

Adipose tissue conditioned media support macrophage lipid-droplet biogenesis by interfering with autophagic flux



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ABSTRACT

Obesity promotes the biogenesis of adipose tissue (AT) foam cells (FC), which contribute to AT insulin resistance. Autophagy, an evolutionarily-conserved house-keeping process, was implicated in cellular lipid handling by either feeding and/or degrading lipid-droplets (LDs). We hypothesized that beyond phagocytosis of dead adipocytes, AT-FC biogenesis is supported by the AT microenvironment by regulating autophagy. Non-polarized (“M0”) RAW264.7 macrophages exposed to AT conditioned media (AT-CM) exhibited a markedly enhanced LDs biogenesis rate compared to control cells (8.3 Vs 0.3 LDs/cells/h, $p < 0.005$). Autophagic flux was decreased by AT-CM, and fluorescently following autophagosomes over time revealed ~20% decline in new autophagic vesicles’ formation rate, and 60–70% decrease in autophagosomal growth rate, without marked alternations in the acidic lysosomal compartment. Suppressing autophagy by either targeting autophagosome formation (pharmacologically, with 3-methyladenine or genetically, with *Atg12* ± *Atg7*-siRNA), decreased the rate of LD formation induced by oleic acid. Conversely, interfering with late autophago-lysosomal function, either pharmacologically with bafilomycin-A1, chloroquine or leupeptin, enhanced LD formation in macrophages without affecting LD degradation rate. Similarly enhanced LD biogenesis rate was induced by siRNA targeting *Lamp-1* or the V-ATPase. Collectively, we propose that secreted products from AT interrupt late autophagosome maturation in macrophages, supporting enhanced LDs biogenesis and AT-FC formation, thereby contributing to AT dysfunction in obesity.

1. Introduction

Obesity promotes marked changes in the cellular composition of adipose tissue (AT). Macrophages were the first immune cells recognized to infiltrate AT, and may comprise up to 50% of the non-adipocyte cells in the obese state [1,2]. However, despite enormous progress in understanding their role, the full function of AT macrophages (ATM) in health and disease remains incomplete, as they seem to play an ever-increasing range of homeostatic and disease-promoting functions, facilitated by extreme adaptivity to various microenvironments. One suggested protective/homeostatic function of ATM involves lipid storage by engulfment of dead adipocytes, re-esterification of free fatty acids (FFA) and/or de-novo lipogenesis, serving a potential

buffering role in AT’s lipid handling [3]. However, massive accumulation of lipids in ATM, especially in the visceral fat depot, results in the biogenesis of adipose tissue foam cells (AT-FC) – i.e., lipid-laden macrophages that contribute to AT insulin resistance and possibly to whole-body glucose intolerance [4–7].

The major role of macrophage’s lipid handling in tissue homeostasis has been well established in atherosclerosis, where aberrant lipid accumulation leading to FC biogenesis in the vessel wall is a major contributor to the atherogenic process [8]. One of the suggested mechanisms involved in this process, is altered autophagy [9]. Autophagy is an evolutionarily-ancient cellular homeostatic “house-keeping” process, through which a double-membraned vesicle, the autophagosome, is formed around intracellular structures/components designated for

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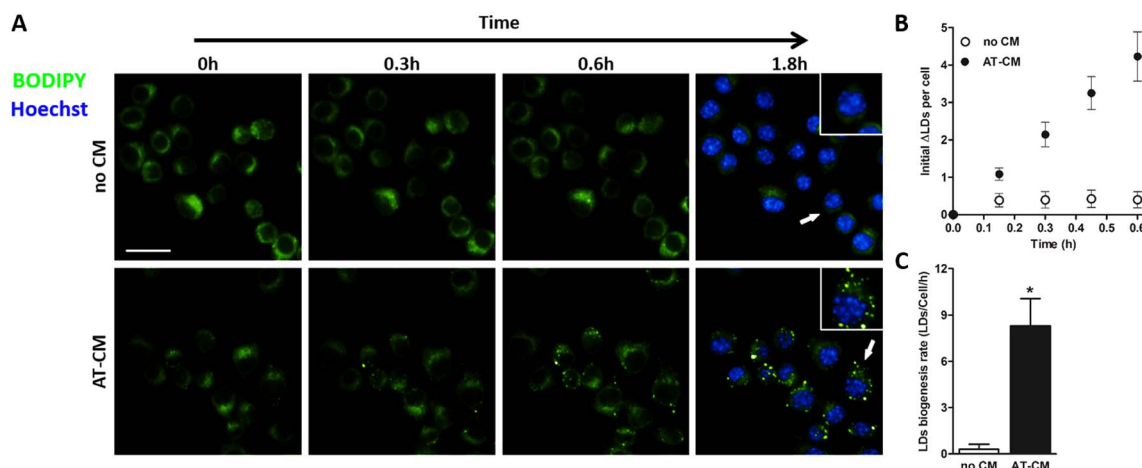


Fig. 1. Adipose tissue conditioned medium enhances lipid droplets (LDs) biogenesis rate in non-polarized macrophages (“M0 state”).

(A) Live cell imaging (Operetta high throughput imaging system) of RAW264.7 murine macrophages. Macrophages were treated with or without adipose tissue conditioned medium (AT-CM) in the presence of BODIPY to stain intracellular neutral lipids. Hoechst was added after 2 h for nuclear staining. Scale bar, 20 μ m. White arrows indicate the cells enlarged in the insets. (B) Lipid droplets (LD) were identified semi-automatically by Columbus software and the change in LDs number per cell, from the initial captured time lap, is shown. Images representing a total of twenty seven images per experiment, from four independent experiments, are shown over time. (C) LDs biogenesis rate (LD/cell/h) was calculated as the mean slope of the curves summarized in B. *, $p < 0.005$.

degradation in lysosomes [10–12]. Recent studies suggest that autophagy is involved in cellular lipid handling by degrading lipid droplets (LDs), a process termed lipophagy [13,14]. Lipophagy was first demonstrated in hepatocytes, wherein autophagy inhibition during incubation with oleic acid (OA) resulted in massive triglyceride accumulation and increased LD number and size [15]. The occurrence of lipophagy has later been shown in fibroblasts, in neurons and in stellate cells [15–18]. In atherosclerotic macrophages, autophagy depletion enhances FC biogenesis and impairs cholesterol efflux, accelerating atherosclerotic plaque formation and progression [19–22]. Whether this applies to ATM in obesity is still controversial, as it remains unclear how the obesogenic environment affects macrophage's autophagic function. Two recent reports suggest that obesity inhibits autophagy in macrophages, while another study (mainly reporting enhanced lysosomal biogenesis) proposed the opposite [23–25]. Moreover, pharmacological inhibition of lysosomal acidification, which inhibits late autophagosome degradation, increased ATM lipid content [3], suggesting a similar scenario to FC in the atherosclerotic plaque. This seeming discrepancy could potentially arise from, or at least intertwine with, ATM's immunological switch from M2-predominating to M1 phenotype [26]. Indeed, M2-polarized macrophages display a higher autophagic activity compared to M1 [23]. Taken together, how obesity alters ATM autophagy, and what is the impact of such change on macrophage lipid handling remains unclear.

In the present study we hypothesized that the altered micro-environment in AT affects macrophage autophagy and lipid handling via auto-paracrine mechanisms, independently of macrophages' phagocytosis of dead adipocytes, which occurs in obese AT [27]. To address this question, non-polarized macrophages (“M0 state”) were exposed to conditioned media of AT (AT-CM) from obese mice or to OA, and the effects on autophagic activity and LD dynamics were studied. Adapting a reductionist approach, we rationalized that using M0 macrophages would serve to isolate the effect of AT-CM from the effects of (pre)-polarization of the macrophages to either M1 or M2. Utilizing OA at a concentration that mimics the effect of AT-CM would allow isolating the role of free fatty acids from other secreted factors from the AT to the medium [28]. Pharmacological and molecular approaches were then used to determine the functional contribution of autophagy alterations to macrophage LD biogenesis and degradation.

2. Methods

2.1. Preparation of adipose tissue conditioned-media (AT-CM)

The study was approved in advance by Ben-Gurion University Institutional Animal Care and Use Committee, and was conducted according to the Israeli Animal Welfare Act following the guidelines of the Guide for Care and Use of Laboratory Animals (National Research Council, 1996). Six-week-old male C57BL/6 mice were obtained from Harlan Laboratories (Rehovot, Israel). After 2 weeks acclimation mice were fed either normal chow (NC) diet or a High-fat diet (HFF) (58.7% calories derived from fat, 25.5% carbohydrate, and 15% protein, D12492; Research Diets, New Brunswick, NJ, USA) for 10 weeks. Mice were killed with isoflurane and epididymal fat pads were minced into 2–3 mm³ fragments and incubated at 100 ± 5 mg/ml in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose, 10% Fetal Bovine Serum (FBS), 50 U/ml penicillin, 50 μ g/ml streptomycin and 4 mM glutamine (Biological industries, Beit HaEmek, Israel), for 24 h, after which explants were washed and re-incubated in DMEM containing 0.1% FBS for additional 24 h. These media were collected (AT-CM, or AT-CM_{NC} and AT-CM_{HFF} for Supplemental Table 1) and kept in -80 °C until use. An adipocytokine screen of this CM has been recently reported elsewhere [28].

2.2. Cell culture

RAW264.7 mouse macrophage cell line (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM 4.5 g/l glucose containing 10% FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin and 4 mM glutamine. For live imaging 2.5×10^4 cells/well or 1×10^4 cells/well (for CM and pharmacological inhibitors, or for siRNA transfection, respectively) were seeded into 96-well uClear, black plate (Greiner Bio One, Kremsmünster, Austria). 2×10^6 cells/well were seeded into 6-well plates for western blot analysis or for lipid extraction, 1×10^5 cells/well for siRNA transfection, and 3×10^4 cells/well were plated into 4-well chamber slide (ibidi, Martinsried, Germany) for confocal microscopy. 24 hour post-seeding, the medium was replaced. For CM experiments, DMEM containing 0.1% FBS (control) or AT-CM was diluted at 1:1 ratio with DMEM without FBS. For live imaging of autophagosomes and western blot analysis macrophages were pre-treated with AT-CM for 6 h.

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