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.

Key Words

- ▶ NFAT transcription factor
- ▶ adipose tissue inflammation
- ▶ insulin resistance
- ▶ macrophage polarization
- ▶ obesity

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Introduction

Obesity and type 2 diabetes mellitus are associated with low-grade, chronic inflammation and macrophage infiltration in adipose tissue (AT) (Weisberg *et al.* 2003, Hotamisligil 2006, 2017, Lumeng & Saltiel 2011, Kusminski *et al.* 2016, Deng *et al.* 2017). A number of studies have demonstrated that inhibiting macrophage

infiltration in white AT can improve glucose tolerance in the context of obese mice (Osborn & Olefsky 2012, Aouadi *et al.* 2013). Also, reduction of macrophage infiltration and inflammation-related gene expression in white AT are associated with weight loss in obese humans (Clement *et al.* 2004, Canello *et al.* 2005).

Classically activated M1 macrophages are activated by T helper (Th)-1 cytokines, such as interferon- γ (IFN γ) 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 IFN γ , tumor necrosis factor alpha (TNF α) and interleukin-1 beta (IL-1 β) 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/EBP β , IRF-4, IRF5, PPAR γ 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, IFN γ (Sica *et al.* 1997), TNF α (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, IFN γ 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 IFN γ , 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 IFN γ (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 IFN γ and TNF α 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 (10–12 h) food withdrawal. Blood samples were taken from the tail vein before (0 min) and 15, 30, 60, 90 and 120 min after glucose administration (2 g 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 4 h. Blood samples were taken from the tail vein before (0 min) and 15, 30, 60 and 90 min 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 $\Delta\Delta Ct$ 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 (150mM NaCl, 0.5% NP-40, 0.1% SDS and 50mM Tris-Cl, pH 7.5) with 1mM 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 24h and then embedded in paraffin. Liver tissue sections (7 μ m 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 μ m thick) were stained with Oil Red O.

Measurement of IFN γ 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 IFN γ levels were measured with a mouse IFN γ 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 100mm 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 100nM insulin in RPMI 1640 medium without serum for 20min. 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, 25mM HEPES, 5mM 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 flow cytometry were all from 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 IFN γ 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 200g 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 IFN γ or with IL-4, respectively, to differentiate into M1 or M2 macrophages.

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 $\pm$ 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*^{-/-}

Figure 1

Nfatc3^{-/-} mice were protected from HFD-induced insulin resistance. (A and C) *Nfatc3*^{-/-} mice and WT counterparts were fed a HFD starting at an age of 6 weeks. Body weights were measured every week; the gained body weights were calculated, which were not significant different between WT and *Nfatc3*^{-/-} mice. (B) Representative images of mice after a HFD feeding for 16 weeks. (D) At 16 weeks of HFD, body fat accumulation was measured by nuclear magnetic resonance and shown as ratio of total body weight, and there were no significant differences between WT and *Nfatc3*^{-/-} mice. (E) Glucose tolerance test. Glucose levels were measured after i.p. injection of 2 g/kg glucose. ** $P < 0.01$ vs *Nfatc3*^{+/+}. $n = 6-8$ per group from two independent experiments. (F) Insulin tolerance test. Glucose levels were measured after i.p. injection of 0.75 U/kg insulin. * $P < 0.05$ or ** $P < 0.01$ vs *Nfatc3*^{+/+}. $n = 6-8$ per group from two independent experiments.

mice was comparable to that of obese WT mice (Fig. 2B). Moreover, the expression of monocyte chemoattractant 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-1 β and TNF α 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 IFN γ was decreased in the supernatants of cultured eWAT explants isolated from *Nfatc3*^{-/-} mice compared to those from WT mice, both on HFD (Fig. 2F).

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.

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

(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 IFN γ for CD4⁺ Th1 cells (F) and for the expression of IL-4 for CD4⁺ Th2 cells (G).

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 IFN γ (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⁺ IFN γ 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⁺ IFN γ ⁺ 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 IFN γ 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

Figure 4
Nfatc3^{-/-} mice were protected from obesity-induced hepatic steatosis and inflammation. (A) Representative images of the livers from WT and *Nfatc3*^{-/-} mice on HFD for 16 weeks were shown. Hematoxylin and eosin (H&E)-stained sections (B) and oil red O staining (C) from the liver of HFD-fed *Nfatc3*^{-/-} and HFD-fed WT mice. Representative images were shown at 20 $\times$ magnification. Similar results were seen in four independent samples. (D and E) Gene expressions of MCP-1 and TLR4 in the liver of HFD-fed *Nfatc3*^{-/-} and HFD-fed WT mice were analyzed by real-time quantitative RT-PCR. **P* < 0.05 vs *Nfatc3*^{+/+}. *n* = 3 per group.

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 IFN γ 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 IFN γ 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 IFN γ , as shown by the lower levels of induced IL-1 β and TNF α

mRNA, but they were sensitive to IL-4, as demonstrated by higher levels of Arg1 and PPAR γ 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 TNF α 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 IFN γ 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 IFN γ 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, IFN γ and TNF α , 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

Figure 5
BMDMs from *Nfatc3*^{-/-} mice were more inclined to differentiate into M2 macrophages. Bone-marrow-derived macrophages (BMDMs) from normal diet-fed *Nfatc3*^{-/-} and WT mice were differentiated into M1 or M2 lineage. Markers of M1 (A) or M2 (B) macrophages were analyzed by real-time quantitative RT-PCR. **P* < 0.05 or ***P* < 0.01 vs *Nfatc3*^{+/+}. *n* = 3 per group.

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 PPAR γ regulates alternative activation of macrophages and is associated with increased insulin sensitivity (Odegaard *et al.* 2007), it is likely that PPAR γ caused the higher proportion of M2 macrophages in the AT of *Nfatc3*^{−/−} mice. Indeed, we found increased PPAR γ 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 IFN γ and IL-1 β inhibit insulin signaling and promote insulin resistance in adipocytes (McGillcuddy *et al.* 2009), decreased IFN γ and IL-1 β 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 (TNF α and IL-1 β) in response to LPS and IFN γ . 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 IFN γ by CD4+ T cells (Deng *et al.* 2013). IFN γ then activates M1 macrophages, which secrete numerous cytokines and chemokines, including TNF- α , IL-1 β 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+ IFN γ + 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 IFN γ -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.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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