Article

Adipose Tissue Caveolin-1 Upregulation in Obesity Involves TNF-α/NFκB Mediated Signaling

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Abstract: Obesity is characterized by chronic low-grade inflammation. Caveolin-1 (CAV1), a structural and functional protein found in adipose tissues (AT), is augmented in obese individuals. We aimed to define the inflammatory mediators that influence *CAV1* gene regulation and associated mechanism in obesity. Using subcutaneous AT from 27 (7 lean/20 obese) normoglycemic individuals, *in vitro* human adipocyte models, and *in vivo* mice models, we found elevated CAV1 expression in obese AT and a positive correlation between the gene expression of CAV1, tumor necrosis factor alpha (TNF-α), and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). *CAV1* gene expression was associated with that of proinflammatory cytokines/chemokines, and their cognate receptor ($r \geq 0.447$, $p \leq 0.030$) but not with anti-inflammatory markers. CAV1 expression was correlated with CD163, indicating a prospective role for CAV1 in adipose inflammatory microenvironment. Unlike wild-type animals, mice lacking TNF-α exhibited reduced levels of CAV1 mRNA/proteins, which were elevated by administering exogenous TNF-α. Mechanistically, TNF-α induces *CAV1* gene transcription by mediating NF-kB binding to its two regulatory elements located in the CAV1 proximal regulatory region. The interplay between CAV1 and TNF-α signaling pathway is interesting and has potential as a target for therapeutic interventions in obesity and metabolic syndromes.

Keywords: Caveolin 1; Obesity; Adipose tissue; Metabolic inflammation; Cytokines; TNF-α; NF-κB

1. Introduction

Obesity is a complex multifactorial metabolic disorder involving the genetic, socioeconomic, and environmental factors [1]. Despite significant efforts made to raise awareness, obesity remains a major global burden and is a precursor of several diseases, including type 2 diabetes mellitus (T2DM) and cancer, further contributing towards lower quality of life and higher mortality rates [2]. In Kuwait, obesity has reached endemic levels [3]; a study conducted in 2014 reported that approximately eight of ten Kuwaiti adults were either overweight or obese, highlighting the need for urgent action plans and strategies [4].

Adipose tissue (AT) is a plastic and dynamic endocrine organ that functions as a primary fat and triacylglycerol deposit [5]. AT also possesses the plasticity to adjust to a surplus of energy through progressive adipocyte hypertrophy and proliferation. AT also does so *via* the recruitment and differentiation of precursor cells and mechanisms associated with active vascularization and extracellular matrix remodeling [6]. Furthermore, AT is an endocrine organ that produces and secretes hormones, adipokines, cytokines, and chemokines [5]. In obese individuals, aberrant AT and residential immune cells intensify local inflammatory responses and augment metabolic dysfunctions [7].

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Caveolae are cholesterol-rich lipid rafts located in the plasma membrane as inward cytoplasmic invaginations, protruding into the cytoplasm. Although caveolae are present in many types of cells, they are particularly abundant in adipocytes, accounting for approximately 30% of the plasma membrane [8]. Caveolae are implicated in several essential cellular functions, such as endocytosis, transcytosis, maintenance of plasma membrane integrity, lipid homeostasis, signal transduction, and mechano-protection [9; 10]. Caveolin-1 (CAV1) is an integral structural and functional protein of the caveolae that helps in the recruitment of various signaling molecules and plays an indispensable role in the insulin signaling pathway [11; 12]. A previous study reported an increase in CAV1 expression in the AT of obese individuals, with or without T2DM, and an association between CAV1 and several inflammatory markers, suggesting a prospective role of CAV1 in obesity-related chronic low-grade inflammation, called matabolic inflammation [13].

In obese individuals, dysfunctional adipocytes secrete proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as well as chemokines, especially CCL2 which plays a major role in recruiting immune cells to ATs, resulting in obesity-associated metabolic inflammation [14]. This is associated with the onset of various conditions, including hyperinsulinemia, dyslipidemia, T2DM, and vascular abnormalities [15; 16]. TNF-α, by activating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), contributes positively toward maintaining a proinflammatory microenvironment in the adipocyte, which further results in significant changes in the expression of adipocyte-specific genes and affects their function [17; 18]. Although, previous studies have reported the effects of TNF-α on insulin resistance and the destabilization of insulin receptor–CAV1 interactions [19], the association between this cytokine and CAV1 expression in normoglycemic obese individuals has not been studied before.

In the context of obesity and metabolic syndromes, CAV1 regulation and association with inflammatory markers are not well defined. Therefore, in the present study, we aimed to evaluate the correlations between CAV1 and proinflammatory cytokine transcripts in non-diabetic obese individuals. Then, we thoroughly investigated the role of TNF-α-mediated CAV1 upregulation using pre-adipocytes isolated from lean and obese individuals as an *in vitro* model. Herein, we show, for the first time to our knowledge, that the simultaneous increase in CAV1 expression in ATs of obese individuals correlates with inflammatory and metabolic markers in this population. We further demonstrate the reduced adipose CAV1 expression in TNF-α global knock-out (KO) mice and the positive modulatory changes in its expression by exogenous TNF-α in this mouse model. We also show that TNF-α acts by activating the NF-κB binding sites located in CAV1 upstream regulatory regions.

2. Materials and Methods

2.1. Study Population and Anthropometric Measurements

The study cohort comprised a total of 27 male and female non-diabetic adult individuals, enrolled at the gymnasium facility at the Dasman Diabetes Institute in Kuwait. Using the standard formula for calculating BMI, i.e., body weight (kg) / height^2 (m^2), the cohort was divided into seven lean (BMI < 25 kg/m^2) and 20 obese (BMI ≥ 30 kg/m^2) participants. The exclusion criteria were as follows: individuals with a history of chronic disease or medications, malignancy, and pregnancy. Anthropometric measurements, such as height, weight, and waist circumference were measured, as described previously [20; 21]. Whole-body compositions, including body fat percentage, soft lean mass, and total body water were measured using an IOI 353 Body Composition Analyzer (Jawon Medical, South Korea). The biochemical and demographic characteristics of the participants are summarized in **Table 1**. This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Dasman Diabetes Institute, Kuwait. A written informed consent was obtained from each participant prior to the study.

Table 1. Anthropometric measurements and clinical characteristics of the study participants.

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Phenotype	Lean	Obese	Lean vs Obese
	(*n* = 7) (Mean ± SD)	(*n* = 20) (Mean ± SD)	*(P-value)*
Age (years)	43.43 ± 7.45	45.45 ± 12.64	0.695
Weight (kg)	61.37 ± 12.27	93.49 ± 15.45	**<0.0001**
Height (cm)	1.65 ± 0.12	1.65 ± 0.11	0.92
BMI (kg/m^2)	22.41 ± 2.61	34.40 ± 3.47	**<0.0001**
Body fat (%)	27.39 ± 6.27	39.31 ± 4.17	**<0.0001**
Triglycerides (mmol/L)	0.66 ± 0.24	1.32 ± 0.90	0.069
Total cholesterol (mmol/L)	5.40 ± 1.03	5.08 ± 1.27	0.554
HDL cholesterol (mmol/L)	1.67 ± 0.59	1.17 ± 0.29	0.071
LDL (mmol/L)	3.43 ± 0.88	3.35 ± 1.08	0.856
FBG (mmol/L)	5.24 ± 0.53	5.20 ± 0.63	0.86
HbA1C (%)	5.70 ± 0.50	5.59 ± 0.67	0.696
Insulin (mU/L)	6.58 ± 3.33	11.15 ± 7.49	0.211
HOMA-IR	1.51 ± 0.69	2.59 ± 1.86	0.223
WBC	5.40 ± 1.59	6.235 ± 1.93	0.345

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FBG, fasting blood glucose; HbA1C, glycated hemoglobin C; HOMA-IR, homeostatic model assessment for insulin resistance; WBC, white blood cells.

2.2. Collection of Subcutaneous AT

We collected AT samples (approximately 500 mg) *via* abdominal subcutaneous fat pad biopsy by following standard surgical procedures, as described previously [20]. Briefly, the periumbilical area was decontaminated using alcohol-soaked gauzes, which was then locally anesthetized using 2% lidocaine. Fat tissue was collected through a small superficial skin incision (0.5 cm). Next, the biopsy tissue was further incised into smaller sections, washed with cold phosphate buffered saline (PBS), preserved in RNAlater solution, and was stored at −80 °C until use [22].

2.3. Blood Collection and Biochemical Measurements

Peripheral blood samples (~10 mL each) were obtained from the participants after an overnight fast; the samples were collected in vacutainer EDTA tubes, plasma was collected by centrifugation, aliquoted and stored at –80 °C until assayed. Fasting blood glucose (FBG) levels and lipids profile, including total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides, were measured using Siemens Dimension RXL Chemistry analyzer (Diamond Diagnostics, Holliston, MA, USA). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated from basal (fasting) glucose and insulin concentrations using the following formula: HOMA-IR = fasting insulin (μU / L) × fasting glucose (nmol / L) / 22.5. HbA1c was measured using Variant device (BioRad, Hercules, CA, USA). White blood cells were counted using a hematocytometer. All assays were performed as per the instructions provided by the manufacturers.

2.4. RNA Extraction, cDNA Synthesis, and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from AT using RNeasy kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. The first cDNA strand was synthesized from 0.5 μg RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). Real-time RT-qPCR was performed as described previously [23]. cDNA samples (50 ng each) were amplified using TaqMan Gene Expression Master Mix (Applied Biosystems) and gene-specific 20× TaqMan gene expression assays (Applied Biosystems), containing forward and reverse primers (**Supplementary Table S1**), along with the target-

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specific TaqMan MGB probes labeled with FAM dye at the 5'-end and NFQ-MGB at the 3'-end of the probe using 7500 Fast Real-Time PCR System (Applied Biosystems). Each cycle involved denaturation for 15 s at 95°C; uracil-DNA glycosylases cycle for 2 min at 50°C; annealing/extension for 1 min at 60°C, and AmpliTaq gold enzyme activation for 10 min at 95°C. Gene expression relative to lean AT (control) was calculated using the comparative cycles to threshold (C_T) method, as previously described [24]. The results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, and data (mean±SEM) were expressed as fold changes in expression relative to controls, as indicated [25].

*2.5. Cell Culture, DNA Transfections, and **Luciferase** Reporter Assays*

Pre-adipocytes isolated from lean and obese individuals were purchased from Zen-Bio (USA). Cells were grown in DMEM/F12 media (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Corporation) and 100 U/mL of penicillin–streptomycin (Gibco, Carlsbad, CA, USA), and were incubated at 37°C in 5% CO_2. Cells were treated with TNF-α (10 ng/mL; Sigma, Street Saint Louis, MO, USA), or vehicle. After 24h of treatment, the cells were harvested for total RNA and protein preparations, following the manufacturers' instructions. For luciferase reporter assays, cells were plated at a density of 5,000 cells/well in 96 well plates and were transiently transfected using lipofectamine LTX (ThermoFisher, USA) with pRMT-Luc-CAV1, containing CAV1 proximal promoter or pRMT-Luc vector. Transfection efficiency was monitored by analyzing Renilla–luciferase activity, as described previously [26]. After 24h, the cells were washed twice with ice-cold PBS, lysed, and assayed in accordance with the Dual Luciferase Kit protocol (Promega, Madison, WI, USA). Luciferase activity was detected using assayed GloMax Navigator Microplate Luminometer (Promega, USA). To remove background, normalized activity of the luciferase reporter vector alone was subtracted from its activity in the presence of OLF-1. Relative luciferase units (RLUs) represent luciferase activity normalized to Renilla activity [27].

2.6. Chromatin Immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) assays were performed, as described previously [28], with minor modifications. Pre-adipocytes from lean and obese individuals were seeded on three 175 mm tissue culture flasks with 1 x 10^6 cells/flask. After 48h, 50 mg of chromatin was immunoprecipitated in each cell line using 0.5 µg of anti-NF-κB-specific antibodies (L8F6, cell signaling Technology, Danvers, MA, USA), anti-Histone-3 acetyl K14 antibodies (H3K14^{ac}, ab176799, Abcam, Cambridge, MA, USA), or non-specific rabbit IgG control antibodies (ab172730, Abcam). Briefly, the cells were crosslinked with 4% formaldehyde (Fisher Scientific, USA) and sonicated using Covaris-E220 ultrasonicator (Covaris, Wohurn, MA, USA) for a total of 30 x 15-second pulses (1 min rest between the pulses), and lysates were cleared by centrifugation at 13,000 rpm for 30 minutes at 4 ^oC. The sheared chromatins were incubated with the described antibodies and the immune complexes were captured using protein G-Sepharose Dynabeads (ThermoFisher, Waltham, MA, USA), as described previously [29]. NF-κB, H3K14^{ac}, or IgG-bound chromatins were quantified as a percent chromatin input. Quantitative PCR (qPCR) analyses were performed using the forward 5'- GAGGTGAAGAGAAGCCAGGAAT-3' and reverse 5'- CCCAATCTCAGGACCCCAAT-3' primers. To confirm a true association, each ChIP sample was examined for the enrichment of a chromatin locus immunoprecipitated with a specific antibody and compared with the same chromatin locus immunoprecipitated with a non-specific IgG. Data are represented as mean±SEM from three independent biological experiments [30].

2.7. Immunohistochemistry (IHC) Assays

IHC staining was performed, as described previously [31; 32]. Paraffin-embedded sections (4 µm thick) of subcutaneous AT were deparaffinized in xylene and rehydrated

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through descending grades of ethanol (100%, 95%, and 75%) to water. Antigen retrieval was then performed by placing slides in a target retrieval solution (pH 6.0; Dako, Denmark) in the pressure cooker, boiling for 8 min and cooling for 15 min. After washing in PBS, endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min and non-specific antibody binding was blocked with 5% non-fat milk for 1h, followed by 1% bovine serum albumin solution for 1h. The slides were incubated at room temperature overnight with primary antibodies, such as rabbit polyclonal anti-CAV1 antibody (3238s, cell signaling Technology, USA) and TNF-α antibody (ab9635, Abcam). After washing with PBS (0.5% Tween), slides were incubated for 1h with goat anti-rabbit conjugated with horseradish peroxidase polymer chain DAKO EnVision Kit (Dako, Denmark), and color was developed using a 3,3'-diaminobenzidine (DAB) chromogen substrate. The specimens were washed, counterstained, dehydrated, cleared, and mounted, as described elsewhere [33]. For analysis, digital photomicrographs of the entire AT sections (20X; Pannoramic Scan, 3DHistech, Hungary) were used to quantify the immunohistochemical staining using ImageJ software (NIH, USA). CAV1 and TNF-α antibody specificity was validated using spleen tissue, as shown in **Supplementary Figure S1**.

2.8. Western blotting

Western blot analysis was performed, as described previously [23]. Briefly, cells were harvested and lysed in RIPA buffer. Cell lysates were quantified using Quick start Bradford assay (Bio-Rad, USA) and equal amounts of protein were resolved on 8–12% polyacrylamide gels and transferred to Nitrocellulose membranes (Bio-Rad, Germany). After blocking, membranes were blotted with the following primary antibodies (CAV1, cat. #3238s; β-actin, 4970L; Cell Signaling Technology, USA) and the corresponding horseradish peroxidase-linked secondary antibody (cat. #7074P2, Cell Signaling Technology). Proteins were visualized using SuperSignal West Femto ECL kit (Thermo Scientific, USA). Images were captured using ChemiDoc MP imaging system (Bio-Rad, Germany).

2.9. Mice

Male mice (6–7 weeks old; 23.64±2.76 grams) were housed at the Animal Core Facility, Dasman Diabetes Institute, Kuwait. The mice were housed in temperature-controlled rooms (22°C) with a 12h light/dark cycle, with access to standard laboratory food and water *ad libitum.* All experiments on animals were approved by the ethics committee for the use of Laboratory Animals in Teaching and in Research, Dasman Diabetes Institute, Kuwait, in accordance with the guidelines of the Animal Research: Reporting of In vivo Experiments (ARRIVE). Wildtype control mice (B6129SF2/J, Strain #:101045, RRID: IMSR_JAX:101045) and TNF-$\alpha^{-/-}$ mice (B6;129S-Tnftm1Gkl/J, Strain #:003008, RRID: IMSR_JAX:003008) were acquired from the Jackson Laboratory, USA. Both genotypes were injected intraperitoneally with either physiological saline or 100 μg/Kg TNF-α to delineate their CAV1 mRNA expression levels. AT samples from these mice were obtained, as previously described [7].

2.10. Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (La Jolla, San Diego, CA, USA) and SPSS for Windows version 19.01 (IBM SPSS Inc., Armonk, NY, USA) as described preiously [34]. Data are shown as mean ± standard deviation, unless otherwise indicated. Unpaired Student's *t*-test was used to compare the means between groups. Pearson correlation was used to determine associations between different variables. For all analyses, a p-value < 0.05 was considered significant.

3. Results

3.1. Demographic and Clinical Characteristics of the study population

The demographic and clinical characteristics of the 27 participants included in this study are shown in **Table 1.** The mean age of the study participants was 44 years; weight,

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BMI, and body fat percentage were significantly higher in the obese participants compared to the lean individuals ($p < 0.0001$ for all). The mean values of total plasma cholesterol and LDL levels were comparable between the two groups, whereas, in obese individuals, plasma triglycerides and HDL levels were marginally higher and lower, respectively ($p = 0.069$ and $p = 0.071$, respectively). All participants had a normal or a prediabetic status with FBG and HbA1c measurements at 5.24 ± 0.53 mmol/L and 5.7 ± 0.50%, respectively, for lean individuals, and 5.20 ± 0.63 mmol/L and 5.59 ± 0.67%, respectively, for obese subjects. Expectedly, HOMA-IR values were also elevated, averaging between 1.51 ± 0.69 and 2.59 ± 1.86 for lean and obese participants, respectively. The plasma insulin concentrations were below 20 mU/L, i.e., within normal fasting concentrations, in both groups.

3.2. Elevated CAV1 Gene and Protein Expression in Subcutaneous AT From Obese Individuals

RT-qPCR analysis showed a significant increase (1.7-fold) in CAV1 mRNA expression in subcutaneous ATs isolated from obese subjects, as compared to lean individuals (**Figure 1A)**. Similarly, IHC analysis of CAV1 protein expression in AT sections showed higher protein levels in obese as compared to lean participants **(Figure 1B)**. The quantification of CAV1 protein expression in AT showed a 2-fold increase in CAV1 expression, during IHC analysis of FFPE-stained AT from obese individuals, as compared to lean individuals **(Figure 1C)**.

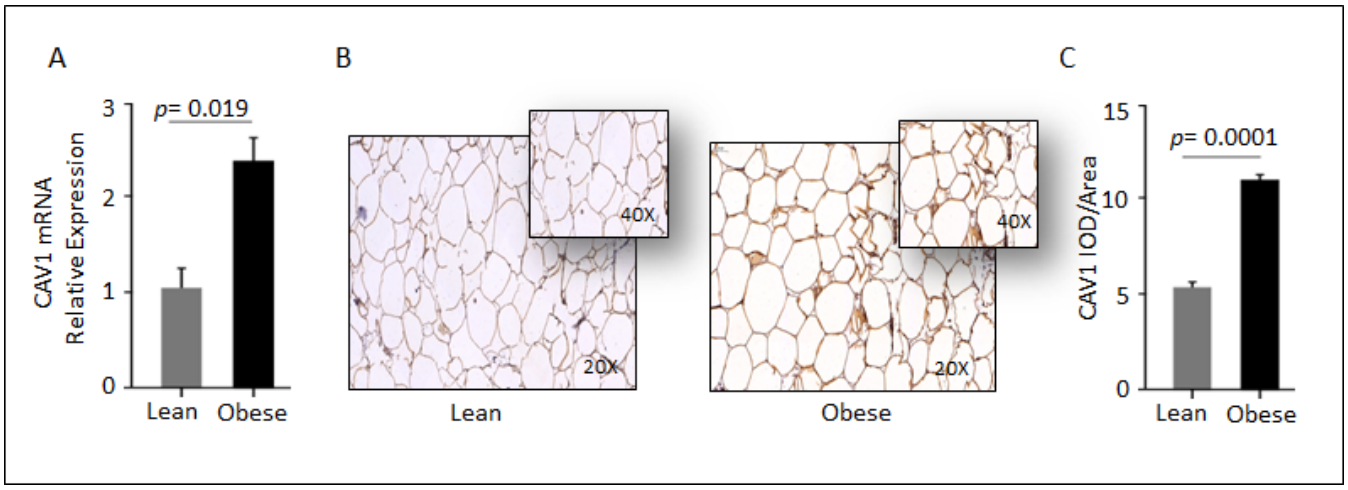

Figure 1. Increased CAV1 mRNA and protein levels in AT. **A.** *CAV1* mRNA expression in AT isolated from lean and obese individuals, as determined by RT-qPCR assay **B.** Representative images of IHC staining for CAV1 protein in tissue sections of AT obtained from lean and obese individuals. Obese sections show stronger staining than lean sections (Images, magnification, x20; enlarged area of image in top right corner, magnification, x40). **C.** IOD/Area quantification of CAV1 expression in AT from lean and obese individuals. CAV1 protein expression in obese individuals is 2-fold higher, as compared to lean participants. Abbreviations: AT, adipose tissue; CAV1, caveolin-1; IHC, immunohistochemistry; IOD, Integrated optical density; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

3.3. Correlations Between CAV1 Expression and Inflammatory Markers in Obese AT

To evaluate the role of CAV1 in obesity-associated metabolic inflammation, we analyzed the association between *CAV1* gene expression and the expression of various inflammatory cytokines in obese participants. As shown in **Table 2**, RT-qPCR analyses revealed a notable positive correlation between *CAV1* gene expression levels and that of several C-C motif chemokines, such as CCL2 and CCL8 and their receptors CCR1 and CCR2 ($0.491 \leq r \leq 0.618$; $p < 0.035$; **Table 2**), which are known to be expressed in AT-residential macrophages and lymphocytes and are implicated in obesity-induced low-grade chronic inflammation [35]. Moreover, a significant correlation was observed between the transcript levels of *CAV1* and members of the angiostatic C-X-C motif chemokines —

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CXCL9, CXCL10, and CXCL11 ($p = 0.016$, $p = 0.029$, $p = 0.017$, respectively). When studying the correlation between *CAV1* and interleukins, a significant correlation was observed between *CAV1* expression and that of interleukin 1 receptor type 1 (IL1RL1) ($p = 0.009$); no significant correlation between *CAV1* gene expression and any other interleukin was detected (**Table 2**). Remarkably, *CAV1* transcripts levels were not associated with that of the β-cell maturation and differentiation markers IL-5 or IL-13, the Th1 cell marker IL-12A, or with the anti-inflammatory IL-10 (**Table 2**). Together, these results indicate that CAV1 is only associated with proinflammatory, and not anti-inflammatory markers. When evaluating the correlation between *CAV1* and M1/M2 macrophage markers, we found a significant correlation between *CAV1* and CD163; no such relationship was found with other macrophage subtypes. Moreover, RT-qPCR analyses show a positive correlation between *CAV1* and the toll-like receptors (TLRs) signaling cascade; such as TLR3 (r = 0.509; $p = 0.031$) and TLR4 (r = 0.488; $p = 0.058$); however, no such correlation was found with the anti-inflammatory TLR9, which is highly expressed in AT (**Table 2**) [36; 37; 38]. Additionally, we were not able to detect any correlation between *CAV1* expression and that of the signal transduction mediator downstream the TLR signaling pathway **(Table 2)**. Finally, no correlation was observed between CAV1 expression and the studied inflammatory factors in lean individuals (Data not shown).

Table 2. Correlation between caveolin-1 gene expression and that of various cytokines or chemokines in non-diabetic obese participants, *$p < 0.05$.

Obese Subjects					
Pearson Correlation (20)			Pearson Correlation (20)		
	r-value	p-value		r-value	p-value
CC chemokine ligands			CXC chemokine ligands		
CCL2	**0.491***	0.0327	CXCL9	**0.544***	0.0161
CCL3	0.194	0.471	CXCL10	**0.500***	0.0293
CCL8	**0.618****	0.0096	CXCL11	**0.552***	0.0175
CCL15	-0.029	0.905	M1-M2 macrophage transition		
CCL18	0.077	0.748	CD16	0.112	0.647
CCR1	**0.574***	0.016	CD68	-0.004	0.989
CCR2	**0.538***	0.034	CD86	0.128	0.601
Cytokines / Interleukins			CD163	**0.447***	0.048
TNF-α	**0.547***	0.0251	Transcription factors related to inflammation		
IL2	0.256	0.277	NFκB	**0.498***	0.033
IL5	0.053	0.826	Toll-like receptors (TLRs) signaling cascade		
IL6	0.023	0.925	TLR2	0.043	0.879
IL8	-0.069	0.794	TLR3	**0.509***	0.0311
IL10	-0.026	0.915	TLR4	**0.488***	0.058
IL12A	0.015	0.955	TLR7	0.292	0.212
IL13	0.046	0.855	TLR8	0.008	0.975
IL23A	0.083	0.729	TLR9	0.05	0.835
IL1RL1	**0.593***	0.0094	TLR10	0.019	0.937
IL2RA	-0.012	0.96	IRF4	0.263	0.262

3.4. Correlations Between CAV1 Gene Expression and TNF-α Signaling Pathway

A significant correlation was observed between the proinflammatory cytokine *TNF-α* and *CAV1* at the transcriptional level in the AT obtained from obese individuals (r = 0.547; $p < 0.025$; **Table 2**, **Figure 2A**). In addition, *TNF-α* was independently associated with *CAV1* ($\beta = 0.06$; $p = 0.032$) in a multiple stepwise regression analysis for the parameters showing a significant correlation with the elevated levels of *CAV1* gene expression. At a similar trend, the transcripts of the TNF-α downstream effector *NF-κB* significantly correlated with that of *CAV1* (r = 0.498; $p = 0.033$; **Table 2** and **Figure 2B**).

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Next, we also performed IHC analysis using TNF-α-specific antibodies to further study the notable association at the protein level; firstly, we observed a 3.5-fold increase in TNF-α protein levels in samples obtained from obese individuals, as compared to those from lean participants (p = 0.0001; **Figure 2C and D**). Secondly, we found that protein expression of CAV1 and TNF-α were significantly correlated (p < 0.0001; **Figure 2F**).

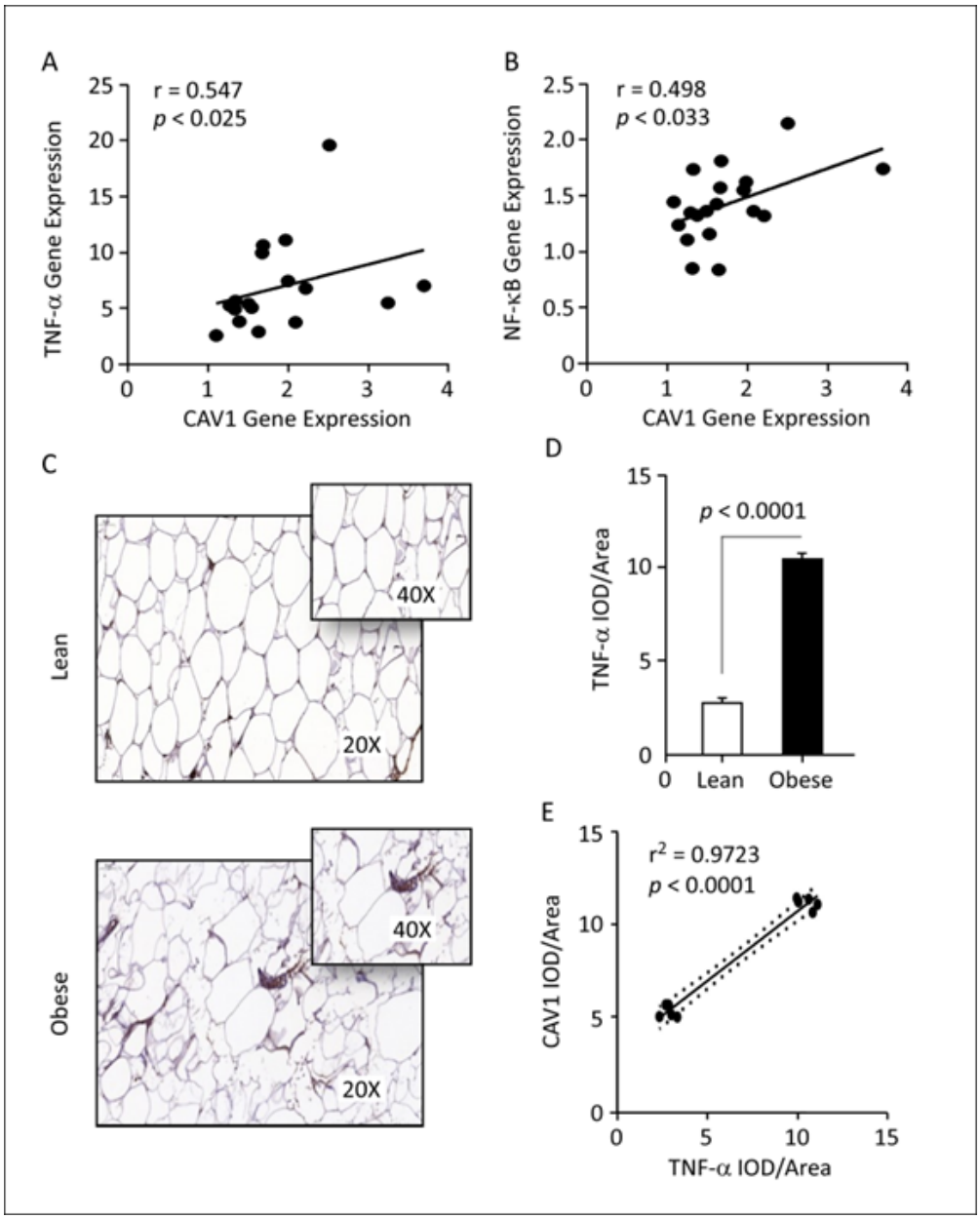

Figure 2. Gene expression and immunohistochemical results in AT isolated from lean and obese individuals. Correlation between CAV1 and **A.** TNF-α and **B.** NF-κB transcripts expression. **C.** Representative images of IHC staining for TNF-α protein in tissue sections of AT obtained from lean and obese individuals. Obese sections show stronger staining than lean sections (Images, magnification, x20; enlarged area of image in top right corner, magnification, x40). **C.** IOD/Area quantification of TNF-α expression in AT from lean and obese individuals. TNF-α protein expression in obese individuals is 3-fold higher compared to lean participants. **D.** IOD/Area quantification of TNF-α expression in AT from lean and obese individuals. **E.** CAV1 and TNF-α immunohistochemistry IOD/Area correlation. *Immunohistochemical analysis was performed on samples from five individuals and each point is the average of five different images. Abbreviations: AT: adipose tissue; CAV1: caveolin-1; TNF-α: tumor necrosis factor-alpha; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; IHC, immunohistochemistry, IOD: integrated optical density.

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3.5. Adipose CAV1 mRNA Levels in Murine Models

Data obtained from humans indicated that the TNF-α pathway was the main regulator of CAV1 expression; therefore, we aimed to determine the expression levels of CAV1 in the subcotaniuos ATs obtained from wildtype and TNF-α KO mice that were intravenously injected with either normal saline or TNF-α. We found that the administration of TNF-α in wildtype or TNF-α KO mice enhanced the expression of CAV1 mRNA and proteins in the AT (**Figure 3**). Furthermore, these results revealed that in the absence of TNF-α, CAV1 expression levels are notably reduced and that the exogenous administration of TNF-α induces CAV1 expression *in vivo*.

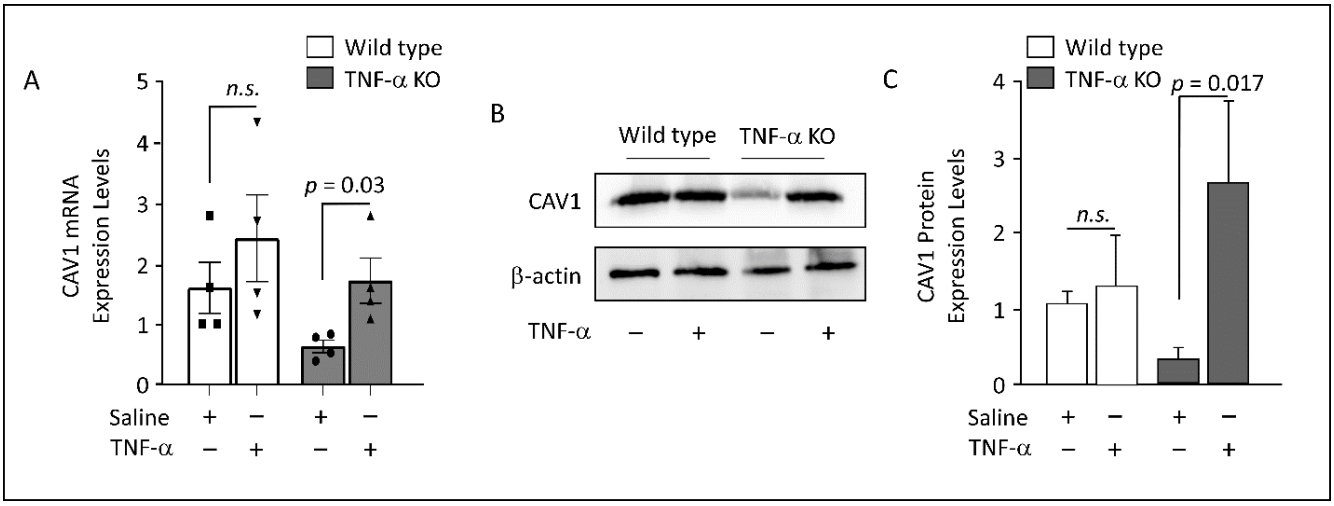

Figure 3. Exogenous administration of TNF-α induces CAV1 expression *in vivo*. **A.** The levels of mRNA and **B.** protein expression of CAV1 in adipose tissue obtained from wildtype and TNF-α knockout mice injected intravenously with saline or TNF-α solution (100 μg/Kg). Data are presented as mean ± SEM values of four animals/group. Two-tailed unpaired Student's t-test was used to determine significance. **B and C.** Western blots were developed with antibodies against CAV1 and β-actin, as indicated. CAV1 expression was significantly elevated in response to TNF-α treatments. Two-tailed unpaired Student's t-test was used to determine significance. Statistical analyses were relative to untreated wildtype animals.

3.6. In Vitro Analysis of CAV1 mRNA and Protein Expression in TNF-α-Treated Pre-Adipocytes

In vivo correlation studies indicate an interplay between CAV1 and proinflammatory factors, particularly TNF-α. Therefore, we next performed the *in vitro* analyses to specifically determine the impact of TNF-α on CAV1 expression and delineate the regulatory mechanism involved. Toward this end, differentiated pre-adipocytes obtained from lean and obese individuals were treated with vehicle or TNF-α. Notably in vehicle-treated cells, *CAV1* mRNA and protein levels were significantly elevated in obese adipocytes, as compared to lean adipocytes. Secondly, treatment with TNF-α significantly augmented the expression of CAV1 in adipocytes, as compared to control **(Figure 3).** It is noteworthy that the expression levels of CAV1 mRNA and protein were comparable post treatment with TNF-α. Taken together, these results show that TNF-α treatment independently enhances the CAV1 expression in human adipocytes.

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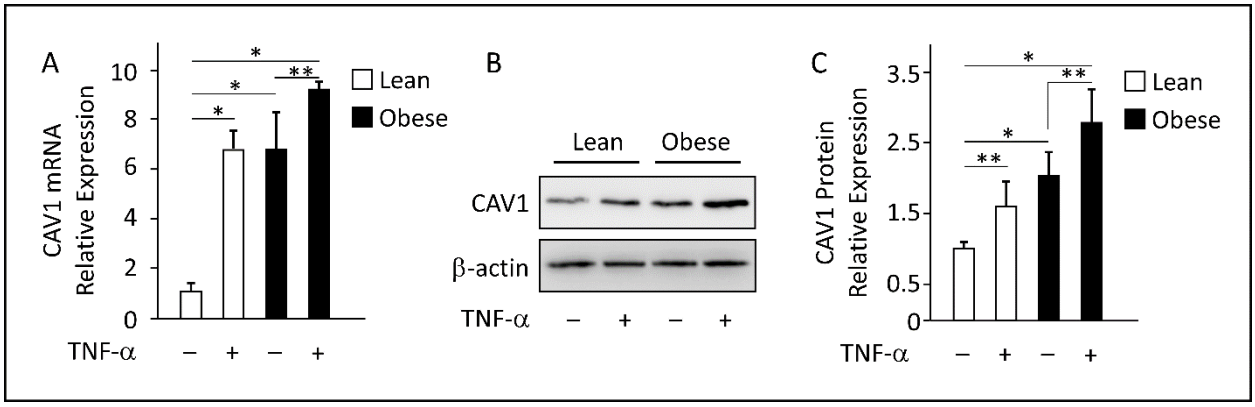

Figure 4. TNF-α augments the expression of CAV1 in differentiated pre-adipocytes isolated from lean and obese individuals. **A.** Relative to untreated lean adipocytes, *CAV1* mRNA expression levels were significantly elevated in obese adipocytes, and in response to TNF-α treatment for 24h. Data are presented as mean±SEM values obtained from three indepnedent experiments (n = 3); Two-tailed unpaired Student's t-test was used to determine significance. **B and C.** Western blots were developed with antibodies against CAV1 and β-actin, as indicated. CAV1 basal protein expression was significantly elevated in obese compared to lean adipocytes, and was significantly induced in response to TNF-α treatments of both lean and obese adipocytes. Two-tailed unpaired Student's t-test was used to determine significance. *$p < 0.001$; **$p < 0.05$.

3.7. ChIP Analyses and Luciferase Reporter Assays

Since CAV1 gene expression is correlated with that of TNF-α and NF-κB, and TNF-α induces CAV1 gene expression, we performed ChIP experiments to assess NF-κB promoter binding. *In silico* analysis of CAV1 proximal regulatory region revealed two adjacent NF-κB binding sites spanning the 1068 bp to 1094bp upstream of the translation state side, suggesting that TNF-α signaling could target CAV1 (**Figure 5A**). Hence, we performed ChIP analysis on control and TNF-α treated pre-adipocytes isolated from lean and obese humans. After performing ChIP-qPCR with NF-κB antibody, we observed a statistically significant enrichment of chromatin fragments in TNF-α treated lean (9.1-fold) and obese pre-adipocytes (22.3-fold), as compared to that in lean control samples (**Figure 5B**). Thus, upon TNF-α treatment, NF-κB appeared to bind to its consensus sites located in the 5' regulatory region of CAV1.

To determine if histone acetylation levels were changed, ChIP was performed with antibodies against acetylated H3K14 (H3K14^{ac}), indicative of actively transcribed chromatin. ChIP-qPCR analysis revealed significantly higher levels of H3K14^{ac} binding to the CAV1 regulatory region in TNF-α-treated lean (10-fold) and obese pre-adipocytes (13-fold), compared to untreated lean control cells. Thus, TNF-α facilitates histone acetylation of CAV1 proximal promoter.

To confirm the ChIP-qPCR data and to determine whether TNF-α acts through NF-κB to enhance promoter activity, we performed a luciferase reporter assay. We cloned 250 bp of the CAV1 regulatory region flanking the two NF-κB binding sites into a luciferase reporter construct (**Figure 5C**). We then transfected the construct into lean and obese pre-adipocytes, following which, we treated them with either a vehicle, or TNF-α. We found a significant increase in luciferase reporter activity in both lean and obese samples treated with TNF-α, as compared to untreated controls, indicating that TNF-αinduces NF-κB signaling and promote *CAV1* gene expression (**Figure 5D**).

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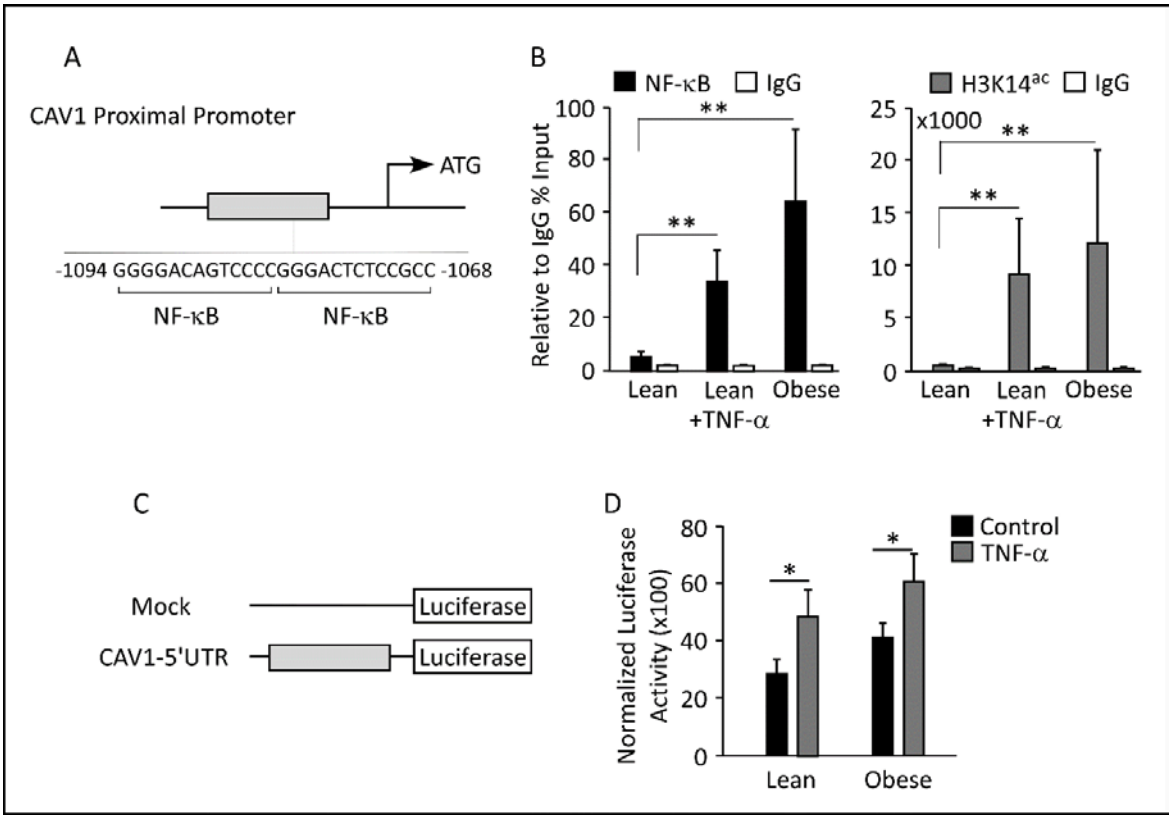

Figure 5. Treatment with TNF-α is associated with an enhanced CAV1 promoter activation. **A.** Schematic representation of the human CAV1 locus showing the regulatory region (vertical box) containing NF-κB binding consensus located 1000 bp upstream of the translation start site (arrow). **B.** ChIP was performed in pre-adipocytes from lean individuals, either treated with TNF-α or without treatment, and obese individuals, using antibodies against NF-κB or H3K14^{ac} and analyzed by qPCR with primers flanking the NF-κB binding site, as indicated. White bars indicate precipitation with IgG-nonspecific antibodies. Relative enrichment was calculated as the percent chromatin input normalized to IgG from three biological replicas (n = 3). $^{**}p < 0.01$. **C.** A schematic representation of *CAV1* gene mapping the proximal regulatory region and showing the cloned 250 bp DNA fragment spanning the NF-κB binding sites into Luciferase-pRMT-Luc reporter vector. **D.** Luciferase reporter assays: pre-adipocytes isolated from lean and obese individuals, were transfected with a CAV1-5'UTR-Luc construct and were either untreated (black bars) or were treated with TNF-α. Luciferase reporter activity was notably higher in samples treated with TNF-α. Luciferase activities are presented as fold change relative to pRMT-Luc reporter. Data are presented as mean ± SD of three biological replicas (n = 3); Two-tailed unpaired Student's t-test was used to determine significance. $^{*}p < 0.05$.

4. Discussion

In the present study, we aimed to investigate the association between CAV1 and pro-inflammatory cytokines in the AT from normoglycemic lean and obese individuals, to determine the independent predictors that may influence *CAV1* gene regulation, and to further elucidate the role of CAV1 in obesity and the regulatory mechanism involved. We identified a crosstalk between CAV1 and a wide range of proinflammatory cytokines and chemokines, particularly, the TNF-α signaling cascade. Human *in vivo* analyses of AT showed that the mRNA and protein expression of CAV1 were positively correlated with that of TNF-α. Furthermore, *in vitro* analysis revealed that CAV1 is notably upregulated by the TNF-α signaling cascade through the binding of NF-κB to the CAV1 promoter, as illustrated in **Figure 6**. Moreover, intravenous administration of TNF-α augmented the CAV1 expression levels in murine ATs. This suggests a strong association between CAV1 and different proinflammatory cytokines in obesity-mediated inflammation, particularly with TNF-α as an independent predictor.

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Several studies have provided strong evidence demonstrating the role of CAV1 in the pathophysiology of various diseases, particularly regarding increase of CAV1 expression in obesity. However, the exact mechanism by which CAV1 expression is induced remains to be elucidated [11; 39]. Briand *et al*. reported that CAV1 expression is crucial to increase caveolae density, accommodate larger lipid droplets, and improve glucose utilization to promote cell expansion in both *in vitro* and mice models. In humans, they found an initial increase in CAV1 expression in response to overfeeding [40]. Our results are in consensus with the above findings; in the present study, the mRNA and protein expression of CAV1 was significantly augmented in obese participants and, although not significant, increased plasma triglyceride levels were seen in these subjects. Thus, CAV1-enriched adipocytes in obese individuals might have a greater capacity for lipid storage, which is a characteristic clinical trait of obesity. This notion was previously supported by Catalán *et al*. who found significantly higher CAV1 expression in visceral and subcutaneous AT of obese individuals [13].

Previous studies, along with our observations, show that TNF-α augments CAV1 expression in human obese AT. However, we, for the first time, demonstrated the underlying mechanism by showing direct binding of NF-κB to the CAV1 proximal promoter, a process that is enhanced by the TNF-α signaling cascade. In addition, CAV1 transcripts and proteins levels were notably reduced in TNF-α KO mice, which were rescued post-exogenous TNF-α administration. In obesogenic states, TNF-α is synthesized by both adipocytes and infiltrated macrophages, acting as an endocrine and paracrine mediator through interaction with type I and II TNF-α receptors and activating NF-κB mediated signaling [41; 42]. The crosstalk between CAV1 and TNF-α in human and animal models have been previously reported. In human airway smooth muscles, Sathish et al. showed that CAV1 is associated with inflammation and its mRNA expression is induced by TNF-α and IL-1β, which was attributed to MAP kinases and NF-κB signaling [43]. Alternatively, in patients with T2DM and diabetic neuropathy, Zhu *et al*. reported a negative correlation between monocytes-CAV1 and TNF-α plasma levels [44]. In the rat pancreatic β-cell model — INS-1 — CAV1 protein levels were significantly elevated post co-treatment with TNF-α and IL-1β [45]. Palacios-Ortega et al. [L4] found that although TNF-α limited the degree of differentiation of the mouse 3T3-L1 pre-adipocytes and decreased the protein expression of CAV1, IR, and GLUT-4 during differentiation, it did not significantly reduce the mRNA expression of these genes, indicating a merely delayed state of adipocytic differentiation, as opposed to complete blockage in their activity. Moreover, TNF-α showed a continued increase in CAV1 expression in late mature adipocytes. Similarly, in the present study, in lean differentiated pre-adipocytes induced with TNF-α, the levels of *CAV1* mRNA expression were higher than the protein expression, suggesting a prospective post-transcriptional and/or post-translational regulation mechanisms (**Figure 4**).

Studies have found that macrophage infiltration occurs at a later stage of AT expansion and contributes toward sustained states of chronic inflammation [46], characterized by elevated expression of chemokines that are predominantly regulated by the NF-κB stimulatory signaling [47]. In our study, we found a positive correlation between CAV1 expression and CXCLs (CXCL9, CXCL10, and CXCL11); this could be explained by the proinflammatory role and heightened presence of CXCLs in obesity [48]. TLRs are immune receptors well-known for their role in mediating inflammation and triggering obesity and other metabolic syndromes; the activation of TLRs result in a signal transduction cascade leading to the activation of NF-κB [36]. Our team has already shown that the expression of several TLRs (TLR2, TLR4, TLR8, and TLR10) and their downstream signaling molecules are augmented in obesity, with or without T2DM, and these higher expression levels of inflammatory mediators are directly associated with insulin resistance, BMI, M1 macrophage polarization markers, and chemokine/cytokine expression [33; 49; 50]. In the present study, we found that CAV1 had a positive correlation with TLR3 and TLR4, both of which are known for inducing proinflammatory responses. CAV1 adipose expression was also positively associated with TLRs' downstream mediator NF-κB, along with IL1R1, further strengthening the inkling that CAV1 has associations specifically with

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proinflammatory cytokines and related pathways. AT macrophages contribute towards the inflammatory microenvironment of adipocytes and are multifaceted as they possess both pro- (M1) and anti- (M2) inflammatory properties. The ability of macrophages to switch their polarized state, i.e., from M2 to M1, under the activation of chemotactic molecules has been reported in murine models. In the present study, CD163, an alternatively activated or anti-inflammatory AT macrophage, was also positively correlated with CAV1. The accumulation of $CD11c^{+}CD163^{+}$ macrophages in both visceral and subcutaneous AT has been previously shown in obesity [51]. The hypertrophied state of adipocytes in our obese participants may explain these elevated levels of CD163.

Considering these intricately interwoven activities occurring in both T2DM and obesity, we infer that CAV1 may be the central factor directly or indirectly exerting a modulating effect on the increased serum triglyceride and free fatty acid levels, hyperinsulinemia, impaired glycogen storage, and a proinflammatory microenvironment in adipocytes. Thus, providing a rationale behind its abundance in adipocytes and positive correlations with proinflammatory factors in adipocytes of obese normoglycemic subjects included in our study. Although, the present study provides important insights, there are a few limitations. Because obesity is a multifactorial condition that is regulated by various factors, the sample size of the study is small to generalize these findings, considering the differences in diet, lifestyle, and genetics across geographical locations. In the future, multicenter studies with participants from various backgrounds, intaking different diets would be helpful to validate our findings.

5. Conclusions

In conclusion, we found augmented CAV1 mRNA and protein levels in ATs of obese human participants, and a positive correlation between CAV1 and proinflammatory cytokines. TNF-α/NF-κB signaling cascade is the key mediator for CAV1 upregulation in adipocytes. Over the past decades, the CAV1 protein, owing to its role in regulating various signaling cascades, has shown immense potential as a therapeutic target for obesity and obesity-associated metabolic inflammation. Future studies with a larger sample size are warranted to decipher the exact crosstalk between CAV1 and TNF-α and the mechanism by which CAV1 plays an important role in transmitting signals from the cell surface via intracellular signaling pathways that regulate inflammation in obesity. Targeting these pathways in humans may lead to a reduction in inflammatory states, thereby reducing insulin resistance, comorbidities, and improving the quality of life of patients with obesity.

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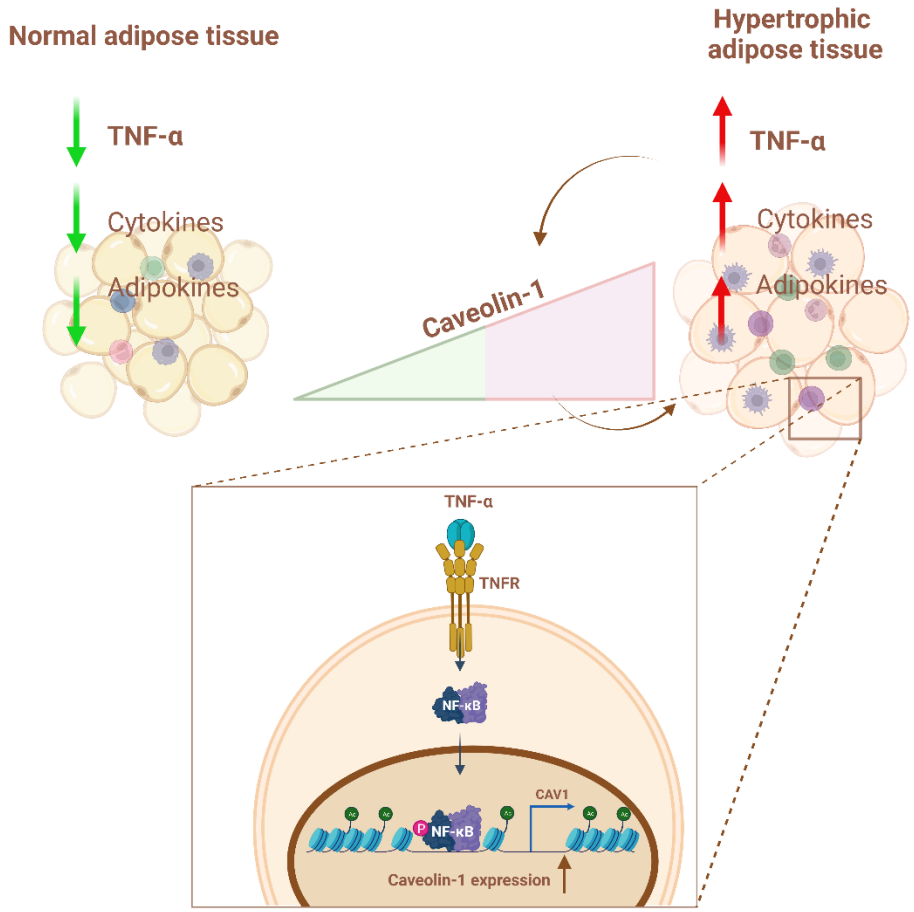

Figure 6. In adipocytes, CAV1 gene expression is regulated by TNF-α/TNFR signaling pathways though the activation of the downstream NF-κB pathway, which in turn binds to its consensus site at CAV1 regulatory promoter and enhances histone acetylation.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Caveolin-1 (CAV1) and TNF-α antibodies specificity validation using spleen tissues. Table **S1**: List of TaqMan Assays used for RT-PCR.

Author Contributions: AAM, MB, RA and FAM conceived the study design. AAM, and FAM were involved in data acquisition, data analysis, and interpretation. AAM and LM performed the cell cultures and ChIP assays. SK, RT and SS performed human and animal tissue RNA experiments. DH and RN performed luciferase assays, and Western blotting. LM performed Sanger sequencing. AAM, DH, and FAM wrote the manuscript. All authors contributed to the drafting and critical review of the manuscript. All authors agreed to the final manuscript content and its submission to the journal.

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Institutional Review Board Statement: The study protocol was approved by the Ethical Review Committee of Dasman Diabetes Institute, ensuring compliance with the guidelines of the Declaration of Helsinki, and of the US Federal Policy for the Protection of Human Subjects (project # RA CB-2021-007 and RA-2013-009). Written informed consent was obtained from the participants before the collection of blood and fat samples. The identities of the participants were protected from public exposure. Samples and data were processed anonymously.

Informed Consent Statement: Written informed consent was obtained from all participants involved in the study.

Data Availability Statement: The data are available upon request.

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Conflicts of Interest: The authors declare no conflict of interest.

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