Original Article

MOLECULAR METABOLISM

Macrophage alternative activation confers protection against lipotoxicity-induced cell death

Lingling Dai [1,2,*], Prerna Bhargava [1,3], Kristopher J. Stanya [1], Ryan K. Alexander [1], Yae-Huei Liou [1], David Jacobi [1,4], Nelson H. Knudsen [1], Alexander Hyde [1], Matthew R. Gangl [1], Sihao Liu [1,5], Chih-Hao Lee [1,]**

ABSTRACT

Objective: Alternative activation (M2) of adipose tissue resident macrophage (ATM) inhibits obesity-induced metabolic inflammation. The underlying mechanisms remain unclear. Recent studies have shown that dysregulated lipid homeostasis caused by increased lipolysis in white adipose tissue (WAT) in the obese state is a trigger of inflammatory responses. We investigated the role of M2 macrophages in lipotoxicity-induced inflammation.

Methods: We used microarray experiments to profile macrophage gene expression regulated by two M2 inducers, interleukin-4 (Il-4), and peroxisome proliferator-activated receptor delta/gamma (Pparδ/Pparγ) agonists. Functional validation studies were performed in bone marrow-derived macrophages and mice deprived of the signal transducer and activator of transcription 6 gene (*Stat6*; downstream effector of Il-4) or *Pparδ/Pparγ* genes (downstream effectors of Stat6). Palmitic acid (PA) and β-adrenergic agonist were employed to induce macrophage lipid loading *in vitro* and *in vivo*, respectively.

Results: Profiling of genes regulated by Il-4 or Pparδ/Pparγ agonists reveals that alternative activation promotes the cell survival program, while inhibiting that of inflammation-related cell death. Deletion of *Stat6* or *Pparδ/Pparγ* increases the susceptibility of macrophages to PA-induced cell death. NLR family pyrin domain containing 3 (Nlrp3) inflammasome activation by PA in the presence of lipopolysaccharide is also increased in $Stat6^{-/-}$ macrophages and to a lesser extent, in $Pparδ/γ^{-/-}$ macrophages. In concert, β-adrenergic agonist-induced lipolysis results in higher levels of cell death and inflammatory markers in ATMs derived from myeloid-specific $Pparδ/γ^{-/-}$ or $Stat6^{-/-}$ mice.

Conclusions: Our data suggest that ATM cell death is closely linked to metabolic inflammation. Within WAT where concentrations of free fatty acids fluctuate, M2 polarization regulated by the Stat6-Ppar axis enhances ATM's tolerance to lipid-mediated stress, thereby maintaining the homeostatic state.

Keywords Obesity; Adipose tissue macrophage; Alternative activation; Lipotoxicity; Meta-inflammation

1. INTRODUCTION

Obesity and related metabolic syndrome are major medical and economic burdens worldwide. Chronic low-grade inflammation is often observed in obesity and is a key contributor to associated pathologies such as insulin resistance, type 2 diabetes, and atherosclerosis [1–3]. White adipose tissue (WAT) is a primary energy storing tissue that has been recognized as an important endocrine organ modulating metabolic-related inflammation (or meta-inflammation) through crosstalk with resident immune cells, such as macrophages and

[1]Department of Genetics and Complex Diseases, Division of Biological Sciences, Harvard T.H. Chan School of Public Health, 665 Huntington Ave, Boston, MA 02115, USA [2]Phase I Clinical Trial Center & Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, Hunan, PR China

[3] Current address: Institute for Medical Engineering & Science, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.
[4] Current address: l'institut du thorax, INSERM, CNRS, UNIV Nantes & CHU Nantes, Nantes, France.
[5] Current address: Gene Expression Laboratory, Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd., La Jolla, CA 92037, USA.

*Corresponding author. Department of Genetics and Complex Diseases, Harvard T.H. Chan School of Public Health, 665 Huntington Ave, Bldg1, Rm 409, Boston, MA, 02115, USA. E-mail: ldai@hsph.harvard.edu (L. Dai).

**Corresponding author. Department of Genetics and Complex Diseases, Harvard T.H. Chan School of Public Health, 665 Huntington Ave, Bldg1, Rm 409, Boston, MA, 02115, USA. E-mail: clee@hsph.harvard.edu (C.-H. Lee).

Abbreviations: M2, alternative activation of macrophage; M1, classic activation of macrophage; WAT, white adipose tissue; ATM, adipose tissue resident macrophage; Ppar, peroxisome proliferator-activated receptor; Stat6, signal transducer and activator of transcription 6; PA, palmitic acid; Nlrp3, NLR family pyrin domain containing 3; BMDM, bone marrow derived macrophage; ILC2, type 2 innate lymphoid cell; SVF, stromal vascular fraction; OCR, oxygen consumption rate; Angptl4, angiopoietin-like 4; Sgms1, sphingomyelin synthase 1; Plin2, perilipin 2; Acadvl, acyl-CoA dehydrogenase, very long chain; Slc25a20, solute carrier family 25 member 20; Mogat1, monoacylglycerol O-acyltransferase 1; Mgl1, macrophage galactose-type lectin-1; Arg1, arginase 1; Bcl2, B-cell lymphoma 2; H2-Eb1, histocompatibility 2, class II antigen E beta

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lymphocytes. In lean individuals, adipose tissue macrophages (ATMs) have an alternatively activated (M2) phenotype that limits inflammation and sustains homeostasis [1,3]. With the onset of obesity, classically activated M1 macrophages preferentially accumulate in WAT and exhibit a pro-inflammatory phenotype characterized by the production of cytokines such as interleukin-1β (Il-1β) and tumor necrosis factor-α (Tnf-α) [1–3]. These M1 derived pro-inflammatory mediators have been shown to cause metabolic dysregulation and insulin resistance [1–3]. While this M1/M2 paradigm is instrumental for describing immune phenotypes in physiological and pathological states, recent studies have implicated a dynamic and complex ATM polarization process [4]. In addition, it has been shown that metabolically activated macrophages driven primarily by free fatty acids (FA) do not express M1 surface markers, despite increased production of Il-1β and Tnf-α in these cells [5].

Several stimuli from the adipose microenvironment have been reported to regulate the ATM phenotype. It has been suggested that at the lean state, eosinophils and type 2 innate lymphoid cells (ILC2s) constitutively produce Th2 cytokines interleukin-4 (Il-4) and interleukin-13 (Il-13), respectively, which promote M2 polarization [6,7]. In the pathological context of obesity, adipocyte hypertrophy and cell death increase the production of chemokines, such as C-X-C motif chemokine ligand 12 (Cxcl12) and monocyte chemotactic protein-1 (Mcp-1) to recruit Ly6C^{hi}Ccr2^{+} monocytes into WAT [8,9], where CD8^{+} cytotoxic T cells have been found to secrete interferon-γ (Ifn-γ) that contributes to macrophage M1 polarization [3,10,11]. In addition to the classical M1 activation, dysregulated lipid homeostasis caused by increased lipolysis in WAT has also been shown to be a trigger of inflammatory responses in ATMs as described above. Recent studies have demonstrated that saturated FA and their metabolites, notably ceramides, can act on NLR family pyrin domain containing 3 (Nlrp3) inflammasome formation, resulting in autocatalytic activation of caspase-1 and production of mature Il-1β to promote inflammation [12,13]. Interestingly, WAT lipolysis by fasting or β-adrenergic stimulation can also drive ATM accumulation in lean individuals [14]. This raises the question of how M2 ATMs regulate FA-induced immune responses.

Macrophage M2 polarization requires activation and cooperation of several transcriptional factors [3,15]. Signal transducer and activator of transcription 6 (Stat6), the canonical effector of Th2 signaling, has been proposed to regulate mitochondrial oxidative metabolism to fuel M2 activation [3,15,16]. In mice, Stat6 controls the expression of nuclear receptors peroxisome proliferator-activated receptor delta (*Pparδ* or *Pparβ*) and gamma (*Pparγ*), both of which can further modulate the function of M2 macrophages [9,17,18]. Pparδ/Pparγ are well-known lipid sensing nuclear receptors. A variety of endogenous lipids, including unsaturated FAs, saturated FAs and hydroxyeicosatetraenoic acids can physically bind to Pparδ and Pparγ, which, in turn, activate the transcriptional programs for FA oxidation, mitochondrial biogenesis, and anti-inflammatory response in macrophages [15,19]. *Stat6*$^{-/-}$, myeloid-*Pparδ*$^{-/-}$, and myeloid-*Pparγ*$^{-/-}$ mice are more prone to high fat diet induced WAT inflammation and insulin resistance [17,20–22]. The metabolic function of the Stat6-Ppar signaling cascade implicates a role for M2 macrophages in mediating FA homeostasis within WAT.

The current study aims to identify potential physiological functions of M2 polarization in ATMs. Through expression profiling and genetic approaches, we find that the Stat6-Ppar axis plays an important role in protecting macrophages against lipotoxicity-induced cellular dysfunction. This is mediated by transcriptional regulation of cell death/pro-survival genes, in addition to their known function in FA metabolism and mitochondrial respiration. Dysregulation of M2 signaling, such as in *Stat6*$^{-/-}$ and *Pparδ/Pparγ*$^{-/-}$ macrophages, increases susceptibility to palmitic acid (PA)-induced cell death, which contributes to the initiation of metabolic inflammation in WAT.

2. MATERIALS AND METHODS

2.1. Animal experiments

2.1.1. Mouse models

Stat6$^{-/-}$ mice and the C57BL/6J controls as well as lysozyme-cre mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Pparδ*$^{f/f}$ and *Pparγ*$^{f/f}$ in the C57BL/6J background were generated as described previously [23,24]. *Pparδ/γ*$^{f/f}$ mice were obtained by crossing the *Pparδ*$^{f/f}$ and *Pparγ*$^{f/f}$ alleles. These mice were used to create myeloid-specific *Pparδ*$^{-/-}$, *Pparγ*$^{-/-}$ and *Pparδ/γ*$^{-/-}$ mice, respectively (Mac-*Pparδ*$^{-/-}$, Mac-*Pparγ*$^{-/-}$ and Mac-*Pparδ/γ*$^{-/-}$), by crossing to lysozyme-cre. All mice were housed in a 12 h light/12 h dark cycle with temperatures of 18–23 °C and 40–60% humidity on standard chow diet and allowed food and water *ad libitum* unless otherwise stated. Mice were sacrificed by CO_2 asphyxiation. All animal studies were approved by the Harvard Medical Area Standing Committee on Animals.

2.1.2. Metabolic studies

In vivo studies were conducted in 3-month-old male mice on normal chow diet (n = 4–7). After 14 h fasting, animals were injected intraperitoneally with 1 mg/kg of CL316,243 (Santa Cruz Biotechnology, Dallas, TX, USA, Cat# sc-203895) or saline and sacrificed 2.5 h after injection.

2.1.3. Stromal vascular fraction (SVF) and ATMs isolation

For SVF isolation, perigonadal adipose tissue was excised and digested with digestion buffer (DMEM, 1 g/L glucose, 2 mg/ml collagenase type II and 2% albumin) at 37 °C for 30 min [17]. After filtering through 250 μm nylon mesh, digested adipose tissue was spun down at 400 g for 5 min. The supernatant was discarded and cells were resuspended in washing buffer (DMEM, 1 g/L glucose and 2% albumin). After filtering through 70 μm filter, cells were spun down at 400 g for 5 min and cell pellet was collected. Lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2–7.4) was added for 5 min to remove red blood cells. Cells were subsequently filtered through 40 μm filter, spun down, and collected for analysis.

For ATMs isolation, SVF was further incubated with an anti-F4/80 antibody (Invitrogen, Grand Island, NY, USA) for 30 min at 4 °C. After spinning down at 400 g for 5 min, SVF was resuspended in isolation buffer (PBS, 0.1% BSA, 2 mM EDTA, pH 7.4) containing Dynabeads (Invitrogen). After gently rotating for 30 min at 4 °C, F4/80 positive cells were selected by magnetic Dynabeads according to manufacturer's protocol.

2.1.4. Blood sampling and analysis

Blood glucose measurements were performed using the One Touch Ultra Blood Glucose Diabetic Test Strips (LifeScan, Milpitas, CA, USA). For blood chemistry, blood samples were taken from mouse hearts immediately after sacrifice and placed on ice for 30 min. Samples were subsequently centrifuged at 2,000 g for 10 min to remove the clot and serum was collected and store at −80 °C for analysis. Serum free FAs were measured using commercially kits from Wako Chemicals (Richmond, VA, USA) and triglycerides (TG) were tested by

