Original Research Article

Fetuin-A regulates adipose tissue macrophage content and activation in insulin resistant mice through MCP-1 and iNOS: Involvement of IFNγ-JAK2-STAT1 pathway

Dipanjan Chattopadhyay, Snehasis Das, Suktara Guria, Soumyadeep Basu, Sutapa Mukherjee*

Endocrinology and Metabolism Laboratory, Department of Zoology, Siksha Bhavana (Institute of Science), Visva-Bharati (A Central University), Santiniketan – 731235, West Bengal, India

*Corresponding author. Tel.: +91 3463 261268
E-mail address: sutapa.mukherjee2010@gmail.com (S. Mukherjee)

Running title: FetA regulates MCP-1 and iNOS through IFNγ

Keywords:
Adipose tissue macrophage/ Fetuin-A/ MCP-1/ IFNγ/ Inflammation/ Insulin resistance
ABSTRACT

In the context of obesity-induced adipose tissue inflammation, migration of macrophages and their polarization from predominantly anti-inflammatory to proinflammatory subtype is considered a pivotal event in the loss of adipose insulin sensitivity. Two major chemoattractants, monocyte chemoattractant protein-1 (MCP-1) and Fetuin A (FetA), have been reported to stimulate macrophage migration into inflamed adipose tissue instigating inflammation. Moreover, FetA could notably modulate macrophage polarization, yet the mechanism(s) is unknown. The present study was undertaken to elucidate the mechanistic pathway involved in the actions of FetA and MCP-1 in obese adipose tissue. We found that FetA knockdown in high fat diet (HFD) fed mice could significantly subdue the augmented MCP-1 expression and reduce adipose tissue macrophage (ATM) content thereby indicating that MCP-1 is being regulated by FetA. Additionally, knockdown of FetA in HFD mice impeded the expression of inducible nitric oxide synthase (iNOS) reverting macrophage activation from mostly proinflammatory to anti-inflammatory state. It was observed that the stimulating effect of FetA on MCP-1 and iNOS was mediated through interferon γ (IFNγ) induced activation of JAK2-STAT1-NOX4 pathway. Furthermore, we detected that the enhanced IFNγ expression was accounted by the stimulatory effect of FetA upon the activities of both cJun and JNK. Taken together, our findings revealed that obesity-induced FetA acts as a master upstream regulator of adipose tissue inflammation by regulating MCP-1 and iNOS expression through JNK-cJun-IFNγ-JAK2-STAT1 signaling pathway. This study opened a new horizon in understanding the regulation of ATM content and activation in conditions of obesity-induced insulin resistance.
INTRODUCTION

As sentinel cells of the body, macrophages play a pivotal role in host defense mechanisms. Armed with multiple pattern recognition receptors including toll-like receptors (TLRs), C-type lectins (CTLs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) etc., macrophages can appropriately sense danger signals emanating from a variety of pathogens and damaged tissue (1). Not only that, these heterogeneous group of cells exhibit a wide range of plasticity and complex molecular program in coordinating efficient responses to an array of cues arising within the tissue microenvironment (2). The adipose tissue (AT) provides an outstanding case of study that was initiated by the seminal observations made independently by two groups about the accumulation of adipose tissue macrophages (ATM) with increasing adiposity in mice and humans (3,4) followed by reports on genetic or chemical ablation of ATM improving insulin sensitivity (5,6). Extensive research over the years has highlighted ATM as key mediator of inflammation, insulin resistance and AT dysfunction during progression of obesity and type 2 diabetes (7). ATM biology in an obese setting has come of age but nevertheless, it has made us appreciate the amazing heterogeneity, inherent complexities of macrophage activation, the bewildering array of metabolic cues that can potentially regulate ATM profile and importantly, cross-talk with adipocytes much of which is yet to be defined. A couple of recent excellent reviews in the subject have put forth that factors and/or metabolic triggers that initiate ATM activation and induce sterile inflammation within AT remain largely unknown (8,9).

Hypertrophied adipocytes secrete a glut of negative signals that can contribute to the onset of AT inflammation in conditions of excess energy. Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C chemokine family, is a potent chemotactic factor for monocytes/macrophages (10). The augmented levels of adipocyte MCP-1 in obese subjects was reduced upon weight loss (11). Higher MCP-1 expression promoted increased macrophage infiltration into obese AT (12). An elegant experiment based on fluorescently-labeled monocyte tracking technique showed that in conditions of either MCP-1 or its receptor gene KO, the reduction in ATM content was upto 40% only (13) asserting the involvement of other factors as well. An
adipokine that is worth mentioning in this context is Fetuin-A (FetA). It has been reported that FetA acts through TLR4 and induces a strong inflammatory response in hypertrophic adipocytes (14). We noticed that being upregulated in hyperlipidemic condition, FetA served as a potent chemoattractant leading to increased macrophage influx into inflamed AT in a synergistic manner with MCP-1, and also caused ATM polarization from anti-inflammatory M2 to proinflammatory M1 subtype in obese insulin resistant mice (15). The close link between MCP-1 and inflammation was revealed by adipose-specific Mcp-1 knockdown mice displaying lowered plasma concentrations of several proinflammatory adipokines viz. leptin, plasminogen activator inhibitor-1 and resistin compared to wild-type controls (16). We believe that the two chemotactic signals, FetA and MCP-1, copiously generated in lipid-enriched AT milieu, can significantly influence ATM content and activation. Considering the multifarious dimensions of FetA action (17), we hypothesized that FetA might regulate MCP-1 level in AT and the interplay between the duo is a critical determinant of AT inflammation.

The contribution of ATM to the pathogenesis of obesity-induced insulin resistance can be adjudged from the massive increase in ATM number (~25-fold) over normal representing upto one-half of total AT cell population in obese diabetic state (18). This is associated with altered phenotype as well. While the inflammatory ATM is characterized by M1-like classically activated subtype identified as F4/80+/CD11b+/CD11c+ and overproducing TNFα, IL-6, IL-1β, iNOS, C-C chemokine receptor 2 (CCR2), the receptor for MCP-1, and reactive oxygen species (ROS), metabolically healthy ATM present alternatively activated M2 profile with F4/80+/CD11b+/CD206+ cells expressing Arginase-1, IL-10 and other type 2 effectors (9). In fact, there exists a continuum of phenotypes between the two extremes of proinflammatory M1 and anti-inflammatory M2 in response to diverse stimuli (19) and what tip-offs the balance is actually the decisive factor.

Interferon γ (IFNγ) is known to be the main cytokine responsible for M1 activation and acts through its receptor recruiting Janus kinase (JAK) adaptor proteins leading to dimerization and nuclear translocation of Signal transducers and activators of transcription 1 (STAT1) (20). The present study revealed the key role of FetA as an upstream regulator of MCP-1 and iNOS through the IFNγ-JAK2-STAT1 axis and
knocking down FetA in high fat diet fed mice had desirable outcomes; it could reduce macrophage infiltration and revert ATM polarization to predominant anti-inflammatory state thereby restoring insulin sensitivity to a significant extent.

MATERIALS AND METHODS

Chemicals and reagents

Cell culture materials were procured from Gibco-BRL/Life Technologies, USA. For the purpose of in vitro study, murine RAW 264.7 cell line was acquired from National Centre for Cell Science (NCCS), Pune, India. Western blot experiments were performed with antibodies against MCP-1, α-tubulin, Arginase 1, IFNγ, NOX4, CD86, CD206, pcJun (Santa Cruz Biotechnology Inc, Dallas, Texas, USA); CD11c, pSTAT1 (Thermo Scientific, MA, USA) and iNOS, pJAK2 (Abclonal, MA, USA). Quantification of desired protein concentrations was carried out using ELISA kits (Ray Biotech, Norcross, GA, USA) specific for MCP-1, TNFα, IL-6, IL-10, IL-1β and insulin. For producing FetA knockdown mice, we used morpholino from Gene Tools Philomath, USA. Fludarabine and SP600125, the inhibitors of STAT1 and JNK, respectively, were procured from Cayman Chemical, MI, USA. IFNγ and NOX4 siRNAs were purchased from abm, Canada. Recombinant IFNγ protein was purchased from Sigma-Aldrich, St. Louis, MO, USA. FITC-tagged CD11c, CD86, CD206, F4/80 primary antibodies were purchased from BD Bioscience, New Jersey, USA. Alkaline phosphatase-conjugated rabbit anti-goat, goat anti-mouse and goat anti-rabbit; FITC-tagged anti-rabbit and anti-mouse; Rhodamine-tagged anti-mouse (Sigma-Aldrich, St. Louis, MO, USA) and HRP-tagged anti-rabbit (Vector Laboratories, Burlingame) were used as secondary antibodies. Boyden chamber system for performing cell migration assay was procured from Millipore Burlington, USA. Customized qPCR primers of IFNγ, MCP-1 and iNOS were provided by Edison Life Science, Kolkata, India. All other chemicals used were analytical grade.

Animal maintenance and treatments
Adult male Swiss albino mice (20–25 g) were acclimatized at ideal environment (25 ± 2 °C and relative humidity 55 ± 5% with 12 h alternate light and dark cycle) and provided with drinking water and standard diet (SD) ad libitum. Animal handling, maintenance and experiments were conducted very attentively and meticulously in the Department of Zoology, Visva-Bharati (A Central University), Santiniketan, India. This was done in strict adherence to the protocols prescribed by the Institutional Animal Ethics Committee (IAEC), Visva-Bharati University under the aegis of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (Permit No.1819/GO/Re/S/15/CPCSEA). After week long acclimatization, the mice were divided into two groups: one group was continuously supplied with Standard Diet (SD) and the other group was provided with High Fat Diet (HFD) for a duration of 3 months as described in detail in our previous reports (14, 21, 22). In vivo morpholino oligos specific for FetA was injected dissolved in physiological saline (25 nM) for 5 days in a row to generate FetA knockdown mouse model (23). As its corresponding control, a subset of HFD mice was given control Vivo Morpholino (VMO) supplied by the manufacturer (Gene Tools, Philomath, USA). On the 10th day following the final injection, the mice were sacrificed. Furthermore, to mimic the effect of FetA in vivo, FetA was dissolved in sterile phosphate-buffered saline (PBS, 0.05M, pH 7.4) and was administered to SD mice at a dose of 0.7 mg/gram body weight for a period of 5 days (22, 23). The control subset of mice received same volume of sterile PBS. Additionally, one subset of HFD mice were administrated subcutaneously with JNK inhibitor, SP600125 at a dose of 30 mg/kg body weight as described previously (24, 25) for a period of 5 days to observe the effect of JNK in regulating FetA’s impact on macrophage migration and polarization. The mice were euthanized by CO₂ inhalation after completion of the respective treatments.

Isolation of stromal vascular fraction (SVF) from adipose tissue

Mice epididymal and retroperitoneal fat was collected in normal saline, cleaned and homogenized in PBS, pH 7.4 at 50 mM supplemented with 0.5% BSA. The tissue suspensions were centrifuged at 500g for 5 min. and subjected to digestion with 1 mg/ml collagenase at 37°C in a shaking water bath for 30 min. The cell suspension was then filtered through a 100 µm sieve and re-centrifuged at 300g for 5 min to
isolate floating adipocytes from the stromal vascular fraction (SVF) pellet which were used for further experimental procedures (26).

**Cell culture and treatments**

For primary culture, isolated SVF was washed in PBS, suspended in fetal bovine serum (FBS)-free culture media in 6-well plates and kept in 5% CO$_2$ incubator for 4 h at 37 °C. RAW 264.7 cells were cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and FBS (10%) at 37°C in a humidified chamber with 5% CO$_2$. Isolated SVF and RAW 264.7 cell lines were treated without (Con) or with (50-200 μg/ml) FetA conjugated with 0.25 mM saturated fatty acid, palmitate in serum-free media for specified time period following our previous publication (22). For further experiments we treated SVF and RAW 264.7 cell line with Fludarabine (50 μM) and SP600125 (10 μM), inhibitors of STAT1 and JNK, respectively. Gene silencing experiments were carried out by transfecting RAW 264.7 cells with IFNγ and NOX4 siRNAs using RNAifectine following manufacturer’s instructions. Negative control siRNAs were used for confirmation.

**Blood and serum analyses**

At the onset of treatment and throughout the treatment tenure, blood glucose level was monitored regularly with Accu-Chek glucometer (Roche, Basel, Switzerland). Oral glucose tolerance test (OGTT) was done by measuring blood glucose levels before and after oral glucose gavage at a dose of 1 g/kg body weight. The efficiency of whole-body insulin functioning was evaluated by insulin tolerance test (ITT) injecting insulin at a dose of 0.75 IU/kg body weight. The sera of mice were used for quantification of insulin and MCP-1 levels.

**Immunoblotting**

The isolated cells were suspended in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), sonicated on ice and centrifuged at 10,000g for 20 min at 4°C. The supernatant was collected and protein concentration was determined by Lowry’s method (27). Equal amount of denatured protein samples were loaded in 7.5% or 10% polyacrylamide
gels and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Non-fat skimmed milk (5%) was used to block protein free regions on the membrane after which the membrane was dipped in desired primary antibody solution. Following overnight incubation at 4°C, alkaline phosphatase-tagged secondary antibody was applied for the detection of protein bands using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as substrate of the tagged enzyme. ImageJ software was used to quantify the intensity of protein bands on the membrane.

**ELISA**

The concentrations of insulin, MCP-1, IL-6, IL-1β, TNFα and IL-10 proteins were measured using respective commercially available ELISA kits (Ray Biotech, Norcross, GA, USA) following the manufacturer’s protocols.

**Quantitative RT-PCR**

Initially, RNA was isolated from the cells using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) and then cDNA was synthesized with the help of Revert Aid First-Strand cDNA Synthesis kit (Thermo Scientific, MA, USA). Customized primers listed in Table 1 were used for SYBR Green-based amplification of desired genes in real-time thermal cycler (Applied Biosystems, Foster City, CA, USA). The value of gene expression was quantified by measuring the fluorescence of double stranded DNA binding dye SYBR Green with respect to the reference gene EF1α. Melting curve analysis was done to ensure the specificity of product.

**Immunohistochemistry**

Paraformaldehyde fixed-paraffin embedded adipose tissue sections of SD, HFD, and HFD+FetA^KD^ mice were incubated firstly with CD11c primary antibody followed by its corresponding horseradish peroxidase (HRP)-tagged secondary antibody for detecting the intensity of M1 macrophage marker.
**Immunofluorescence**

Small pieces of adipose tissues were collected from SD, HFD and HFD+FetA<sup>KD</sup> mice and fixed in paraformaldehyde, followed by washing with PBS, pH 7.4. For preventing non-specific binding, glycine (22.52 mg/ml) and fatty acid-free 1% BSA in PBST was added at room temperature and kept for 1 h. Following this, rhodamine-tagged CD206 and FITC-tagged CD86 antibodies were used and the adipose tissue was incubated in a humidified chamber for a period of 4 h. After completion of the incubation period, images were captured with a laser scanning confocal microscope (TCS-SP8, Leica Microsystems GmbH, Wetzlar, Germany).

**FACS**

For *in vitro* experiments, RAW 264.7 cells were treated with FetA (100 µg/ml) in absence or presence of cells transfected with IFNγ siRNA. After washing cells were resuspended in 50 µl of PBS containing EDTA (2 mM) and BSA (0.5%). The cells were treated with FITC-tagged CD11c primary antibody for 1 h. For detection of Arginase 1, the cells were incubated with rabbit *anti*-Arginase 1 for 4 h followed by FITC-conjugated anti-rabbit secondary antibody for 1 h. After incubation, cells were washed in PBS and analysed by flow cytometry (BD FACSARia<sup>TM</sup> II and FACSDiva Software). Similar procedure was followed for analysis of FITC-tagged CD86 and CD206 markers in SVF primary culture incubated with FetA in presence or absence of JNK inhibitor SP600125. In addition to M1 and M2 macrophage specific markers, all treatment groups were treated with rhodamine-conjugated antibody against F4/80 which is a common marker of macrophage.

**Cell migration assay**

Cell migration studies were undertaken in SVF isolated from SD, HFD, HFD+FetA<sup>KD</sup> and HFD+SP600125 mice using QCM 24-well colorimetric cell migration assay kit (Merck-Millipore, MA, USA). The cells were incubated for 4 h and then the media was placed in the lower chamber with RAW 264.7 cells in the upper chamber for 4 h incubation. After completion of the incubation period, the migration of cells was observed under bright field microscope followed by quantification of staining intensity.
in an ELISA plate reader at 560 nm wavelength following the manufacturer’s protocol.

**Statistical analyses**

The data were subjected to Student’s t-test (for single-factorial designs) and one-way ANOVA (for multi-factorial designs). The means were compared by performing Tukey’s post hoc multiple range test using Prism version 8 for Windows (GraphPad Software, Inc.). All values were expressed as means ± standard error of the mean (SEM) and minimal statistical significance was considered at $P < 0.05$.

**RESULTS**

*FetA silencing reduces MCP-1 and iNOS, and lowers M1/M2 ratio within inflamed adipose tissue*

To discern the effect of FetA on macrophage migration, we knocked down FetA in high fat diet (HFD) mice using *in vivo* morpholino. The efficiency of FetA knockdown was assessed by immunoblotting followed by densitometry of the protein expression of FetA in lysates of hepatocytes, adipocytes and SVF isolated from SD, HFD and HFD+FetA$^{KD}$ mice (Supplementary Fig. S1). No significant change in body weight and/or fat mass between HFD mice and HFD+FetA$^{KD}$ mice was noticeable (data not shown). However, it was interesting to note that HFD+FetA$^{KD}$ mice displayed much reduced M1 macrophage load compared to that of HFD mice as evident from immunohistochemistry with *anti*-CD11c (Fig. 1A). Further, immunofluorescence of adipose tissue (AT) clearly revealed that knockdown of FetA in HFD mice drastically reduced the M1 surface marker CD86 but augmented the M2 marker CD206 (Fig. 1B). We then explored the effect of FetA silencing upon different M1/M2 markers in adipose-derived stromal vascular fraction (SVF). FetA knockdown prominently abated iNOS and CD11c, major M1 markers and in parallel enhanced the expression of Arginase 1, an M2 marker (Fig. 1C). These observations depicted the crucial role essayed by FetA in macrophage polarization. Since one of the best studied
chemokines known to regulate macrophage migration is monocyte chemoattractant protein-1 (MCP-1) (10), we checked the effect of FetA knockdown upon MCP-1. It was striking to note that serum MCP-1 protein expression as well as concentration were markedly lowered in HFD+FetAKD mice (Fig. 1D,E). These data provided an insight that FetA effect upon ATM is possibly by regulating MCP-1. To ascertain FetA’s direct involvement in this event, we injected FetA in SD mice and examined the level of MCP-1 by ELISA. Fig. 1F demonstrated that FetA injection elevated MCP-1 level in SD mice while knockdown of FetA considerably reduced MCP-1 in SVF lysate. This was accompanied by notable rise in levels of proinflammatory cytokines TNFα and IL-6 in FetA-injected SD mice as well as in HFD mice whereas decreased cytokine values were recorded in HFD+FetAKD condition (Fig. 1G,H). On the contrary, the concentration of IL-10, the key anti-inflammatory cytokine followed the opposite trend (Fig. 1I).

**FetA tightly regulates IFNγ expression and FetA action is obliterated in IFNγ-silenced macrophages**

Administration of high fat diet to mice resulted in robust expression of IFNγ mRNA and protein in SVF; however, it was interesting to note that FetA knockdown could lower IFNγ expression at both the levels of transcription and translation (Fig. 2A,B). Presence of this type II interferon can induce MCP-1 expression (28,29). It is also known to be a potent stimulator of the inducible isoform of nitric oxide synthase (iNOS) (30,31). Intriguingly, at a concentration of 50 and 100 µg/ml in vitro, FetA upregulated IFNγ, MCP-1 and iNOS proteins in SVF in a dose-dependent manner (Fig. 2C). Not only for protein, it held true for gene expression as well. FetA (100 µg/ml) was shown to enhance significantly MCP-1 and iNOS transcripts in SVF (Fig. 2D). We performed time kinetics experiments after incubating SVF with 100 µg/ml FetA for 2 h, 4 h and 6 h, respectively. Maximal effect of FetA upon IFNγ, MCP-1 and iNOS expression was detected after 4 h (Fig. 2E). This was found to correlate strongly with dose-dependent and time-dependent responses of MCP-1 concentration to FetA (Fig. 2F,G). In order to clearly delineate the involvement of IFNγ, we silenced IFNγ in murine macrophage RAW 264.7 cell line by using siRNA that was confirmed by immunoblotting followed by densitometry and significantly...
subdued levels of MCP-1 and CD11c (Supplementary Figure S2). Our hypothesis was validated as the raised MCP-1 and iNOS protein levels upon FetA treatment were significantly downregulated when IFNγ was silenced (Fig. 2H). This was reflected in FACS analyses showing obliterated FetA action in IFNγ-silenced cells having reduced CD11c+ cell population, a potent M1 marker (Fig. 2I) while Arginase 1+ cell population, an indicator of M2 phenotype was restored (Fig. 2J).

Proinflammatory stimulus of FetA is mediated through IFNγ-JAK2-STAT1 pathway

To get a deeper understanding of the IFNγ signaling pathway, we looked into the Janus kinase (JAK)/Signal transducers and activators of transcription (STAT) pathway which is activated by IFNγ (32). This pathway is also shown to regulate NADPH oxidase 4 (NOX4) (33). Administration of FetA to RAW 264.7 cells markedly enhanced the expression of pJAK2, pSTAT1 and NOX4 proteins (Fig. 3A). However, when given to IFNγ-silenced cells, FetA effect was markedly subdued (Fig. 3B). In order to validate STAT1’s direct involvement in the pathway, we treated SVF with a potent inhibitor of STAT1, fludarabine (34,35). In presence of fludarabine, FetA failed to upregulate the protein expression of NOX4 and iNOS but not of IFNγ (Fig. 3C). Inhibition of STAT1 also suppressed MCP-1 level even in presence of FetA (Fig. 3D). Not only this, the proinflammatory stimulus of FetA was significantly inhibited when fludarabine was administered as shown by lowered TNFα, IL-6 and IL-1β levels (Fig. 3E-G) while anti-inflammatory IL-10 concentration was recovered to a significant extent (Fig. 3H). In a similar vein, inactivating STAT1 resulted in suppression of CD86 and CD11c M1 markers (Fig. 3I) while restoring CD206 and Arginase 1 M2 markers in SVF even in presence of FetA (Fig. 3J). Additionally, FetA failed to augment MCP-1 level when NOX4 was silenced in RAW 264.7 cells (Fig. 3K).

Inhibition of JNK attenuates FetA action and reverts ATM polarization

It was fascinating to observe that in primary culture of SVF isolated from SD mice AT, treatment of FetA in vitro could trigger JNK and cJun activation (Fig. 4A). Furthermore, we treated SVF isolated from SD mice with a strong inhibitor of JNK,
SP600125 (24,25) and noticed that protein expression of all the downstream molecules pcJun, IFNγ, pJAK2, pSTAT1, iNOS and NOX4 were strikingly reduced even in presence of FetA (Fig. 4B) that was reflected in MCP-1 level as well (Fig. 4C). These results clearly depict FetA action being governed by JNK. Inhibition of JNK lowered FetA-induced CD11c while elevating Arginase 1 expression (Fig. 4D). The polarity shift was evident from FACS data displaying lesser M1 phenotype marker CD86+ cells and increased M2 marker CD206+ cell population in presence of SP600125 (Fig. 4E,F).

**HFD mice record similar IFNγ signaling, macrophage migration, cytokine and marker profiles in conditions of either FetA knockdown or JNK inhibition**

To substantiate that FetA is regulating macrophage polarization through JNK-cJun-IFNγ-JAK2-STAT1 signaling cascade, we checked the total pathway in vivo by treating HFD mice with JNK inhibitor, SP600125 and in another subset of HFD mice, knockdown of FetA was accomplished. It was captivating to observe that conditions of either FetA knockdown or JNK inactivation in vivo elicited somewhat similar regulation of the pathway in HFD mice (Fig. 5A). Similar trend was also observed for the downstream MCP-1 (Fig. 5B). Next, we tried to analyze the cell-to-cell communication by using Boyden chamber system where incubation media of SVF isolated from SD, HFD, HFD+FetA KD and HFD+SP600125 mice were kept at the bottom and RAW 264.7 cells were kept in the upper chamber. This incubation was carried out for 4 h following which the upper chamber was taken under bright field microscope for photography. Fig. 5C-D demonstrated that macrophage migration and staining intensity was least in HFD mice with FetA knockdown and less in JNK inhibited-HFD mice compared to their only HFD fed counterparts. Additionally, both these conditions could significantly revert the cytokine profile of HFD mice (Fig. 5E-H) and the expression of relevant molecular markers (Fig. 5I).

**FetA knockdown and JNK inhibition revives insulin sensitivity in HFD mice**

As both FetA knockdown and JNK inhibition effected inflammatory status in AT, we then sought to test whole-body insulin sensitivity by performing OGTT and ITT.
Significant revival of insulin sensitivity was possible in HFD+FetA\textsuperscript{KD} mice that was followed by JNK inhibition in HFD mice (Fig. 6A,B). Serum insulin level also revealed the same pattern (6C). Taken together, the data depicted that both FetA knockdown and JNK inhibition could restore insulin sensitivity and asserted that FetA action in governing AT inflammation is mediated through JNK.

**TLR4 suppression significantly blocks the proinflammatory effects of FetA**

We next concentrated on the known signaling of FetA involving TLR4; in obese mice, FetA acts as an endogenous ligand for TLR4 and through TLR4-mediated pathway mounts strong inflammatory responses in obese HFD mice (14). TLR4-silenced RAW 264.7 cell line was utilized for this purpose and the extent of TLR4 knockdown was validated (Fig. 7A). The data clearly revealed that in TLR4-knockdown RAW 264.7 macrophages, FetA could not stimulate the inflammatory pathway controlling macrophage migration and polarization as evident from expression of different connecting signaling molecules (Fig. 7B). MCP-1 level was significantly decreased in TLR4-silenced cells even in presence of FetA (Fig. 7C). The involvement of TLR4 observed in vitro was substantiated in adipose-derived SVF prepared from SD mice. TLR4 activity was inhibited by CLI-095 and primary culture of these cells displayed low abundance of key inflammatory molecules namely, pJNK, IFN\textgamma, pJAK2, pSTAT1, iNOS and MCP-1 even in presence of FetA as revealed by western blot analyses (Fig. 7D). Moreover, in CLI-095-treated cells even in presence of FetA, MCP-1 level was decreased (Fig. 7E). These results altogether imply the crucial involvement of TLR4 in FetA-mediated AT inflammation.

**DISCUSSION**

The present study was performed in stromal vascular fraction (SVF) isolated from adipose tissue (AT). A heterogeneous population of varied cells viz. mesenchymal stem cells, preadipocytes, fibroblasts, endothelial cells and immune cells comprise
AT-derived SVF wherein macrophages are the highly dynamic and dominant cell type (18). Additionally, we have used the murine macrophage cell line RAW 264.7. The role of the hepatoadipokine Fetuin-A (FetA) in regulating a plethora of events during inflammation is well recognized (36,17). Based on the premise that adipocyte-derived FetA could promote macrophage migration into inflamed AT and cause polarity shift of ATM from predominantly M2-like to M1-like phenotype (15), the present study attempts to define the mechanism by which FetA could possibly exert this effect.

In conditions of obesity, the accumulation and polarization of macrophages within lipid-enriched AT is instrumental in generating an inflammatory milieu (3,4,37,38). Inflamed AT copiously secretes monocyte chemoattractant protein-1 (MCP-1) the deficiency of which not just reduced macrophage infiltration into AT but consequentially improved insulin sensitivity (12). In contrast, overexpression of adipose MCP-1 was reported to exacerbate ATM recruitment thereby worsening insulin resistance (39). In our preliminary experiments, both immunoblotting and ELISA confirmed the presence of significantly higher MCP-1 levels in mice fed HFD which was expected; however, strikingly enough, MCP-1 protein was substantially lowered in HFD+FetA<sup>KD</sup> mice. The deficiency of FetA reverted AT profile to anti-inflammatory even when challenged with high dietary fat. This crucial evidence of a close correlation between FetA and MCP-1 proteins set up our hypothesis that FetA is an upstream regulator of MCP-1. Our subsequent experiments were designed to verify the hypothesis and understand how FetA could influence ATM content and activation in hyperlipidemic obese AT by regulating the critical chemoattractant MCP-1.

Macrophages display an astonishing range of plasticity in responding to various endogenous cues arising within the tissue microenvironment (2). Compared to normal lean physiology, the dynamics of ATM in obesity is radically altered under the influence of a host of cytokines and chemokines as it becomes increasingly skewed towards the classically activated proinflammatory M1-like phenotype. A chief AT-derived inflammatory factor implicated in metabolic abnormalities inherent in obesity is the type II interferon, IFNγ (40). It provides the primary stimulus for M1 activation inducing multiple gene transcripts including MCP-1 (28,29), NADPH oxidases (NOX)
and inducible nitric oxide synthase (iNOS) (41). A number of studies have shown potent induction of MCP-1 at gene and protein levels in response to IFNγ stimulation in several cell types (42-46). Earlier, preponderance of M2-like ATM was observed in SVF isolated from obese IFNγ-KO mice with modestly attenuated systemic inflammation relative to obese wild-type control littermates (47). On a similar note, we found that silencing IFNγ not only downregulated MCP-1 and iNOS, but shifted macrophage polarity from M1 to M2 even in the presence of FetA which is known to cause M2 to M1 phenotypic switch in obesity (15). The present study revealed the intricate relationship between IFNγ and FetA in two aspects: firstly, FetA could induce gene and protein expression of IFNγ, MCP-1 and iNOS in a dose-dependent and time-dependent manner and secondly, FetA knockdown in HFD mice downregulated IFNγ expression at dual levels of transcription and translation.

One of the major signaling pathways mediating the biological processes of inflammation and immune response, JAK-STAT pathway is known to modulate adipogenesis, AT development and function (48). Since diet-induced obesity in mice significantly enhanced the gene and protein expression of IFNγ in adipose-derived SVF, we sought to explore the signaling pathway triggered by IFNγ in non-adipocyte AT immune cells. At the cellular level, IFNγ responses are mediated through its receptor activating Janus kinases (JAKs) by phosphorylation which in turn, activate and phosphorylate the signal transducer and activator of transcription 1 (STAT1) in the cytosol (49). Homodimers of pSTAT1 translocate to the nucleus, bind to specific enhancer elements and initiate transcription of multiple IFNγ-inducible genes. The tight regulation of IFNγ by FetA was corroborated by the observation that FetA could trigger JAK-STAT signaling in normal RAW 264.7 murine macrophages but not when IFNγ was silenced. There are compelling evidences that STAT1 is robustly activated by IFNγ and implicated in regulating genes for fat cell development, insulin sensitivity, lipid and carbohydrate metabolism in AT (50). According to a recent report, STAT1 deficiency in mice relieves AT inflammation (51). NADPH oxidase 4 (NOX4), a major source of reactive oxygen species (ROS), is known to be enhanced in AT during obesity (52) and reported to regulate MCP-1 (53). NOX4 is reported to be governed by IFNγ-JAK2-STAT1 pathway (33). In the present study, STAT1 inhibition with fludarabine not only blocked FetA’s stimulation of iNOS, NOX4 and MCP-1 proteins but also changed cytokine profile to more anti-inflammatory subtype.
This provided definitive evidence that FetA-induced AT inflammation was substantially resolved in the presence of fludarabine. It was noteworthy that in NOX4-silenced macrophages, administration of FetA failed to augment MCP-1, thus providing clear evidence of a functional IFNγ-JAK2-STAT1-NOX4-MCP-1 axis responsible for macrophage infiltration with FetA as upstream regulator. Furthermore, FetA also seemed to regulate macrophage polarization through IFNγ-JAK2-STAT1-iNOS axis. A signature M1 marker iNOS is responsible for selectively regulating M1 macrophage gene expression and dedifferentiation (54).

In our pursuit of the probable mechanism by which FetA could be regulating IFNγ, we considered cJun because it has been shown previously that cJun binds to the proximal promoter of IFNγ gene and enhances its expression (55). JNK is reported to be responsible for stimulating the activity of cJun (56). This was confirmed by the fact that FetA treatment could potentially exacerbate JNK and cJun activation; suppression of JNK by its inhibitor SP600125 eventually blocked FetA’s effect. Several reports have demonstrated that disrupting JNK1 function, which is activated in HFD mice, restores insulin sensitivity by reducing inflammation (57-59). We treated HFD mice with JNK inhibitor, SP600125 and interestingly enough, noted that it elicited responses akin to that of FetA knockdown in HFD mice. Boyden chamber culture set up was used to comprehend the actual nature of crosstalk in vivo with RAW 264.7 cells in the upper chamber and incubation media of SVF isolated from SD, HFD, HFD+FetA KD and HFD+SP600125 mice at the bottom. Macrophage migration was inhibited mostly in HFD+FetA KD followed by HFD+SP600125 and thus, suppression of JNK obliterated FetA action to a significant extent. Whole-body insulin sensitivity was considerably revived in FetA knockdown or JNK inhibition in HFD mice. Earlier reports have shown remarkable improvement of insulin sensitivity in FetA-null mice and how it protects against insulin resistance associated with aging (60,61). There is evidence of partial protection from HFD-induced insulin resistance in Tlr4-knockout mice that was ascribed to downregulated inflammatory gene expression in liver and fat tissue (62). Since FetA is known to activate TLR4 signaling thereby generating inflammatory responses in hyperlipidemic condition (14), we conducted experiments to look into this aspect. When TLR4 was suppressed in RAW 264.7 cells or adipose-derived SVF, the pJNK, IFNγ, pJAK2, iNOS and MCP-1 levels were markedly inhibited even in the presence of FetA.
indicating that FetA is working through TLR4 in regulating macrophage migration as well as polarization to induce AT inflammation.

In summary, our data ascertained FetA as an upstream master regulator of ATM content and activation; FetA is tightly regulating MCP-1 and iNOS through IFNγ. This mechanistic insight attains credence in the light of recent evidences that complete disruption of IFNγ signaling restored insulin sensitivity and metabolic homeostasis in obese mice (51). Future endeavors towards targeting FetA might present a promising therapeutic option to resolve lipid-induced AT inflammation and thereby improve insulin sensitivity.

ACKNOWLEDGEMENTS

Dipanjan Chattopadhyay acknowledges financial assistance as fellowship from SERB, Govt. of India (No. SB/SO/AS/064/2013). Snehasis Das is grateful to UGC, Govt. of India (Award letter no. 2061530649) for senior research fellowship. Suktara Guria thanks CSIR, Govt. of India (09/202(0098)/2019-EMR-1) and Soumyadeep Basu thanks SERB, Govt. of India (No. ECR/2017/002470) for junior research fellowships. The authors owe their gratitude to Prof. Samir Bhattacharya for active cooperation in extending laboratory facilities and to Dr. Alpana Mukhuty for technical assistance in confocal microscopy. The authors gratefully acknowledge the Head, Department of Zoology (supported by UGC-CAS No. F.5-11/2012[SAP-II], DST-FIST No. SR/FST/LS II-031/2013 [C] and DST-PURSE, Govt. of India), Siksha Bhavana, Visva-Bharati, Santiniketan for providing all infrastructural facilities.

DECLARATION OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

**FUNDING**

This work is supported by Science and Engineering Research Board (SERB), Govt. of India (SB/SO/AS-064/2013) and UGC-FRPS Start-Up Grant for New Faculty, Govt. of India (F.30-7/2014(BSR)).

**CRediT Contribution:**

D.C.: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Validation; Visualization; Writing-original draft. S.D.: Methodology; Investigation; Data curation; Formal analysis; Validation; Visualization. S.G.: Investigation; Formal analysis; Validation, S.B.: Investigation; Formal analysis; Validation. S.M.: Conceptualization; Methodology; Data curation; Formal analysis; Visualization; Funding acquisition; Project administration; Supervision; Writing - original draft; review & editing.

**REFERENCES**

1. Palm, N.W. and Medzhitov, R. (2009) Pattern recognition receptors and control of adaptive immunity. *Immunol. Rev.* **227**, 221-233 https://doi.org/10.1111/j.1600-065X.2008.00731.x

2. Okabe, Y. and Medzhitov, R. (2014) Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell.* **157**, 832-844 https://doi.org/10.1016/j.cell.2014.04.016

3. Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. and Ferrante Jr, A.W. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Investig.* **112**, 1796–1808 https://doi.org/10.1172/jci19246
4. Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J. et al. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Investig.* **112**, 1821–1830 [https://doi.org/10.1172/jci19451](https://doi.org/10.1172/jci19451)

5. Patsouris, D., Li, P.P., Thapar, D., Chapman, J., Olefsky, J.M., and Neels, J.G. (2008) Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell Metab.* **8**, 301-309 [https://doi.org/10.1016/j.cmet.2008.08.015](https://doi.org/10.1016/j.cmet.2008.08.015)

6. Feng, B., Jiao, P., Nie, Y., Kim, T., Jun, D., Van Rooijen, N. et al. (2011) Clodronate liposomes improve metabolic profile and reduce visceral adipose macrophage content in diet-induced obese mice. *PLoSOne.* **6**, e24358 [https://doi.org/10.1371/journal.pone.0024358](https://doi.org/10.1371/journal.pone.0024358)

7. Russo, L. and Lumeng, C.N. (2018) Properties and functions of adipose tissue macrophages in obesity. *Immunology.* **155**, 407-417 [https://doi.org/10.1111/imm.13002](https://doi.org/10.1111/imm.13002)

8. Orliaguet, L., Ejlalmanesh, T. and Alzaid, F. (2020) Metabolic and molecular mechanisms of macrophage polarisation and adipose tissue insulin resistance. *Int. J. Mol. Sci.* **21**, 5731 [https://doi.org/10.3390/ijms21165731](https://doi.org/10.3390/ijms21165731)

9. Orliaguet, L., Dalmas, E., Drareni, K., Venteclef, N. and Alzaid, F. (2020) Mechanisms of macrophage polarization in insulin signaling and sensitivity. *Front. Endocrinol.* **11**, 62 [https://dx.doi.org/10.3389%2Ffendo.2020.00062](https://dx.doi.org/10.3389%2Ffendo.2020.00062)

10. Deshmane, S.L., Kremlev, S., Amini, S. and Sawaya, B.E. (2009) Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res.* **29**, 313-326 [https://doi.org/10.1089/jir.2008.0027](https://doi.org/10.1089/jir.2008.0027)

11. Christiansen, T., Richelsen, B. and Bruun, J.M. (2005) Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes.* **29**, 146-150 [https://doi.org/10.1038/sj.ijo.0802839](https://doi.org/10.1038/sj.ijo.0802839)

12. Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R. et al. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin Resistance, and hepatic steatosis in obesity. *J.Clin. Investig.* **116**, 1494-1505 [https://doi.org/10.1172/JCI26498](https://doi.org/10.1172/JCI26498)
13. Oh, D.Y., Morinaga, H., Talukdar, S., Bae, E.J. and Olefsky, J.M. (2012) Increased macrophage migration into adipose tissue in obese mice. *Diabetes.* **61**, 346-354. [https://doi.org/10.2337/db11-0860](https://doi.org/10.2337/db11-0860)

14. Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S. et al. (2012) Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat. Med.* **18**, 1279–1285. [https://doi.org/10.1038/nm.2851](https://doi.org/10.1038/nm.2851)

15. Chatterjee, P., Seal, S., Mukherjee, S., Kundu, R., Mukherjee, S., Ray, S. et al. (2013) Adipocyte fetuin-A contributes to macrophage migration into adipose tissue and polarization of macrophages. *J. Biol. Chem.* **288**, 28324–28330. [https://doi.org/10.1074/jbc.c113.495473](https://doi.org/10.1074/jbc.c113.495473)

16. Sundaram, S. and Yan, L. (2019) Adipose-specific monocyte chemotactic protein-1 deficiency reduces pulmonary metastasis of Lewis lung carcinoma in mice. *Anticancer Res.* **39**, 1729-1738. [https://doi.org/10.21873/anticanres.13279](https://doi.org/10.21873/anticanres.13279)

17. Bourebaba, L. and Marycz, K. (2019) Pathophysiological implication of Fetuin-A glycoprotein in the development of metabolic disorders: a concise review. *J. Clin. Med.* **8**, 2033. [https://doi.org/10.3390/jcm8122033](https://doi.org/10.3390/jcm8122033)

18. Bora, P. and Majumdar, A.S. (2017) Adipose tissue-derived stromal vascular fraction in regenerative medicine: a brief review on biology and translation. *Stem Cell Res. Ther.* **8**, 1-10. [https://doi.org/10.1186/s13287-017-0598-y](https://doi.org/10.1186/s13287-017-0598-y)

19. Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004) The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25**, 677-686. [https://doi.org/10.1016/j.it.2004.09.015](https://doi.org/10.1016/j.it.2004.09.015)

20. Martinez, F.O. and Gordon, S. (2014) The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000 Prime Rep.* **6**, 13. [https://doi.org/10.12703/P6-13](https://doi.org/10.12703/P6-13)

21. Ghosh, S. and Mukherjee, S. (2018) Testicular germ cell apoptosis and sperm defects in mice upon long-term high fat diet feeding. *J. Cell. Physiol.* **233**, 6896–6909. [https://doi.org/10.1002/jcp.26581](https://doi.org/10.1002/jcp.26581)

22. Chattopadhyay, M., Mukherjee, S., Chatterjee, S.K., Chattopadhyay, D., Das, S.,
Majumdar, S. S. et al. (2018) Impairment of energy sensors, SIRT1 and AMPK, in lipid induced inflamed adipocyte is regulated by fetuin A. Cell. Signal. 42, 67–76 https://doi.org/10.1016/j.cellsig.2017.10.005

23. Das, S., Chattopadhyay, D., Chatterjee, S.K., Mondal, S.A., Majumdar, S.S., Mukhopadhyay, S. et al. (2021) Increase in PPARγ inhibitory phosphorylation by fetuin-A through the activation of Ras-MEK-ERK pathway causes insulin resistance. Biochim. Biophys. Acta, Mol. Basis Dis. 1867, 166050 https://doi.org/10.1016/j.bbadis.2020.166050

24. Shen, H., Wu, N., Wang, Y., Han, X., Zheng, Q., Cai, X. et al. (2017) JNK inhibitor SP600125 attenuates paraquat-induced acute lung injury: an in vivo and in vitro study. Inflammation. 40, 1319–1330 https://doi.org/10.1007/s10753-017-0578-0

25. Bennett, B.L., Sasaki, D.T., Murray, B.W., O’Leary, E.C., Sakata, S.T., Xu, W. et al. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc. Natl. Acad. Sci. U.S.A. 98, 13681–13686 https://doi.org/10.1073/pnas.251194298

26. Lumeng, C. N., DeYoung, S. M., Bodzin, J.L. and Saltiel, A.R. (2007) Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes. 56, 16–23 https://doi.org/10.2337/db06-1076

27. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275 https://doi.org/10.1016/S0021-9258(19)52451-6

28. Valente, A. J., Xie, J. F., Abramova, M. A., Wenzel, U. O., Abboud, H. E., and Graves, D. T. (1998) A complex element regulates IFN-γ-stimulated monocyte chemoattractant protein-1 gene transcription. J. Immunol. 161, 3719-3728.

29. Zhou, Z.H.L., Han, Y., Wei, T., Aras, S., Chaturvedi, P., Tyler, S.et al. (2001) Regulation of monocyte chemoattractant protein (MCP)-1 transcription by interferon-gamma (IFN-γ) in human astrocytoma cells: postinduction refractory state of the gene, governed by its upstream elements. FASEB J. 15, 383-392 https://doi.org/10.1096/fj.00-0373com

30. Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W. et al. (1993) Macrophage nitric oxide synthase gene: two upstream regions
mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9730-9734 [https://doi.org/10.1073/pnas.90.20.9730](https://doi.org/10.1073/pnas.90.20.9730)

31. Xie, Q.W., Whisnant, R. and Nathan, C. (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J. Exp. Med.* **177**, 1779-1784 [https://doi.org/10.1084/jem.177.6.1779](https://doi.org/10.1084/jem.177.6.1779)

32. Schroder, K., Hertzog, P.J., Ravasi, T. and Hume, D.A. (2004) Interferon-γ: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75**, 163-189 [https://doi.org/10.1189/jlb.0603252](https://doi.org/10.1189/jlb.0603252)

33. Manea, A., Tanase, L.I., Raicu, M. and Simionescu, M. (2010) JAK/STAT signaling pathway regulates Nox1 and Nox4-based NADPH oxidase in human aortic smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **30**, 105–112 [https://doi.org/10.1161/ATVBAHA.109.193896](https://doi.org/10.1161/ATVBAHA.109.193896)

34. Frank, D.A., Mahajan, S. and Ritz, J. (1999) Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat. Med.* **5**, 444–447 [https://doi.org/10.1038/7445](https://doi.org/10.1038/7445)

35. Feng, Z., Zheng, W., Tang, Q., Cheng, L., Li, H., Ni, W. et al. (2017) Fludarabine inhibits STAT1-mediated up-regulation of caspase-3 expression in dexamethasone-induced osteoblasts apoptosis and slows the progression of steroid-induced avascular necrosis of the femoral head in rats. *Apoptosis.* **22**, 1001–1012 [https://doi.org/10.1007/s10495-017-1383-1](https://doi.org/10.1007/s10495-017-1383-1)

36. Trepanowski, J.F., Mey, J. and Varady, K.A. (2015) Fetuin-A: A novel link between obesity and related complications. *Int J Obes.* **39**, 734–741 [https://doi.org/10.1038/ijo.2014.203](https://doi.org/10.1038/ijo.2014.203)

37. Glass, C.K. and Olefsky, J.M. (2012) Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab.* **15**, 635–645 [https://doi.org/10.1016/j.cmet.2012.04.001](https://doi.org/10.1016/j.cmet.2012.04.001)

38. Johnson, A.M.F. and Olefsky, J. M. (2013) The origins and drivers of insulin resistance. *Cell.* **152**, 673–684 [https://doi.org/10.1016/j.cell.2013.01.041](https://doi.org/10.1016/j.cell.2013.01.041)
39. Kamei, N., Tobe, K., Suzuki, R., Ohsugi, M., Watanabe, T., Kubota, N. et al. (2006) Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J. Biol. Chem.* **281**, 26602–26614 [https://doi.org/10.1074/jbc.m601284200](https://doi.org/10.1074/jbc.m601284200)

40. Wentworth, J.M., Zhang, J.G., Bandala-Sanchez, E., Naselli, G., Liu, R., Ritchie, M. et al. (2017) Interferon-gamma released from omental adipose tissue of insulin-resistant humans alters adipocyte phenotype and impairs response to insulin and adiponectin release. *Int J Obes.* **41**, 1782–1789 [https://doi.org/10.1038/ijo.2017.180](https://doi.org/10.1038/ijo.2017.180)

41. Nathan, C.F., Murray, H.W., Wiebe, M.E. and Rubin, B.Y. (1983) Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* **158**, 670-689 [https://doi.org/10.1084/jem.158.3.670](https://doi.org/10.1084/jem.158.3.670)

42. Barker, J. N., Jones, M. L., Swenson, C. L., Sarma, V., Mitra, R. S., Ward, P. A. et al. (1991) Monocyte chemotaxis and activating factor production by keratinocytes in response to IFN-gamma. *J. Immunol.* **146**, 1192-1197.

43. Schmouder, R. L., Strieter, R. M., and Kunkel, S. L. (1993) Interferon-γ regulation of human renal cortical epithelial cell-derived monocyte chemotactic peptide-1. *Kidney Int.* **44**, 43-49 [https://doi.org/10.1038/ki.1993.211](https://doi.org/10.1038/ki.1993.211).

44. Grandaliano, G., Valente, A. J., Rozek, M. M., and Abboud, H. E. (1994) Gamma interferon stimulates monocyte chemotactic protein (MCP-1) in human mesangial cells. *J. Lab. Clin. Med.* **123**, 282-289.

45. Winsor, G. L., Waterhouse, C. C., MaCLellan, R. L., and Stadnyk, A. W. (2000) Interleukin-4 and IFN-gamma differentially stimulate macrophage chemotactrant protein-1 (MCP-1) and eotaxin production by intestinal epithelial cells. *J. Interferon Cytokine Res.* **20**, 299-308 [DOI: 10.1089/107999000312432](https://doi.org/10.1089/107999000312432).

46. Han, Y. L., Li, Y. L., Jia, L. X., Cheng, J. Z., Qi, Y. F., Zhang, H. J. et al. (2012) Reciprocal interaction between macrophages and T cells stimulates IFN-γ and MCP-1 production in Ang II-induced cardiac inflammation and fibrosis. *PLoS One*, **7**, e35506 [DOI: 10.1371/journal.pone.0035506](https://doi.org/10.1371/journal.pone.0035506)

47. O’Rourke, R.W., White, A.E., Metcalf, M.D., Winters, B.R., Diggs, B.S., Zhu, X. et
al. (2012) Systemic inflammation and insulin sensitivity in obese IFN-γ knockout mice. *Metab. Clin. Exp.* **61**, 1152–1161  
https://doi.org/10.1016/j.metabol.2012.01.018

48. Burrell, J.A., Boudreau, A. and Stephens, J.M. (2020) Latest advances in STAT signaling and function in adipocytes. *Clin. Sci.* **134**, 629-639  
https://doi.org/10.1042/CS20190522

49. Ramana, C.V., Gil, M.P., Schreiber, R.D. and Stark, G.R. (2002) Stat1-dependent and-independent pathways in IFN-γ-dependent signaling. *Trends Immunol.* **23**, 96-101  
https://doi.org/10.1016/S1471-4906(01)02118-4

50. Zhao, P. and Stephens, J.M. (2013) Identification of STAT target genes in adipocytes. *JAKSTAT*, **2**, e23092  
https://doi.org/10.4161/jkst.23092

51. Cox, A.R., Chernis, N., Bader, D.A., Saha, P K., Masschelin, P.M., Felix, J.B. et al. (2020) STAT1 dissociates adipose tissue inflammation from insulin sensitivity in obesity. *Diabetes* **69**, 2630–2641  
https://doi.org/10.2337/db20-0384

52. Hansen, S.S., Aasum, E. and Hafstad, A.D. (2018) The role of NADPH oxidases in diabetic cardiomyopathy. *Biochim. Biophys. Acta, Mol. Basis Dis.* **1864**, 1908–1913  
https://doi.org/10.1016/j.bbadis.2017.07.025

53. Ullevig, S., Zhao, Q., Lee, C.F., Kim, H.S., Zamora, D. and Asmis, R. (2012) NADPH oxidase 4 mediates monocyte priming and accelerated chemotaxis induced by metabolic stress. *Arterioscler. Thromb. Vasc. Biol.* **32**, 415–426  
https://dx.doi.org/10.1161/ATVBAHA.111.238899

54. Xue, Q., Yan, Y., Zhang, R. and Xiong, H. (2018) Regulation of iNOS on immune cells and its role in diseases. *Int. J. Mol. Sci.* **19**, 3805  
https://doi.org/10.3390/ijms19123805

55. Samten, B., Townsend, J.C., Weis, S.E., Bhoumik, A., Klucar, P., Shams, H. et al. (2008) CREB, ATF and AP-1 transcription factors regulate IFN-γ secretion by human T cells in response to mycobacterial antigen. *J. Immunol.* **181**, 2056–2064  
https://doi.org/10.4049/jimmunol.181.3.2056
56. Yarza, R., Vela, S., Solas, M. and Ramirez, M.J. (2016) c-Jun N-terminal kinase (JNK) signaling as a therapeutic target for Alzheimer’s disease. *Front. Pharmacol.* 6, 1–12. https://doi.org/10.3389/fphar.2015.00321

57. Hirosumi, J., Tuncman, G., Chang, L., Görgün, C.Z., Uysal, K.T., Maeda, K. et al. (2002) A central role for JNK in obesity and insulin resistance. *Nature.* 420, 333–336. https://doi.org/10.1038/nature01137

58. Nakatani, Y., Kaneto, H., Kawamori, D., Hatazaki, M., Miyatsuka, T., Matsuoka, T. et al. (2004) Modulation of the JNK pathway in liver affects insulin resistance status. *J. Biol. Chem.* 279, 45803–45809. https://doi.org/10.1074/jbc.m406963200

59. Bennett, B.L., Satoh, Y. And Lewis, A.J. (2003) JNK: A new therapeutic target for diabetes. *Curr Opin Pharmacol.* 3, 420–425. https://doi.org/10.1016/S1471-4892(03)00068-7

60. Mathews, S.T., Singh, G.P., Ranalletta, M., Cintron, V.J., Qiang, X., Goustin, A.S. et al. (2002) Improved insulin sensitivity and resistance to weight gain in mice null for the *Ahsg* gene. *Diabetes.* 51, 2450–2458. https://doi.org/10.2337/diabetes.51.8.2450

61. Mathews, S. T., Rakhade, S., Zhou, X., Parker, G. C., Coscina, D. V., & Grunberger, G. (2006) Fetuin-null mice are protected against obesity and insulin resistance associated with aging. *Biochem Biophys Res Comm.* 350, 437–443. https://doi.org/10.1016/j.bbrc.2006.09.071

62. Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H., & Flier, J. S. (2006) TLR4 links innate immunity and fatty acid–induced insulin resistance. *J. Clin. Investig.* 116, 3015–3025. https://doi.org/10.1172/JCI28898
FIGURE LEGENDS

Figure 1. FetA induces MCP-1 and iNOS expression.

Immunohistochemistry of adipose tissue from SD, HFD and HFD+FetA\textsuperscript{KD} mice with anti-CD11c (A). Adipose immunofluorescence of SD, HFD and HFD+FetA\textsuperscript{KD} mice after dual-immunostaining with anti-CD206 and anti-CD86 and DAPI as nuclear stain (B). Stromal vascular fraction (SVF) isolated from SD, HFD and HFD+FetA\textsuperscript{KD} mice subjected to iNOS, CD11c and Arginase 1 immunoblotting (C). MCP-1 expression by immunoblotting and ELISA-quantified concentration in sera obtained from SD, HFD and HFD+FetA\textsuperscript{KD} mice (D,E). Quantification of MCP-1 concentration in SVF lysates of SD, FetA-injected SD (SD+FetA), HFD only and HFD+FetA\textsuperscript{KD} mouse was determined by ELISA (F). Estimations of TNFα, IL-6 and IL-10 levels in media after incubating SVF isolated from SD, SD+FetA, HFD only and HFD+FetA\textsuperscript{KD} mice for 4 h by ELISA (G-I). All images shown here are representatives of at least three independent experiments; other analyses were carried out with n=6 animals in each group. Data are presented as mean ± SEM. Statistical significance is denoted by *P<0.05 and **P<0.01 vs SD; #P<0.05 and ##P<0.01 vs HFD.

Figure 2. FetA mediates its effect on MCP-1 and iNOS through IFNγ.

Protein and mRNA expression of IFNγ in stromal vascular fraction (SVF) obtained from SD, HFD and HFD+FetA\textsuperscript{KD} mice (A,B). Immunoblotting of IFNγ, MCP-1 and iNOS in SVF lysates of SD mice following incubation with varied doses of FetA (50-200 µg/ml) (C). qRT-PCR of iNOS and MCP-1 in SVF of SD mice treated without or with FetA (D). SVF was isolated from SD mice and incubated without or with FetA (100 µg/ml) at varied time intervals. On termination, expression of IFNγ, MCP-1 and iNOS were analysed by western blotting (E). MCP-1 level was quantified by ELISA in SVF dose and time dependently with FetA treatment (F,G). iNOS and MCP-1 protein expression in RAW 264.7 cells was estimated after FetA (100 µg/ml) treatment in absence or presence of IFNγ-siRNA (H). FACS analyses exhibiting CD11c\textsuperscript{+}F4/80\textsuperscript{+} and Arginase 1\textsuperscript{+}F4/80\textsuperscript{+} population of RAW 264.7 cells incubated with FetA (100 µg/ml) without or with IFNγ-siRNA (I,J). All images shown here are representatives of at least three independent experiments; other analyses were carried out with n=6 animals in each group. Data are presented as mean ± SEM.
Statistical significance is denoted by *$P<0.05$ and **$P<0.01$ vs SD and Control (Con); # $P<0.05$ and ## $P<0.01$ vs HFD.

**Figure 3. Induction of JAK-STAT pathway by FetA instigates macrophage migration and polarization.**

Immunoblots showing protein expression of pJAK2, pSTAT1, and NOX4 in RAW 264.7 cells treated without or with FetA (100 $\mu$g/ml) (A). RAW 264.7 cells were transfected with IFN$\gamma$-siRNA and incubated without or with FetA for 4 h followed by pJAK2, pSTAT1 and NOX4 western blotting (B). SVF isolated from SD mice were incubated without or with FetA (100 $\mu$g/ml) for 4h in absence or presence of Fludarabine, STAT1 inhibitor and protein expression of IFN$\gamma$, iNOS and NOX4 were determined (C). Quantification of MCP-1 in SVF lysate isolated from SD mice treated without or with FetA (100 $\mu$g/ml) for 4 h in absence or presence of Fludarabine by ELISA (D). Concentrations of TNF$\alpha$, IL-6, IL-1$\beta$ and IL-10 by ELISA with media of SVF isolated from SD mice incubated with FetA in absence or presence of Fludarabine (E-H). SVF was isolated from SD mice, treated with FetA in absence or presence of Fludarabine followed by immunoblotting of CD86, CD11c, CD206 and Arginase 1 (I,J). Control (Con) or NOX4-siRNA transfected RAW 264.7 cells were treated in absence or presence of FetA to quantify MCP-1 level by ELISA (K). Images of immunoblotting shown here are representatives of at least three independent experiments; other analyses were carried out with n=6. All the data are presented as mean $\pm$ SEM. *$P<0.05$ and **$P<0.01$ vs Control (FetA$^-$); #$P<0.05$ and ##$P<0.01$ vs FetA$^+$ represent statistical significance.

**Figure 4. FetA augments IFN$\gamma$ through JNK-cJun.**

SVF isolated from SD mice were incubated with FetA for 4h. The cell lysates were analyzed by immunoblotting with anti-pJNK, anti-pcJun and anti-IFN$\gamma$ (A). Immunoblots of pcJun, IFN$\gamma$, pJAK2, pSTAT1, iNOS and NOX4 in SVF isolated from SD mice treated with FetA in absence or presence of SP600125 (B). ELISA was performed to quantify MCP-1 in SVF collected from SD mice incubated with FetA in absence or presence of SP600125(C). Protein expression of M1 marker, CD11c and M2 marker, Arginase 1 were determined by western blotting in SVF without or with FetA in absence or presence of SP600125 (D). FACS data showing
CD86, M1 marker and CD206, M2 marker immunostaining of RAW 264.7 cells untreated or treated with FetA in absence or presence of SP600125 (E,F). Immunoblotting shown here are representatives of at least three independent experiments; other analyses were carried out with n=6. All the data are presented as mean ± SEM. *P<0.05 and **P<0.01 vs Control (Con) and FetA; #P<0.05 and ##P<0.01 vs FetA+ represent statistical significance.

**Figure 5. Comparative analysis of FetA knockdown and JNK inhibition in HFD mice**

SVF were isolated from SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice, cells were lysed, centrifuged and supernatant was subjected to western blotting to detect expression of p-cJun, IFNγ, pJAK2, pSTAT1, iNOS and NOX4 (A). Level of MCP-1 was quantified by ELISA with SVF lysates isolated from SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice (B). Microscopic images exhibiting the migration of RAW 264.7 cells placed in the upper chamber with the conditioned media of SVF collected from SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice kept in lower chamber for a period of 4 h (C). Dye was extracted from the above experiment followed by estimation of optical density (OD) at 560 nm (D). The amount of release of TNFα, IL-6, IL-1β and IL-10 in the media of SVF isolated from SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice were determined by ELISA (E-H). Protein expression of CD11c and Arginase 1 were assessed in SVF obtained from SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice using respective antibodies (I). Immunoblotting and microscopic images shown here are representatives of at least three independent experiments; other analyses were carried out with n=6. All the data are presented as mean ± SEM. *P<0.05 and **P<0.01 vs SD; #P<0.05 and ##P<0.01 vs HFD represent statistical significance.

**Figure 6. Inactivation of FetA and JNK improves insulin sensitivity.**

Investigation of insulin sensitivity in SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice was performed through Oral Glucose Tolerance Test (OGTT) and Insulin tolerance test (ITT) (A,B). ELISA was performed for estimating serum insulin content of SD, HFD, HFD+FetA KD and HFD+SP600125 treated mice (C). Analyses
were carried out with n=6. All the data are presented as mean ± SEM. *P<0.05 and **P<0.01 vs SD; #P<0.05 and ##P<0.01 vs HFD represent statistical significance.

**Figure 7. TLR4 suppression inhibits FetA action.**

Knockdown of TLR4 was assessed in RAW 264.7 cell line using TLR4 siRNA by immunoblotting followed by densitometric analysis (A). Immunoblots of pJNK, IFNγ, pJAK2, pSTAT1, iNOS and MCP-1 in RAW264.7 macrophages with or without FetA (100 μg/ml) in absence or presence of TLR4 siRNA using respective antibodies followed by its densitometric quantitation (B). In absence or presence of TLR4 siRNA, the concentration of MCP-1 was measured in RAW264.7 macrophages with or without FetA (C). SVF isolated from SD mice treated with or without FetA (100 μg/ml) in absence or presence of CLI-095 (3 μM), a TLR4 inhibitor and subjected to immunoblotting of pJNK, IFNγ, pJAK2, pSTAT1, iNOS and MCP-1 in cell lysates followed by densitometry (D). Determination of MCP-1 concentration in SVF lysates from SD mice with or without FetA (100 μg/ml) in presence or absence of CLI-095 (3 μM) using ELISA (E). All images shown here are representatives of at least three independent experiments; other analyses were carried out with n=6 animals in each group. Data are presented as mean ± SEM. Statistical significance is denoted by*P<0.05 and **P<0.01 vs Con; #P<0.05 and ##P<0.01 vs FetA.
| Serial No. | Gene | Primer Sequences |
|-----------|------|-----------------|
| 1         | MCP-1 | Forward: 5'-GAAGGAATGGGTCCAGACATAC-3'  
Reverse: 5'-CACATTCAAAGGTGCTGAAGAC-3' |
| 2         | IFNγ | Forward: 5'-CTGAGACAATGAACGCTACA-3'  
Reverse: 5'-CAGTTCCCTCCAGATATCCAA-3' |
| 3         | iNOS | Forward: 5'-TTCACCCAGTTGTGCATCGACCTA-3'  
Reverse: 5'-TCCATGGTCACCTCCAACCAAGA-3' |