RESEARCH

NFATc3 deficiency reduces the classical activation of adipose tissue macrophages

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Abstract

Nuclear factors of activated T cells (NFAT) c3 have a prominent role in the regulation of proinflammatory factors in immune cells. The classically activated M1 macrophages are key players in the initiation and maintenance of adipose tissue (AT) inflammation. The role of NFATc3 in obesity and AT inflammation is unknown. We set out to determine how deficiency of NFATc3 effected macrophage polarization, inflammation and insulin resistance in visceral AT of high-fat diet (HFD)-fed mice. Nfatc3−/− and WT mice were fed a HFD for 8–17 weeks. Epididymal white AT (eWAT) F4/80(+) cells were characterized by fluorescence-activated cell sorting and quantitative RT-PCR. Results showed that Nfatc3−/− mice developed HFD-induced obesity similar to WT mice, but insulin sensitivity and glucose tolerance were improved, and liver fat accumulation was reduced in Nfatc3−/− mice compared to WT control mice. Moreover, M1 macrophage content and proinflammatory factors were reduced, whereas the alternatively activated M2 macrophage content was increased in eWAT of HFD-fed Nfatc3−/− mice compared to that of WT mice. In addition, eWAT insulin signaling was improved in HFD-fed Nfatc3−/− mice. Importantly, after bone-marrow-derived macrophages had been isolated from Nfatc3−/− mice and cultured in vitro, treatment of these cells with interferon-γ and lipopolysaccharide resulted in reduction of M1 inflammatory markers, suggesting that NFATc3 promoted M1 polarization by a cell-autonomous mechanism. The results demonstrated that NFATc3 played an important role in M1 macrophage polarization, AT inflammation and insulin resistance in response to obesity through transcriptional activation of proinflammatory genes.

Introduction

Classically activated M1 macrophages are activated by T helper (Th)-1 cytokines, such as interferon-γ (IFNγ) and lipopolysaccharide (LPS). By contrast, the alternatively activated M2 macrophages are activated with interleukin-4 (IL-4) and interleukin-13 (IL-13). The proinflammatory cytokines IFNγ, tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) are implicated in disrupting insulin signaling (Mantovani et al. 2004, De Taeye et al. 2007, Harford et al. 2011). The activation of classically activated M1 macrophages and the reduction of anti-inflammatory M2 macrophages have been implicated in the development of AT inflammation and insulin resistance (Gordon 2003, Lumeng et al. 2007a, Mosser & Edwards 2008, McLaughlin et al. 2017). However, the molecular mechanisms underlying how macrophage infiltration was regulated in obesity-induced inflammation in white AT remain to be completely elucidated.

The transcription factors STAT1, STAT6, C/EBPβ, IRF-4, IRF5, PPARγ and GATA6 regulate transcriptional programs that control M1/M2 macrophage polarizations (Lawrence & Natoli 2011, Okabe & Medzhitov 2014). Candidate regulators can be identified by analysis of promoters of M1-specific genes in macrophages, and among these, IFNγ (Sica et al. 1997), TNFα (Falvo et al. 2000), IL12p40 (Zhu et al. 2003) and iNOS (Ranjan et al. 2014) promoters are regulated by nuclear factor of activated T cells (NFAT). NFAT family consists of five members, NFATc1, NFATc2, NFATc3 (aka NFAT4), NFATc4 (aka NFAT3) and NFAT5, and they have been implicated in regulating transcription of a number of inducible genes in immune cells (Rao et al. 1997, Crabtree & Olson 2002, Hogan et al. 2003). The production of several T cell-derived cytokines including IL-2, IFNγ and IL-4 were significantly diminished in T cells lacking both NFATc1 and NFATc2 (Peng et al. 2001). CD4+ Th1 cells have been implicated as positive regulators of AT inflammation via production of IFNγ, which contributes to the activation of M1 macrophages (Deng et al. 2017). Depletion of Th1 cells with CD3-specific antibodies protected mice from HFD-induced AT inflammation and insulin resistance (Winer et al. 2009). However, mice lacking NFATc3 produce normal levels of serum IL-4 and IFNγ (Oukka et al. 1998), but whether their levels change in obese ATs is unknown. In transgenic mice expressing constitutively active NFATc3 in T cells, NFATc3 enhances expression of Th1 cytokine IFNγ and TNFα but suppresses that of Th2 cytokine IL-4 (Chen et al. 2003). NFATc3 and NFATc4 are required for the Toll-like receptor (TLR)-activated innate inflammatory response and cytokine induction in macrophages (Minematsu et al. 2011). In macrophages responding to LPS stimulation, NFATc3 undergoes rapid nuclear translocation, whereas NFATc2 and NFATc4 are localized to the nucleus and NFATc1 is localized to the cytosol, constitutively, suggesting that NFATc3 is the only member rapidly activated by LPS stimulation (Ranjan et al. 2014). LPS-induced NFATc3 in turn directly activates iNOS expression in bone marrow-derived macrophages (BMDMs) (Ranjan et al. 2014).

However, it remains unclear whether NFATc3 participates in immune dysfunction causing chronic inflammation and insulin resistance in diet-induced obesity. We showed that reduced M1 macrophage polarization in AT and improved insulin sensitivity in Nfatc3−/− mice fed with HFD. We had identified NFATc3 as a positive regulator of obesity-induced inflammation and insulin resistance. This work was presented at Keystone Symposia in Vancouver, Canada in 2014.

Materials and methods

Nfatc3−/− mice that were backcrossed to C57BL/6 mice as described before (Oukka et al. 1998) were obtained from Jackson Laboratory (Stock number 010589). Nfatc3+/− heterozygous mice were bred to generate age-matched Nfatc3−/− and WT littermates. All mice were maintained in the Specific Pathogen Free (SPF) mice facility of Second Xiangya Hospital under a 12-h light/darkness cycle. At age 6 weeks, male mice were fed either a normal diet (ND, SLA-COM) containing 4% (wt/wt) total lipids or a HFD (Research Diets) containing 60% kcal fat for different weeks. Total body fat content was measured by nuclear magnetic resonance with the Minispec Mq7.5 (Bruker, Karlsruhe, Germany). All animal experiments were performed according to the protocols approved by the Animal Care Research Committee of Second Xiangya Hospital of Central South University. Anti-phospho-Akt (S473) and anti-beta-actin antibody were from Cell Signaling Technology.

Intraperitoneal glucose tolerance tests (IGTTs) and insulin tolerance tests (ITTs)

IGTTs were carried out with mice that were subjected to overnight (10–12h) food withdrawal. Blood samples were taken from the tail vein before (0min) and 15, 30, 60, 90 and 120min after glucose administration (2g of glucose/kg body weight). For insulin tolerance (sensitivity) tests, human insulin (0.75 U/kg; Eli Lilly) was administered intraperitoneally to mice that had fasted for 4h. Blood samples were taken from the tail vein before (0min) and 15, 30, 60 and 90min after insulin administration.
Blood glucose levels of samples were determined using an automated blood glucose meter (Roche).

**RNA extraction and gene expression**

RNA from cells or mouse tissues were homogenized in TRIzol (Invitrogen) and then followed by purification and DNase I digestion to remove genomic DNA contamination with RNAeasy column (Qiagen) according to the manufacturer’s protocol. After reverse transcription, gene expression was quantified with ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative gene expression levels were calculated by the use of the ΔΔCt method with 18S or GAPDH being used as the reference gene (Luo et al. 2014). PCR primer sequences will be available upon request.

**Western blots**

Cells or tissues were lysed in lysis buffer (150 mM NaCl, 0.5% NP-40, 0.1% SDS and 50 mM Tris–Cl, pH 7.5) with 1 mM PMSF and protease inhibitor cocktail (Sigma). The cell lysates were separated by 10% SDS-PAGE gels, transferred to a nitrocellulose membrane (Millipore). The membranes were incubated with the primary antibodies anti-AKT, anti-phospho-Akt (S473) and anti-beta-actin, respectively, followed by horseradish peroxidase-labeled donkey anti-rabbit or donkey anti-mouse antibodies. Immunoreactive proteins were visualized with the Molecular Imager Chemi Doc XRS Western Blot Detection System (BIO-RAD), and bands were quantified with Image Lab software as described before (Meng et al. 2017).

**Hematoxylin and eosin staining and Oil Red O staining**

H&E staining and Oil Red O staining were performed as previously described (Liu et al. 2012). Briefly, for H&E staining, liver tissues from HFD-fed Nfatc3−/− and WT mice were fixed in 10% formalin solution for 24 h and then embedded in paraffin. Liver tissue sections (7 μm thick) were stained with hematoxylin-eosin. For Oil Red O staining, liver tissues from HFD-fed Nfatc3−/− and WT mice were flash-frozen by liquid nitrogen. Liver tissue sections (5 μm thick) were stained with Oil Red O.

**Measurement of IFNγ protein levels**

Nfatc3−/− and WT control mice, both on HFD, were killed and epididymal fat pad was isolated immediately and were cultured at 37°C in a humidified incubator (5% CO₂) overnight. The supernatant IFNγ levels were measured with a mouse IFNγ ELISA kit from Dakewe Biotech Co.

**Isolation of adipose stromal vascular cells**

Mouse primary stromal vascular fractions (SVFs) were isolated and cultured as previously described (Liu et al. 2014). Briefly, subcutaneous white fat depots from Nfatc3−/− or WT control mice (both on HFD) were isolated and minced and then digested by type II collagenase (Sigma-Aldrich). The digested tissue was filtered through a 100 mm nylon screen. The collected cells were centrifuged for 5 min and suspended in PBS and used for flow cytometry analysis.

For AKT phosphorylation study in AT and AT explants were incubated at 37°C in a humidified incubator (5% CO₂) with or without 100 mM insulin in RPMI 1640 medium without serum for 20 min. The AT lysates were subjected to Western blot analysis with anti-phospho-AKT (473) antibody.

**Flow cytometry analysis**

SVF cells obtained from eWAT of HFD-fed mice were blocked with rat anti-mouse CD16/CD32 in flow cytometry buffer (PBS; 2% FBS, 25 mM HEPES, 5 mM EDTA) for 15 min on ice. For M1 or M2 macrophages, cells were stained with anti-CD11c or anti-CD206 and anti-F4/80 (antibody for macrophages) followed by anti-mouse CD16/CD32 (Biolegend, San Diego, CA, USA) in flow cytometry buffer for 30 min on ice protected from light. For CD4+ helper T cell subsets, CD4 and IFNγ antibodies were used to stain CD4 Th1 cells, and CD4 and IL-4 antibodies were used to stain CD4 Th2 cells. Cells were washed and pelleted at 200 g for 10 min before being re-suspended in flow cytometry buffer. Samples were run on a flow cytometer (Biolegend) and analyzed with FlowJo 10 software.

**Isolation of BMDMs**

Mouse bone marrow cells were flushed by PBS from the tibia and femur of male mice and differentiated into BMDMs in DMEM media with 20% FBS and 10% L929 cell conditioning media as a source of M-CSF as described before (Dai et al. 2008). Cells were cultured for 7 days at 37°C/5% CO₂ and then were treated with LPS and IFNγ or with IL-4, respectively, to differentiate into M1 or M2 macrophages.
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Statistics
Statistics were performed using the Prism software (GraphPad). Statistical significance of two groups was assessed by unpaired two-tailed Student’s t-test or one-way ANOVA when more than two groups were compared. A value of \( P < 0.05 \) was considered significance. All data are expressed as the mean ± S.E.M.

Results
Deficiency of NFATc3 improves glucose and insulin tolerance in HFD-fed mice

To determine the effect of NFATc3 deficiency on glucose metabolism, Nfatc3−/− mice were placed on either a normal diet or HFD. Interestingly, Nfatc3−/− mice gained weight to a similar extent as did their WT counterparts when fed a HFD (Fig. 1B and C). Because Nfatc3−/− mice weighed less than their WT counterparts at the initiation of the study (~30 g vs ~40 g respectively), Nfatc3−/− mice weighed less than WT mice on a HFD during the study (Fig. 1A). However, in the end of the study, there were no significant differences in the ratio of body fat/body weight between WT and Nfatc3−/− mice (Fig. 1D), indicating that Nfatc3−/− mice had same fat content as WT mice.

To evaluate how NFATc3 deficiency effects glucose homeostasis, we performed GTT and ITT in WT and Nfatc3−/− mice. HFD-induced pronounced glucose intolerance in WT mice, whereas obese Nfatc3−/− mice were partially protected from glucose intolerance (Fig. 1E). Importantly, high-fat feeding decreased insulin-stimulated glucose disposal in WT mice, whereas Nfatc3−/− mice were partially protected from insulin resistance (Fig. 1F). Taken together, these results indicated that deficiency of NFATc3 moderately protected HFD-fed mice from glucose intolerance and insulin resistance.

AT M1 macrophage content and inflammation are decreased in obese Nfatc3−/− mice

The accumulation of M1 macrophages in AT couples AT expansion with systemic insulin resistance (Weisberg et al. 2003, Lumeng et al. 2007a, McLaughlin et al. 2017). Therefore, we next examined whether deficiency of NFATc3 influences the accumulation of M1 adipose tissue macrophages (ATMs). As shown in Fig. 2A, epididymal fat pad weights were increased in WT and Nfatc3−/− mice, both on a HFD, consistent with the observed increase in total body mass. Particularly, the magnitude of epididymal fat pad expansion in obese Nfatc3−/− mice was comparable to that of obese WT mice (Fig. 2B). Moreover, the expression of monocyte chemotactant protein-1 (MCP-1) was significantly decreased in Nfatc3−/− mice relative to WT mice (Fig. 2C). Because M1 ATMs produce inflammatory cytokines that block insulin action (Lumeng et al. 2007b), we next assessed the expression of IL-1β and TNFα in AT and found that their expression levels were significantly decreased in AT of obese Nfatc3−/− mice compared to that of obese WT mice (Fig. 2D and E). Moreover, the secretion of IFNy was decreased in the supernatants of cultured eWAT explants isolated from Nfatc3−/− mice compared to those from WT mice, both on HFD (Fig. 2F).
To illuminate how AT insulin signaling was altered by NFATc3 deficiency, we evaluated insulin-stimulated phosphorylation of AKT in eWAT explants of Nfatc3−/− mice and WT mice, both on HFD for 17 weeks. Indeed, phosphorylation of AKT was significantly increased in AT of Nfatc3−/− mice compared to that of obese WT mice before and after ex vivo treatment with or without 100 nM insulin for 20 min (Fig. 2G and H). These results suggested that HFD-fed Nfatc3−/− mice were protected from insulin resistance in visceral AT presumably due to the reduced levels of IL-1β, TNFα and IFNγ.

Deficiency of NFATc3 alters ATM phenotypes

AT from lean mice contains resident M2 macrophages, whereas the macrophages resident in the expanded AT in obesity are derived either from local proliferation or from circulating monocytes and adopt an M1 phenotype (Lumeng et al. 2008, Shaul et al. 2010, Amano et al. 2014). Because NFATc3 deficiency decreased AT inflammation, we examined whether the phenotype of ATMs was influenced by NFATc3 deficiency. Therefore, we isolated stromal vascular cells from AT of WT or Nfatc3−/− mice (both on HFD) and evaluated the surface expression of CD11c and CD206, markers of M1 and M2 macrophages, respectively, on the total macrophage (F4/80+) population by flow cytometry analysis. HFD feeding prominently increased the population of ATMs expressing CD11c and F4/80 in WT mice but not in Nfatc3−/− mice (Fig. 3C). HFD feeding also increased total F4/80 macrophages and total CD45+ immune cells in WT mice but not in Nfatc3−/− mice (Fig. 3A and C). Conversely, the amount of ATMs expressing F4/80 and CD206 (M2) was increased in Nfatc3−/− mice compared to WT mice (Fig. 3B and D). CD206+ F4/80 macrophages relative to total live cells or to total CD45+ immune cells were both increased in HFD-fed Nfatc3−/− mice (Fig. 3D). Representative dot plots of the F4/80+ ATMs were shown in Fig. 3A and B.

We next examined the expression of characteristic M2

Figure 2

Reduced adipose tissue inflammation and M1 macrophage content in Nfatc3−/− mice on HFD. (A) Representative images of epididymal fat pad (eWAT) from WT and Nfatc3−/− mice on HFD for 16 weeks were shown. (B) eWAT was weighed and the fat-to-body weight ratio was not significantly different between WT and Nfatc3−/− mice. (C, D and E) Gene expressions of MCP-1, IL-1β and TNFα in adipose tissue from HFD-fed Nfatc3−/− and Nfatc3+/+ mice were analyzed by real-time quantitative RT-PCR. *P<0.05 vs WT mice. n=3 per group. (F) The IFNγ concentrations in cultured eWAT supernatants were measured with a mouse IFNγ ELISA kit. (G) Insulin-stimulated phosphorylation of AKT in the adipose tissue of Nfatc3−/− and WT mice was determined by Western blot analysis. (H) The relative phosphorylation levels of AKT shown in (G) were quantified. *P<0.05 or **P<0.01 vs WT mice. n=3 per group. A full color version of this figure is available at https://doi.org/10.1530/JME-18-0070.
Figure 3

Reduced M1 ATMs and increased M2 ATMs in Nfatc3−/− mice fed a HFD. (A and B) ATMs contents were assessed by flow cytometry in SVF cells obtained from eWAT of HFD-fed Nfatc3−/− and WT mice. Cells were gated for F4/80+ ATMs and analyzed for expression of markers of M1 (CD11c) and M2 (CD206) ATMs. (C and D) Data shown as percentage of CD45+ cells or total live SVF cells. *P<0.05 or **P<0.01 vs WT mice. n=3–4 per group. (E) Expression analysis of inflammatory genes in adipose tissue from Nfatc3−/− and WT mice both on HFD. SVF cells obtained from eWAT of HFD-fed mice were analyzed by real-time quantitative RT-PCR. *P<0.05 vs Nfatc3+/+. n=3 per group. (F and G) Cells were gated for CD4+ T cells and analyzed for the expression of IFNγ for CD4+ Th1 cells (F) and for the expression of IL-4 for CD4+ Th2 cells (G).
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genes to evaluate how ATM phenotype was influenced by NFATc3 deficiency. The expression of M2 genes Arg1, IL-4 and IL-13 was significantly increased in Nfatc3−/− mice compared to WT mice on HFD (Fig. 3E). Together, these results suggested that NFATc3 deficiency prevented polarization of M1 ATMs in obesity and consequently altered the equilibrium between M1 and M2 ATMs.

Since CD4+ Th1 cells have been implicated as positive regulators of AT inflammation via production of IFNγ (Winer et al. 2009), we isolated stromal vascular cells from AT of WT or Nfatc3−/− mice (both on HFD) and sorted the surface expression of CD4+ IFNγ for CD4 Th1 cells and that of CD4+ IL-4 for Th2 cells on the total CD4+ T cell population by flow cytometry. The results in Fig. 3F showed that CD4+ IFNγ+ cells accounted for 11.6% among CD4+ cells in SVF cells of WT mice compared to 7.8% in those of Nfatc3−/− mice. In contrast, there were no significant differences in CD4+ IL-4+ cells, which accounted for 12.6% on the total CD4+ T cells of WT SVF compared to 11.8% on those of Nfatc3-deficient SVF (Fig. 3G). The results suggested that reduction of both CD4+ Th1 cells and IFNγ in turn contributed to the reduced M1 macrophages in the AT of HFD-fed Nfatc3−/− mice.

Obesity-induced hepatic steatosis and inflammation are improved by NFATc3 deficiency

Macrophage-mediated AT inflammation can promote insulin resistance in other insulin-sensitive tissues, such as the liver and skeletal muscle (Olefsky & Glass 2010, Lackey & Olefsky 2016). Insulin resistance in the liver can be caused by fatty acid release from AT, resulting in hepatic triglyceride accumulation; also, insulin resistance can be caused by increased inflammatory cytokines produced from the AT (Samuel & Shulman 2016). Therefore, we tested whether deficiency of NFATc3 improved hepatic steatosis in HFD-fed mice. As shown in Fig. 4A, NFATc3 deficiency markedly alleviated diet-induced steatosis in the liver. Histological analysis of the liver showed an apparent decrease in fat accumulation in Nfatc3−/− mice (Fig. 4B). Accordingly, fat staining with Oil Red O showed a reduction in total hepatic fat content in Nfatc3−/− mice on HFD, compared to obese WT mice (Fig. 4C). Because steatosis can induce hepatic inflammation, we next evaluated whether the inflammatory factors were reduced by NFATc3 deficiency. Indeed, decreased MCP-1 and Toll-like receptor 4 (TLR4) mRNA were observed in the liver in Nfatc3−/− mice compared to WT mice, both on HFD (Fig. 4D and E). It has been shown that TLR4 stimulated by LPS drives macrophages to M1 phenotype (Orr et al. 2012).

NFATc3 is required for M1 macrophage activation in BMDMs ex vivo

The Nfatc3-deleted mouse line that we analyzed was not conditional gene knockout; therefore, in order to understand whether NFATc3 functions in a cell-autonomous or non-cell-autonomous manner in AT macrophages, we used an ex vivo system, whereby BMDMs were differentiated into the M1 or M2 lineage. Type 2 cytokines IL-13 and IL-4 secreted by adipocytes and eosinophils activate ATMs toward the anti-inflammatory
M2 phenotype, whereas IFNγ activates ATMs to differentiate into the M1 phenotype (McLaughlin et al. 2017). Therefore, BMDMs were isolated from Nfatc3−/− and WT mice under normal diet and treated with LPS and IFNγ or with IL-4 to promote M1 or M2 differentiation, respectively. Subsequently, the expression of specific markers of each phenotype was determined by quantitative RT-PCR. Remarkably, BMDMs from Nfatc3−/− mice had a dramatically reduced ability to respond to LPS and IFNγ, as shown by the lower levels of induced IL-1β and TNFα mRNA, but they were sensitive to IL-4, as demonstrated by higher levels of Arg1 and PPARγ mRNA (Fig. 5A and B). These results suggested that NFATc3 was required for the differentiation of macrophages into M1, but not M2, phenotype and therefore was an important transcriptional regulator of proinflammatory factors in macrophage in a cell-autonomous manner.

Discussion

NFAT family has diverse activities, such as the activation of genes encoding type I interferon (Bao et al. 2016), inflammatory cytokines, including TNFα and IL-2 (Rao et al. 1997, Crabtree & Olson 2002). Here, we demonstrate a role for NFATc3 in regulating M1 macrophage lineage in ATs in HFD-fed mice. Our results showed reduced CD4+ Th1 cells and IFNγ in AT of HFD-fed Nfatc3−/− mice, suggesting a mechanism for macrophage polarization toward M2 phenotype in these mice. Remarkably, when BMDMs isolated from WT or Nfatc3−/− mice were cultured in vitro and treated by LPS and IFNγ during their differentiation, the induction of M1-specific cytokines was reduced in BMDMs of Nfatc3−/− mice compared to those of WT control mice, suggesting that NFATc3 regulated M1 macrophage polarization in the macrophage in a cell-autonomous manner. The results documented a previously unrecognized role of NFATc3 in promoting M1 macrophage polarization, inflammation and insulin resistance in obesity.

Obesity promotes the recruitment of monocytes from the bone marrow in part by activating the C-C motif chemokine receptor 2 (Ccr2). Deficiency of Ccr2 or its ligand, MCP-1, in mice protects from monocyte infiltration into AT and colonic inflammation (Lumeng et al. 2007a, Odegaard et al. 2007, Kawano et al. 2016). MCP-1 also drives macrophages within the visceral AT to undergo proliferation (Amano et al. 2014). Our results showed reduced MCP-1 in the AT in Nfatc3−/− mice on HFD, suggesting that reduced M1 macrophage polarization in obese ATs in Nfatc3−/− mice may be partly due to reduced MCP-1. NFATc3 is highly expressed on macrophages; previous studies have demonstrated that NFATc3 nuclear localization in macrophages is increased upon LPS stimulation (Ranjan et al. 2014). Moreover, since NFATc3 has been shown to regulate proinflammatory cytokines, including iNOS, IFNγ and TNFα, our results suggested that NFATc3 promoted M1 macrophage polarization in AT and BMDMs through activation of these cytokines. Although our studies suggested a key role of NFATc3 in mediating tissue recruitment of monocytes in obesity, we had not
ruled out the possibility that NFATc3 was an important factor in proliferating macrophages at the local tissue level as local proliferation of macrophages also contributes to AT inflammation (Amano et al. 2014). Our studies showed a profound decrease in the activation of inflammatory signaling in AT, liver and BMDMs in Nfatc3−/− mice on HFD; therefore, NFATc3 pathway may play an important role in both recruitment and local proliferation of monocytes and macrophages in these tissues.

Macrophage polarization toward a M1 or M2 state depends generally on cytokines and lipid mediators (Mantovani et al. 2004). A high ratio of inflammatory M1 ATMs (F4/80+CD11c+) to resident M2 ATMs (F4/80+CD206+) is responsible for both adipose tissue as well as systemic insulin resistance (Lumeng et al. 2007a, Shaul et al. 2010). In comparison with obese WT mice, ATMs were considerably decreased in obese Nfatc3−/− mice, which was associated with a higher M2/M1 ratio. This observation was confirmed by the identification of higher M2 marker mRNA, including ARG1, in the AT and BMDMs isolated from Nfatc3−/− mice. Because PPARγ regulates alternative activation of macrophages and is associated with increased insulin sensitivity (Odegaard et al. 2007), it is likely that PPARγ caused the higher proportion of M2 macrophages in the AT of Nfatc3−/− mice. Indeed, we found increased PPARγ expression in Nfatc3-deficient BMDMs after IL-4 stimulation. In HFD-fed Nfatc3−/− mice, M1 polarization was largely reduced and insulin signaling was well-maintained in the AT; triglyceride accumulation in the liver was largely reduced. These data suggested that NFATc3 regulated macrophage polarization in the AT and that AT inflammation causes inflammation and insulin resistance in other insulin-sensitive tissues (Rosen & Spiegelman 2006, Guilherme et al. 2008, Reilly & Saltiel 2017). Our results also were in line with previous studies that showed lipolysis in AT promotes hepatic triglyceride accumulation and subsequent insulin resistance (Vigouroux et al. 2011, Gan et al. 2015). A duration of high-fat feeding for 17 weeks were chosen because it can induce AT inflammation and insulin resistance in the liver, skeletal muscle and the AT (Kim et al. 2008).

Because IFNγ and IL-1β inhibit insulin signaling and promote insulin resistance in adipocytes (McGillicuddy et al. 2009), decreased IFNγ and IL-1β in the AT of Nfatc3−/− mice led to the improved insulin sensitivity in Nfatc3−/− mice. In addition, Nfatc3-deficient BMDMs displayed an M2-skewed polarization profile at the mRNA level and exhibited a significantly lower expression of proinflammatory cytokines (TNFa and IL-1β) in response to LPS and IFNγ. Therefore, NFATc3 influences macrophage polarization by regulating the expression of polarization-associated genes in macrophages.

Infiltration of CD4+T cells into the AT and development of insulin resistance transpires 5 weeks after HFD feeding in mice, whereas recruitment of macrophages in AT was delayed until 10 weeks of HFD (Kintscher et al. 2008, Stolarczyk 2017). CD4+ T cells react with antigenic peptides associated with class II major histocompatibility complex (MHC) molecules; adipocytes expressing class II MHC activate CD4+ T cells and increase the leptin-induced secretion of IFNγ by CD4+ T cells (Deng et al. 2013). IFNγ then activates M1 macrophages, which secrete numerous cytokines and chemokines, including TNF-α, IL-1β and MCP-1, causing insulin resistance in adipocytes. M1 macrophages also promote Th1 polarization of CD4+T cells by IL-12 production, reinforcing the positive feedback loop between CD4+Th1 cells and M1 macrophages. In Nfatc3−/− mice on HFD, CD4+ IFNγ+ cells were reduced in the AT compared to WT control, suggesting regulation of recruitment of AT CD4+ Th1 cells by NFATc3. Interestingly, T helper cells can also promote cardiac fibrosis by secretion of cytokines and activation of macrophages (Nevers et al. 2017). In mouse, heart failure model induced by pressure overload, activated Th1 T cells promote cardiac fibrosis in an IFNγ-dependent manner by inducing the transition of cardiac fibroblasts into TGF-β-producing myofibroblasts (Groschel et al. 2017), suggesting a potential mechanism for protection of cardiac hypertrophy and heart failure in Nfatc3−/− mice (Wilkins et al. 2002).

Collectively, these results identify NFATc3 as a regulator of macrophage polarization in AT through activation of macrophage proinflammatory genes in HFD-fed mice and shed new insights into the mechanisms by which NFATc3 contributes to macrophage polarization, inflammation and insulin resistance in the AT.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y D and L H conceived and designed the experiments; L H, F H, M H and Y D performed the experiments; L H, F H, M H, M P, Z Z, F L and Y D analyzed the data; M P, Z Z and FL contributed reagents/materials/analysis tools; Y D and L H contributed to the writing of the manuscript.
References


Harford KA, Reynolds CM, McGillicuddy FC & Roche HM 2011 Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue. *Proceedings of the Nutrition Society* 70 408–417. (https://doi.org/10.1017/S0029665111000563)


Lumeng CN & Saltiel AR 2011 Inflammatory links between obesity and metabolic disease. *Journal of Clinical Investigation* 121 2111–2117. (https://doi.org/10.1172/JCI57132)


Okabe Y & Medzhitov R 2014 Toll-like receptor 4 deficiency promotes the alternative activation of adipose tissue macrophages. *Diabetes* 61 2718–2727. (https://doi.org/10.2337/db11-13595)


Oukka M, Ho IC, de la Brousse FC, Hoey T, Grusby MJ & Gilmer LH 1998 The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* 9 295–304. (https://doi.org/10.1016/S1074-7613(00)80612-3)


Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL & Ferrante AW Jr 2003 Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation* 112 1796–1808. (https://doi.org/10.1172/JCI200319246)


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