing lipid turnover in ATMs that may include the nuclear transcription factors PPARγ and –δ, and AMPK, known to be central regulators of mitochondrial capacity, OXPHOS, and FAO in macrophages (115).

Human relevance

Importantly, the majority of studies on ATMs, including ours, have been done in mice or using mouse cells. Although the contribution of macrophages to adipose tissue inflammation and, ultimately, insulin resistance and Type 2 Diabetes is less robust than results from murine models suggest, ATMs do accumulate in obese adipose tissue of humans (116-118) where they adopt a lipid-laden appearance (40). Interestingly, despite conflicting inflammatory

phenotypes found in the obese adipose tissue of humans (119, 120), ATMs of obese individuals have been shown to display similar markers of metabolic activation as mouse ATMs (19, 38), corroborating metabolic activation and lipid handling to be a core feature of ATMs in obese adipose tissue. Studies aimed at further characterization of metabolic and inflammatory activation of freshly isolated ATMs from humans with varying BMI may shed light on the therapeutic potential of targeting ATM metabolism in humans.

Targeting intracellular metabolism of ATMs in humans will be challenging. We already observed pronounced inter-individual differences between human monocytes isolated from lean, healthy, young individuals (**Chapter 2**). Obese individuals with adipose tissue inflammation, the target population for drugs directed at ATMs, will be much more diverse and complex. They will not only greatly vary in bodyweight and degree of adipose tissue inflammation, but may also have comorbidities including hypertension and insulin resistance that involves usage of a variety of drugs as well. Not all these individuals will benefit from treatment strategies targeting ATM metabolism. Nevertheless, our data as well as that of the other studies described in this thesis point to a core role for intracellular metabolism in ATM functioning, and ATM functioning for adipose tissue homeostasis. As such, investing into research aimed at targeting ATM metabolism is hypothesized to be a worthwhile strategy for abating chronic low-grade inflammation in the adipose tissue with the ultimate goal of preventing or attenuating insulin resistance in a group of obese individuals.

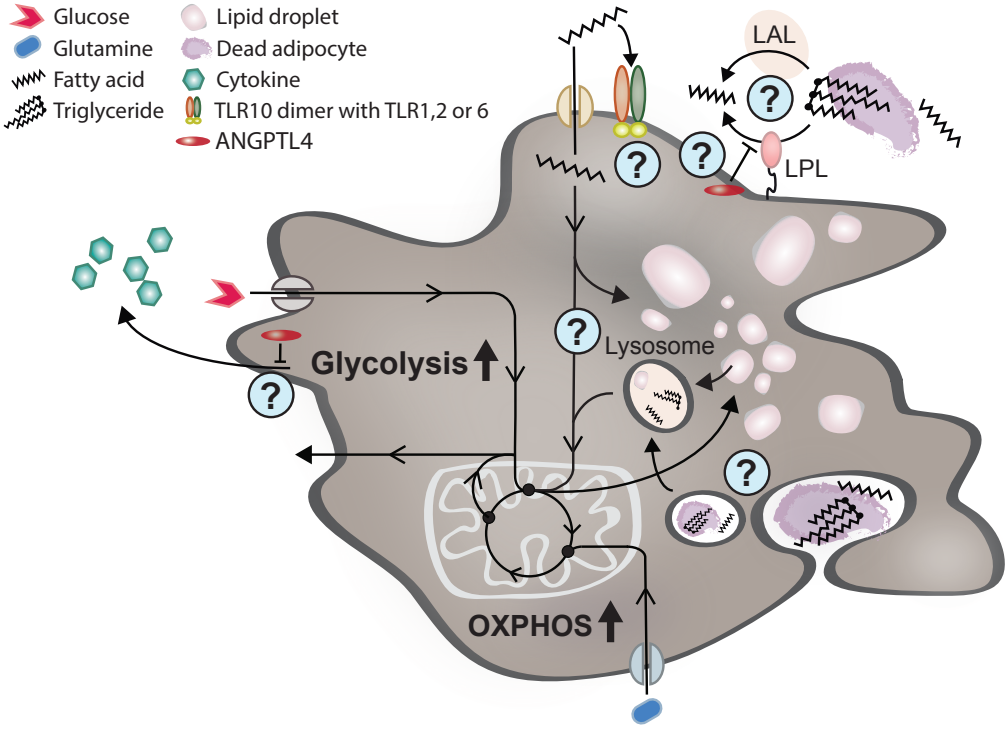


Figure 3 Representation of metabolic reprogramming in ATMs part of obese adipose tissue as well as potential important molecules and pathways involved.
Macrophages part of obese adipose tissue are characterized by a high number of intracellular lipid droplets (LDs) and profound production of pro-inflammatory cytokines. Most likely, lipids play a key role in shaping this adipose tissue macrophage (ATM) phenotype. Hypertrophic, stressed adipocytes in obese adipose tissue increasingly release free fatty acids (FFAs) via lipolysis, may secrete small lipid droplets rich in triglycerides (TGs) and FFAs, or comprise a source of both TGs and FFAs upon cell death. Dead adipocytes have been shown to be degraded extracellularly in acidic compartments, a process that involves lysosomal acid lipase (LAL). Additionally, TGs may require extracellular breakdown mediated by lipoprotein lipase (LPL). The relative contribution of LAL versus LPL for extracellular TG breakdown warrants further study. A role for LPL would point to involvement of angiopoietin-like 4 (ANGPTL4), a well-known inhibitor of LPL, in lipid uptake by ATMs. Indeed, we found ANGPTL4 to be upregulated in macrophages exposed to adipose tissue-conditioned medium or dead adipocytes, yet its role in lipid uptake in obese adipose tissue remains ambiguous. Similarly to FFAs, FAs derived from TG-breakdown may activate Toll-like receptors (TLRs) on the macrophage surface. Activation of TLRs by FAs initiates inflammatory signalling that has been shown to contribute to the pro-inflammatory trait of ATMs part of obese adipose tissue. Expression of *TLR10*, the only anti-inflammatory member of the family of TLRs, could, however, not alleviate the development of adipose tissue inflammation in obese mice. Alternatively, FAs may enter the cell via FA-transporters, or (together with extracellular TGs) can be engulfed via endocytosis or phagocytosis. Once intracellularly, FAs are either stored or oxidized. The lipid-laden appearance of ATMs in obese adipose tissue is suggestive of increased esterification of FAs into TGs and subsequent storage as LDs. Rather than being statically maintained, LDs in ATMs of obese adipose tissue were shown subjected to breakdown in lysosomal compartments. The exact intracellular fate of lipids taken up by ATMs is, however, relatively unclear and requires further investigation. Most likely, increased FA uptake as well as LD-breakdown comprises a prominent source for FA oxidation (FAO) in mitochondria. Unexpectedly, however, FAO was found unaltered in ATMs from obese versus lean mice, despite increased OXHOS. Glucose and glutamine comprise two other nutrient sources that may account for the increased OXPHOS observed in ATMs isolated from obese mice. Not OXPHOS, but glycolysis – that also was sharply increased in ATMs isolated from obese mice – appeared to predominantly fuel cytokine production by ATMs in the obese state. Interestingly, ANGPTL4 was found to inhibit cytokine expression and release by macrophages in an adipose tissue inflammatory environment, which appeared to be unrelated to its role as LPL inhibitor yet requires further investigation.

Therapeutic potential of targeting adipose tissue macrophage metabolism: Future challenges

For targeting intracellular ATM metabolism to become clinical practice, various challenges need to be overcome. This last section of the discussion addresses some of the remaining challenges that warrant future study, and includes some recommendations for research strategies to tackle these challenges.

Understanding the cause of metabolic activation of ATMs

Considering the great complexity of the adipose tissue environment, lipids but also many other *stressors* and *cues* present in the obese adipose tissue environment shape ATM functioning. In our experiments, we have mimicked the adipose tissue environment in vitro using a co-culture system with adipose tissue explants, or adipose tissue explant-conditioned medium. This approach takes the complete adipose tissue environment into account and, in contrast to studies that still use LPS/IFN-γ or GM-CSF versus IL-4/IL-13 or M-CSF stimulation to simulate ATMs from obese versus lean adipose tissue, corroborates metabolic and inflammatory activation of ATMs in vivo (19, 38)(**Chapter 4**).

Future challenges are to deal with the amount of variation that is introduced because of inter-individual differences between adipose tissue donors and due to differences in the amount of adipose tissue used for producing the conditioned medium. Moreover, it remains to be elucidated what factors exactly are involved in driving the ATM phenotype. A combination of insulin, glucose and palmitic acid has been shown to induce the expression of membrane markers representative for metabolically activated ATMs or macrophages stimulated with adipose tissue-conditioned medium (38). However, although these molecules may be elevated in the circulation of patients with metabolic disease (121), dominant presence in the direct surroundings of ATMs is questionable. It would therefore be worthwhile to carefully examine the adipose tissue environment in order to find molecules that on itself, but most likely in combination affect ATMs phenotypes, with particular emphasis on differences between lean and obese conditions. This demands for a combination of chromatography and spectroscopy approaches to measure proteins, lipids, and metabolites possibly in whole tissue lysates but preferably in the interstitial fluid, for example by using open flow microperfusion techniques to allow for unfiltered collection (122). In addition to the discovery of potential new factors involved in shaping the ATM phenotype, this approach yields important information of physiological and relative concentrations of various *stressors* and *cues* and, ultimately, may provide a standardized way to generate and study ATM-alike macrophages in vitro by using a mixture of factors dominantly present in the adipose tissue. In addition, it might bring to light new pathways that underlie macrophage activation in obese adipose tissue and that can be targeted for therapeutic gain.

Understanding metabolic activation of ATMs at different stages of obesity

Macrophages have been shown to increase in number already after three days of HFD-feeding and further accumulate in expanding adipose tissue (68, 123). Rather than a progressive, linear increase in inflammatory activation, ATMs display diverse inflammatory, metabolic, and functional phenotypes at different stages during the development of obesity (124). Accordingly, metabolic activation of ATMs and its relation to function may deviate during the development of obesity and critically depend on changes in the adipose tissue environment. For example, the amount of dead adipocytes will be relatively low at early stages of adipose tissue expansion, but more pronounced when obesity progresses. This is characterized by the appearance of the first CLSs somewhere between eight and twelve weeks of HFD-feeding in mice, whereas sixteen weeks of HFD-feeding results in an adipose tissue full of CLSs (51). Accordingly, clearance of dead adipocytes probably becomes a critical requirement for ATMs only at more advanced stages of obesity. In addition, ATMs may be more capable of effectively clearing adipocytes at early stages of obesity than at later stages when ATM functioning appears to derail (51, 104). It will be a future challenge to delineate macrophage functions required at different stages of adipose tissue expansion, and to unravel specific metabolic reprogramming essential for macrophages to execute these functions. Approaches linking ATM metabolism to function at distinct stages of obesity might include analyses in single cells that enable determination of causal relationships between metabolic routes and ATM functioning. This may also bring forward potential markers predictive for ATM dysfunction that may ultimately be used for predicting individual therapeutic strategies.

One of the current challenges of the field encompasses the interpretation of results obtained by using metabolic inhibitors. Firstly, given the fundamental role for ATP production for every cell, blocking one metabolic pathway will inevitably drive metabolic reprogramming to maintain ATP production. As a consequence, deriving a causal relationship from blocking one metabolic route is precarious: is the decrease in the one metabolic route or the increase in the compensatory route responsible for the observed phenotype? Secondly, unfortunately most metabolic inhibitors used in the field, including 2-DG and etomoxir, display off-target effects that may contribute to the observed effects. Repeating experiments using different inhibitors of one metabolic route (to reduce the chance of off-target effects to be responsible for the observed phenotype) or a combination of inhibitors blocking multiple metabolic routes (leaving one route to be used for ATP production) may take away part of these concerns.

Investigating the therapeutic gain of targeting intracellular metabolism of ATMs specifically

Once specific routes have been identified, the next challenge is to reveal the potential of targeting these routes for therapeutic gain. Myeloid specific knock-out or -in of genes involved in lipid uptake, transport or metabolism have demonstrated the significance of lipid handling capacities for macrophages in the adipose tissue during the development of

obesity. These experimental models, however, do not specifically target ATMs and reveal little about the therapeutic potential of targeting these molecules when obesity already has developed. Would it, for example, be possible to raise mitochondrial capacity for FAO in ATMs of individuals with severe obesity? Our data (**Chapter 4** & **5**) as well as that of others (19, 38) show that lipid metabolism and OXPHOS are already strongly enhanced in ATMs during obesity, and it will be a future challenge to investigate whether mitochondrial capacity can be further increased in these cells. While mitochondria have been shown to become dysfunctional during obesity in various cell types, it is currently unknown whether mitochondrial grid-lock appears in ATMs during obesity. Our findings of high spare respiratory capacity (SRC), a measure of the oxidative potential of mitochondria, in ATMs isolated from obese adipose tissue (data not shown) and macrophages exposed to obese adipose tissue ex vivo (**Chapter 4**) are suggestive of the potential to increase FAO in mitochondria of ATMs, and point to the absence of structural mitochondrial dysfunction that has been shown to prevent repolarization of M1 into M2 macrophages (89). Building upon these data, increasing mitochondrial capacity to burn FAs predominantly may provide a promising therapeutic approach. Yet whether indecisive mitochondria can still be guided to become decisive and remain decisive in the complex adipose tissue environment accommodating various *stressors* and *cues* that may interfere with drug-induced metabolic reprogramming, warrants further study. Moreover, whether raising FAO will be beneficial in ATMs part of obese adipose tissue remains to be elucidated as well. For instance, FAO may drive mitochondrial ROS formation at complex III of the electron transport chain (125), especially when mitochondria get overwhelmed (112). Importantly, palmitate-induced JNK activation and pro-inflammatory cytokine expression by macrophages has been shown enhanced when oxygen tension drops, which could for a great part be attributed to mitochondrial ROS formation (126). Since hypoxia is considered a ubiquitous feature of obese adipose tissue (127), it remains to be investigated whether increasing FAO in ATMs part of obese adipose tissue reduces lipotoxicity and inflammatory activation, or accelerates mitochondrial ROS formation and further fuels the pro-inflammatory trait of ATMs. Lastly, one should critically evaluate the benefit there is to gain from therapeutically reprogramming ATM metabolism at more advanced stages of obesity, that may be accompanied by insulin resistance or even Type 2 Diabetes. Most likely, more progressive stages of obesity require additional treatment strategies that target insulin production in the pancreas or glucose tolerance in other organs than the adipose tissue as well.

The development of methods specifically targeting ATMs in obese individuals may comprise the most difficult yet essential future challenge. So far, drugs carried in liposomes (128), glucan shells (129) or linked to glucose polymers coated with polysaccharides (130) have been shown to effectively and specifically target ATMs upon intraperitoneal injection in obese mice. These methods exploit the intrinsic phagocytic capacity of macrophages to readily take up liposomes or foreign (nano)particles. These methods, however, also critically

depend on macrophage infiltration into the adipose tissue during obesity. Studies have brought forward additional mechanisms contributing to ATM accumulation in obese adipose tissue of both humans and mice, including in situ myelopoiesis and local macrophage proliferation (131-133) that may not be targeted using these approaches. While β-glucan is recognized by Dectin-1, a receptor that is ubiquitously expressed by neutrophils, monocytes and macrophages (134), particles may be coated with ligands of receptors or transporters specifically or highly expressed on the membrane of ATMs part of obese adipose tissue. Conceivably this will enhance ATM-specificity without solely relying on monocyte influx into obese adipose tissue. The use of pro-drugs comprises an alternative approach. For example, the attachment of an esterase-sensitive motive that is selectively hydrolysed in macrophages and monocytes (both expressing the required enzyme carboxylesterase-1) to small drugs, has been shown effective for delivering pharmacological activity only in monocytes and macrophages (135). Hypothetically, liposome- or particle-mediated delivery of a pro-drug that depends on the high metabolic or lysosomal activity of ATMs to become pharmacologically active may be a future opportunity for targeting ATMs in obese individuals. Otherwise, above mentioned existing macrophage-targeted approaches may already sufficiently reach ATMs for achieving clinical benefits.

Importantly, merging immunology and metabolism into a new scientific field named ‘immunometabolism’ has initiated an avalanche of studies that have greatly enhanced our understanding of the impact of intracellular metabolism on immune cell function. Increasing evidence illustrates cell- and location-specific macrophage functions and accompanying metabolic requirements. Currently, we may have reached a tipping point at which immunomodulatory drugs can be clinically tested, yet are short of methods for achieving immune cell specificity. Joining forces of scientists across various scientific fields including immunology, physiology, and chemistry may be critical to catalyse the development of (tissue-)specific macrophage treatment opportunities for conditions that are characterized by macrophage-induced inflammation, among which metabolic diseases.

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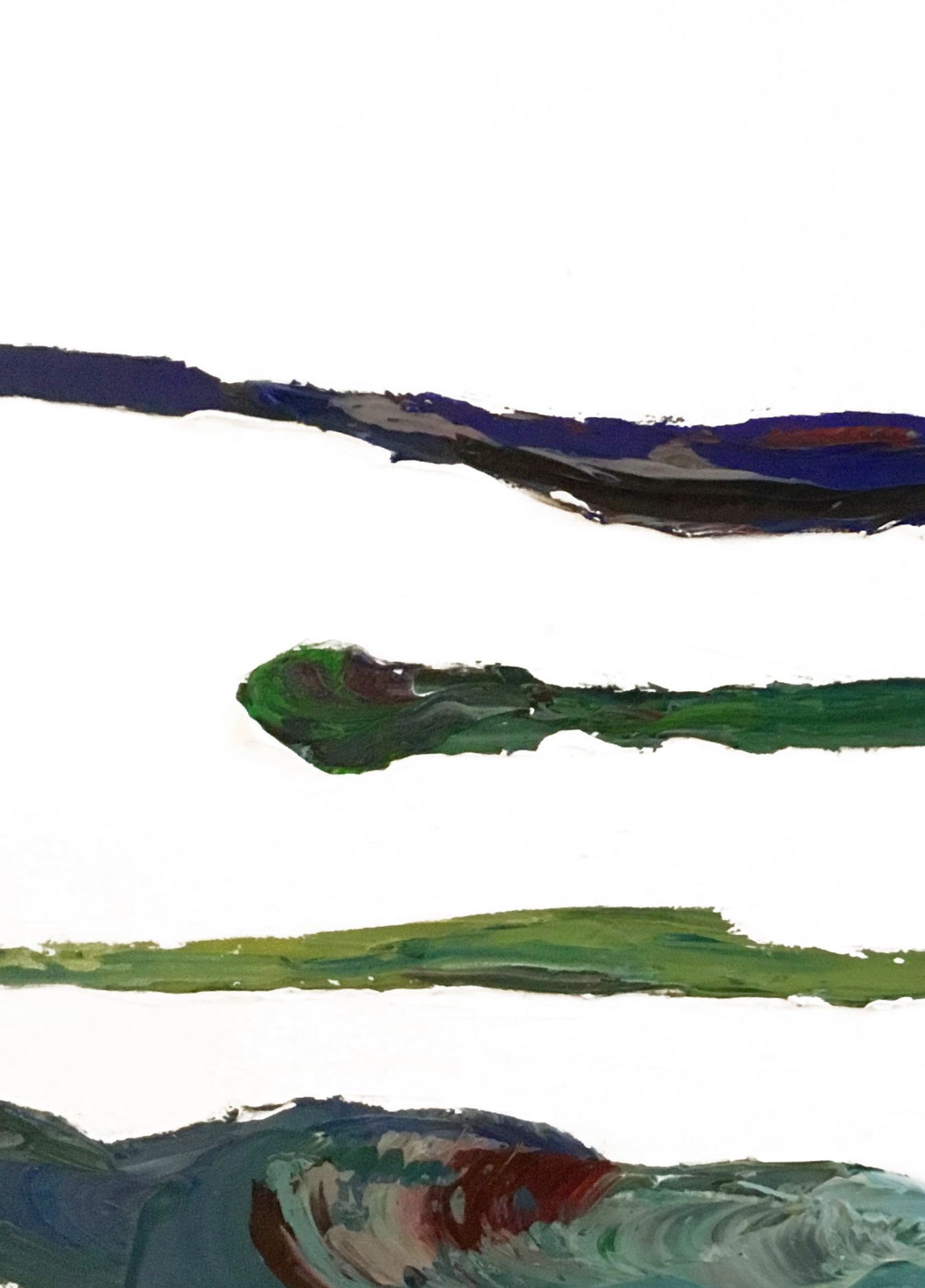


Summary

Macrophages and their monocyte precursors continuously patrol the bloodstream and tissues, ready to eliminate unwelcome visitors such as pathogens or foreign particles. Tissue-resident macrophages are crucial during development and for maintaining tissue homeostasis as well. The engulfment of dying or damaged tissue cells, a process called efferocytosis, is a central part of their role to maintain homeostasis, yet is accompanied by several other tissue-tailored functions. Accordingly, macrophages display great plasticity by adopting unique phenotypes to fulfil tissue-specific needs.

This thesis is particularly devoted to macrophages residing in the adipose tissue. In lean conditions adipose tissue macrophages (ATMs) promote tissue and whole-body homeostasis by buffering lipids released by adipocytes and removing dead or damaged cells, and ensure tissue dynamics by promoting angiogenesis, adipogenesis, and extracellular matrix remodelling. Obese adipose tissue, however, is characterized by low-grade chronic inflammation reflective of homeostatic imbalance. Given their pivotal role for maintaining homeostasis in lean conditions, ATMs are considered key players in the development of adipose tissue inflammation during obesity. Indeed, during obesity ATMs sharply increase in number while simultaneously gaining a pro-inflammatory trait. This pro-inflammatory activation of ATMs is thought to importantly link obesity to the development of insulin resistance and, ultimately, Type 2 Diabetes.

Notwithstanding the considerable progress made, the underlying causes of macrophage activation and phenotypical and functional characteristics of ATMs in obese adipose tissue have not yet been fully unravelled. In this thesis, we have investigated various aspects of activation of macrophage and their monocyte precursors. First, we have examined metabolic reprogramming in monocytes stimulated with various pathogenic stimuli (**Chapter 2**). This research adds to the growing evidence of intracellular metabolism as fundamental driver of immune cell functioning. In contrast to the majority of studies in the field that have focussed on one single stimulant, we have carefully evaluated intracellular metabolism upon activation with different pathogenic stimuli, including whole pathogen lysates and isolated Toll-like receptor (TLR) ligands. In line with the current paradigm, we found glycolysis to be a general characteristic of monocyte activation irrespective of the present stimulus. Interestingly, however, in contrast to the current paradigm, oxidative phosphorylation (OXPHOS), the alternative route for ATP production that occupies mitochondria, was found to be enhanced by most pathogenic stimuli as well. In fact, the most commonly used stimulant for activating monocytes and macrophages, being lipopolysaccharide (LPS), appeared unique in aggravating mitochondrial metabolism. Importantly, such stimulus-specific metabolic reprogramming appeared to have functional consequences, that we evaluated by comparing the two different TLR-ligands LPS (TLR4 ligand) and Pam_3CysSK_4 (P3C: TLR2 ligand). While glycolysis contributed to cytokine release by both LPS and P3C, OXPHOS only

contributed to cytokine production in P3C-stimulated monocytes. Moreover, phagocytosis appeared to rely on OXPHOS but not glycolysis in monocytes stimulated with P3C. Probably consequential to their reduced mitochondrial activity, LPS-stimulated monocytes displayed low phagocytic capacity. Together these findings are suggestive of stimulus-tailored metabolic rearrangements fuelling functional output of monocytes.

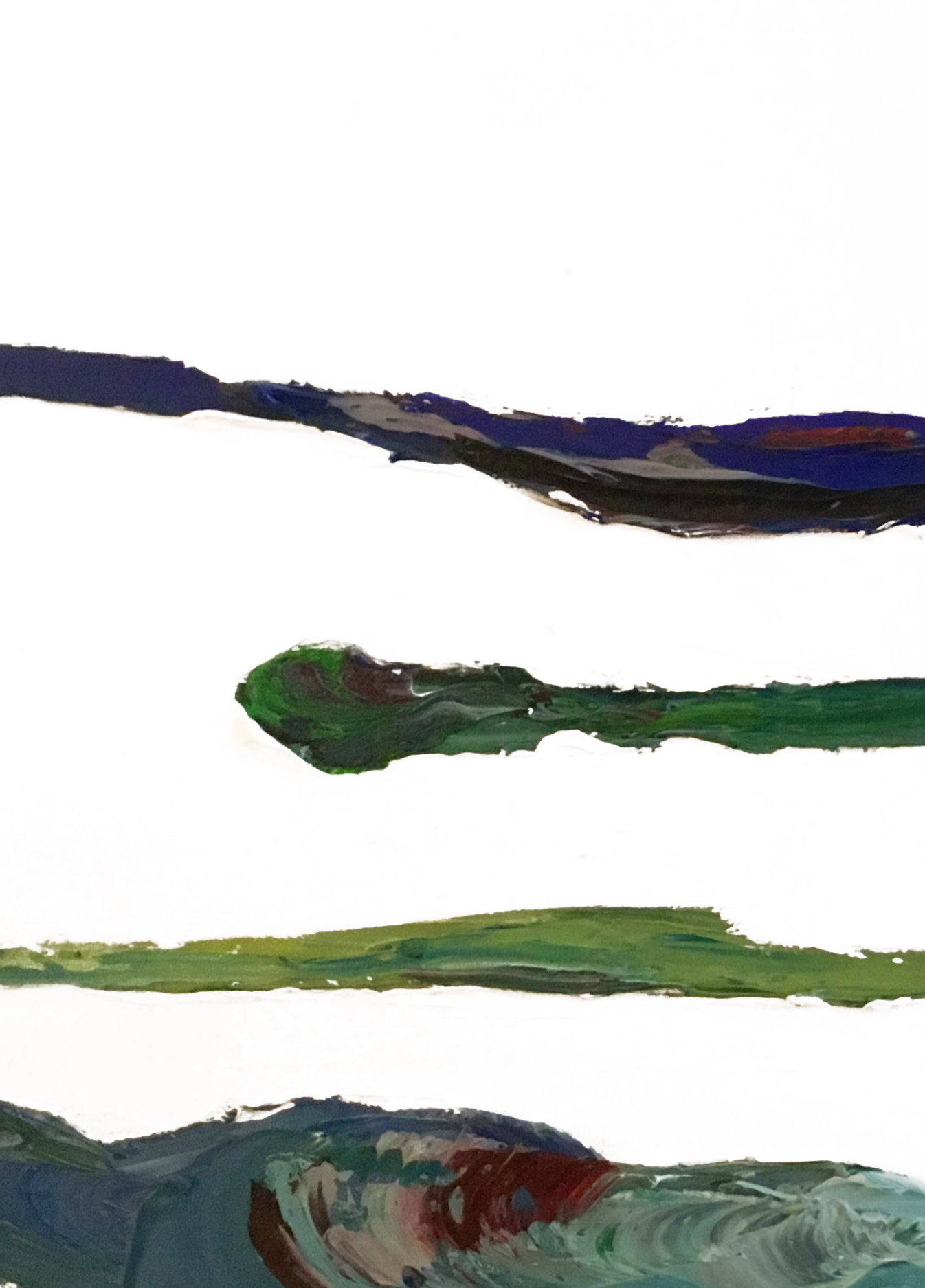
After reviewing various aspects of ATMs in **Chapter 3** – including their origin, activation and function in obese versus lean conditions – we examined metabolic rearrangements in ATMs, and evaluated their contribution to the pro-inflammatory ATM trait apparent in obese adipose tissue (**Chapter 4**). Not surprisingly given the rather challenging environment provided by obese adipose tissue, ATMs were found to be strongly metabolically activated during obesity illustrated by enhanced activation of both glycolysis and OXPHOS. Interestingly, this metabolic activation appeared to be specific for ATMs, and was not manifested in macrophages isolated from the peritoneum of obese versus lean mice. In line with recent studies, we showed that both the metabolic and inflammatory trait of ATMs was pronouncedly different from that displayed by classically (LPS-)activated macrophages. Indeed, the ATM phenotype appeared dose-dependently induced by adipose tissue-derived factors. Using metabolic inhibitors, we identified various metabolic routes including fatty acid oxidation, glycolysis and glutaminolysis to contribute to cytokine release by ATMs isolated from lean mice. Glycolysis, however, contributed the most to cytokine production and was responsible for the increased release of inflammatory cytokines by ATMs from obese mice. Unexpectedly, however, HIF-1α, a key regulator of glycolysis and inflammatory activation, appeared not to be critically involved in the development of a pro-inflammatory ATM trait during obesity.

Because lipids most likely play a central role in shaping the ATM phenotype, we evaluated the role of triglycerides (TGs) versus free fatty acids (FFAs) as driver of pro-inflammatory activation of ATMs in **Chapter 5**. First we confirmed lipid handling to be a fundamental characteristic of ATMs by showing that ATMs, but not other tissue macrophages or circulating monocytes from humans and mice, display enhanced expression of genes involved in lipid uptake and processing. This associated with increased expression of ER stress markers and inflammatory activation of macrophages, pointing to a relation between lipid loading and inflammatory activation of ATMs. Interestingly, both lipoprotein lipase (*Lpl*), that breaks down extracellular TGs into FAs that can be taken up, and its endogenous inhibitor angiopoietin-like 4 (*Angptl4*) were upregulated in macrophages in an adipose tissue environment, suggestive of the presence of a negative feedback mechanism to limit LPL activity and thus excessive uptake of FAs from TGs. Indeed, we observed ANGPTL4 to inhibit inflammatory activation of macrophages in an adipose tissue environment. Intriguingly, however, reduced inflammatory activation of *Angptl4* knock-out macrophages in an adipose tissue environment appeared to be independent of lipid loading which most likely occurred through uptake of FFAs rather than TGs.

In **Chapter 6**, we zoomed into a role for ATMs in efferoctysis of dead adipocytes, that may impose an important source of lipids for ATMs. Indeed, we found profound transcriptional regulation of the efferocytic machinery in ATMs isolated from obese versus lean adipose tissue accompanied by increased expression of genes involved in lipid handling and processing of lipid-derivatives. In vitro, dead adipocytes were readily taken up by macrophages and induced the expression of various genes involved in lipid handling, similar to what we found in ATMs in vivo. Interestingly, macrophages part of obese adipose tissue display pronounced down-regulation of Interferon (IFN)-signalling, whereas effective efferocytosis in vitro was characterized by enhanced IFN signalling. Accordingly, our data are suggestive of a link between impaired IFN signalling and dysfunctional, pro-inflammatory ATMs in obese adipose tissue.

Lastly, in **Chapter 7** we have evaluated a role for TLR10, the sole anti-inflammatory TLR family member, in adipose tissue of humans and mice. Because mice do not express functional *TLR10*, we fed mice expressing human *TLR10* a high-fat diet for 16 weeks. Unexpectedly, *TLR10* did not attenuate the development of adipose tissue inflammation during obesity. Interestingly, however, mice carrying human *TLR10* had reduced adipose tissue weight and adipocyte size, suggestive of a role for TLR10 in adiposity. In humans, obese but not lean individuals carrying single nucleotide polymorphisms (SNPs) in *TLR10* had or tended to have lower circulating leptin and macrophage numbers in the adipose tissue, reflective of a role for TLR10 in the adipose tissue at states of low-grade chronic inflammation specifically.

In conclusion, we have revealed macrophage metabolic reprogramming to be stimulus-driven and location-specific and crucial for fuelling functional output in line with specific environmental demands. In the adipose tissue, lipid handling is central to macrophage functioning, yet ATMs appear to be overwhelmed by lipids during obesity. From a therapeutic point of view, we propose stimulation of FA oxidation to support ATM functioning according to increasing demands of the obese adipose tissue environment, while simultaneously driving them away from glycolysis that appeared to critically underlie their pro-inflammatory trait. Future studies, however, are warranted to clarify the therapeutic potential of raising mitochondrial FA oxidation in ATMs of obese individuals.



Acknowledgements

Dankwoord

Gesjees, gepipetteer, gelach, (gebrekkig) geplan, gestress, gemopper, geknal, geschrijf, gedoe, gepresenteer, gefiets: de afgelopen jaren als PhD in Wageningen hebben een onuitwisbare indruk gemaakt en impact gehad. Mede mogelijk gemaakt door velen die direct of indirect, bewust of onbewust, hebben bijgedragen aan dit proefschrift.

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All (former) **PhDs in Wageningen**; **Antwi**, **Anouk**, **Charlotte**, **Fenni**, **Inge**, **Juri**, **Katja**, **Jvalini**, **Mara**, **Merel**, **Milène**, **Miranda**, **Montse**, **Nikkie**, **Neeraj**, **Parastoo**, **Philip**, **Rogier**, **Roland**, **Sophie**, **Xanthe** and **Ya**, thanks for all the fun, serieus, short and longer talks in and outside the lab, and the best of luck with your experiments, writing, presentations, weddings, new houses and (new) carreers!

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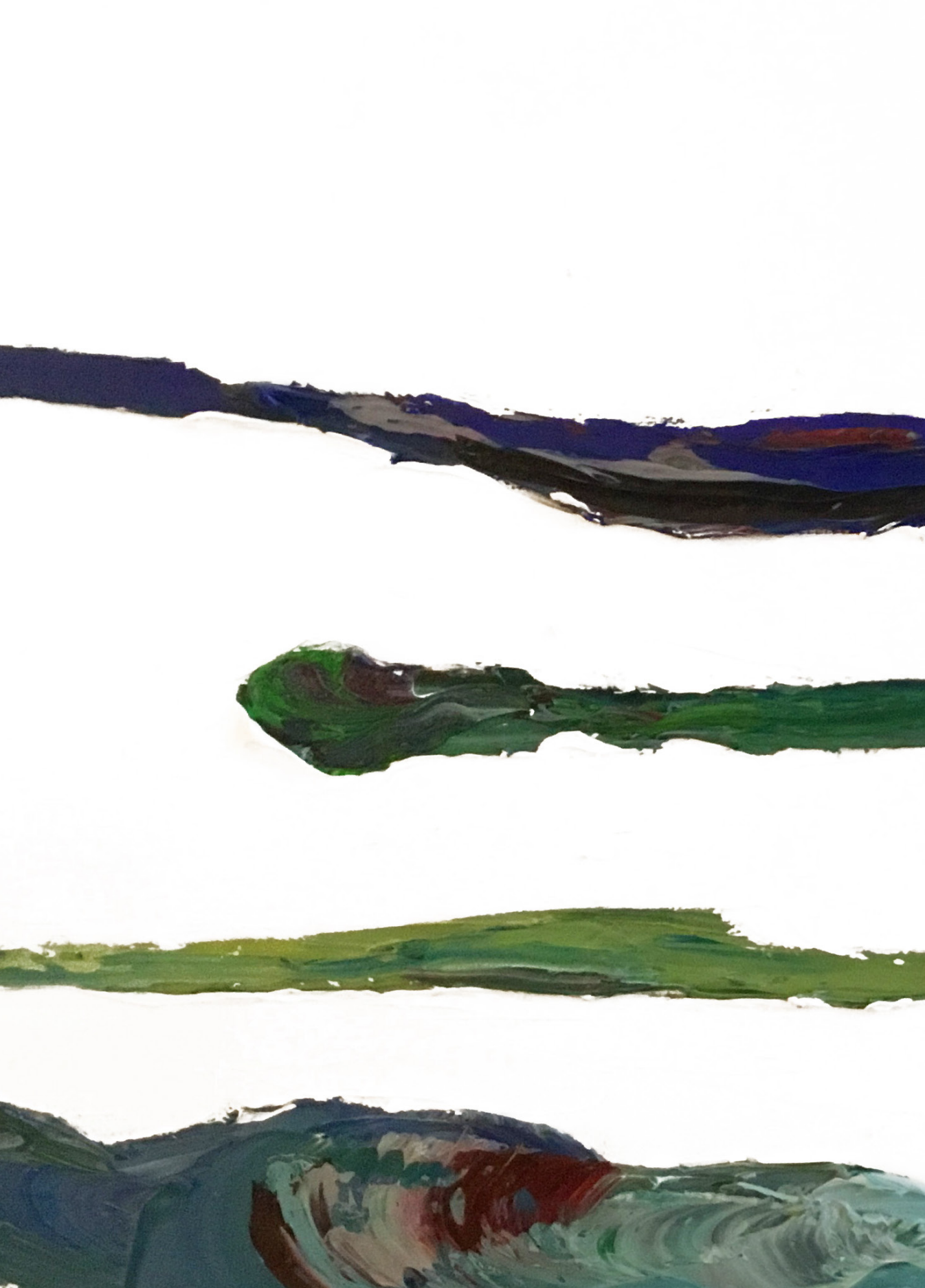
Lily



About the author

ABOUT THE AUTHOR

Lily Boutens was born on September 26 1988 in Amsterdam, the Netherlands. In 2007 she completed secondary school (athenaeum, *cum laude*) at the Hervormd Lyceum Zuid in Amsterdam and started the Beta-gamma bachelor at the University of Amsterdam. Three years later, she had learned a little bit of everything and a little more of Biomedical Sciences, and received her bachelor's degree in 2010 (*cum laude*). While travelling through South America her eye was set on studying the fate of nutrients entering the mouth. As such, upon return in the Netherlands in 2011, Lily moved out of her Netherlands (Amsterdam) to Wageningen for the master Human Nutrition at Wageningen University. During her master's she focused on Molecular Nutrition and performed two internships. One encompassed the search for the effects of orally delivered α-galactosylceramide on metabolism and immune responses in the liver and white adipose tissue, and was supervised by prof. dr. Sander Kersten en dr. Rinke Stienstra at the Nutrition, Metabolism and Genomics group of Wageningen University. The other was at Oxford University, where she studied the role of the Fat mass and Obesity associated (FTO) gene in cellular nutrient sensing, under supervision of prof. dr. Frances Ashcroft and dr. Myrte Merkestein. At that point Lily met all the requirements for receiving her master's degree in 2013 (*cum laude*).

In October 2013 Lily started her PhD project in Wageningen. The scientific consequences of this choice are described in this thesis entitled: '*Understanding macrophage activation in the adipose tissue: at the crossroads of immunology and metabolism*'.

LIST OF PUBLICATIONS

This thesis

Boutens L, Borst JW, Stienstra R. Efferocytosis of adipocytes by macrophages in obese versus lean adipose tissue. *Manuscript in preparation.*

Boutens L, Dijk W, Kersten AH, Stienstra R. Uptake of triglyceride-derived or free fatty acids by macrophages favours inflammatory responses in lipid-rich envrionments: Involvement of ANGPTL4. *Manuscript in preparation.*

Boutens L*, Mirea AM*, van den Munckhof I, Oosting M, Jaeger M, Hijmans A, Netea MG, Joosten LAB^, Stienstra R^. A role for TLR10 in obesity and adipose tissue morphology. *Submitted for publication.*

Boutens L, Hooiveld GJ, Dhingra S, Cramer RA, Netea MG, Stientstra R. Unique metabolic activation of adipose tissue macrophages in obesity promotes inflammatory responses. *Diabetologia*, doi: 10.1007/s00125-017-4526-6.

Lachmandas E*, **Boutens L***, Ratter JM*, Hijmans A, Hooiveld GJ, Joosten LA, Rodenburg RJ, Fransen JA, Houtkooper RH, van Crevel R, Netea MG, Stienstra R. Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. *Nat Micriobiol.* 2016; 19(2): 16246.

Boutens L, Stienstra R. Adipose tissue macrophages: going off track during obesity. *Diabetologia.* 2016; 59(5): 879-94.

* or ^ Equal contribution

LIST OF PUBLICATIONS

Others

Domínguez-Andrés J, Arts RJW, Ter Horst R, Gresnigt MS, Smeekens SP, Ratter JM, Lachmandas E, **Boutens L**, van de Veerdonk FL, Joosten LAB, Notebaart RA, Ardavín C, Netea MG. Rewiring monocyte glucose metabolism via C-type lectin signaling protects against disseminated candidiasis. *PLoS Pathog.* 2017; 13(9): e1006632.

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OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Conferences and symposia

IL-1 mediated inflammation and diabetes	IGMD	Nijmegen, The Netherlands	2013	
Dutch Diabetes Research	NVDO	Oosterbeek, The Netherlands	2013	
Dutch Diabetes Research	NVDO	Oosterbeek, The Netherlands	2014	Oral
European Association for the study of Diabetes	EASD	Vienna, Austria	2014	Poster
Mechanisms of Innate Immunity, Cell Death and Inflammation	ICDI	Ghent, Belgium	2014	Oral
Dutch Diabetes Research	NVDO	Oosterbeek, The Netherlands	2015	
Minisymposium on the occasion of Institute of Food Research visit	VLAG	Wageningen, The Netherlands	2015	Oral
Systems Biology of Innate Immunity	RUMC	Nijmegen, The Netherlands	2016	Poster
100 Years of Phagocytes conference	Cell Symposia	Giardini-Naxos, Italy	2016	Poster
Immunometabolism in Immune Function and Inflammatory Disease	Keystone Symposia	Banff, Canada	2016	Poster
Integrating Metabolism and Immunity conference	Keystone Symposia	Dublin, Ireland	2017	Poster

Course

Microscopy and Spectroscopy in Food and Plant Sciences	VLAG	Wageningen	2014

General courses

PhD week	VLAG	Wageningen	2014
Course on Laboratory Animal Science	UU	Utrecht	2014
Brain Training	WGS	Wageningen	2015
Techniques for writing and presenting a scientific paper	WGS	Wageningen	2016
Career assessment	VLAG	Wageningen	2017

Optionals

Scientific meetings / Journal Clubs TSK	WUR	Wageningen	2013-2017
Weekly science meeting NMG / Pharma group	WUR	Wageningen	2013-2017
Weekly science meeting Internal Medicine	RUMC	Nijmegen	2013-2017
Research proposal	WUR	Wageningen	2013

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