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ARTICLE

The inflammatory receptor *CD40* is expressed on human adipocytes: contribution to crosstalk between lymphocytes and adipocytes

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Abstract
Aims/hypothesis Obesity is associated with adipose tissue inflammation. The CD40 molecule, TNF receptor superfamily member 5 (CD40)/CD40 ligand (CD40L) pathway plays a role in the onset and maintenance of the inflammatory reaction, but has not been studied in human adipose tissue. Our aim was to examine *CD40* expression by human adipocytes and its participation in adipose tissue inflammation.
Methods *CD40* expression was investigated in human whole adipose tissue and during adipocyte differentiation by real-time PCR, Western blot and immunohistochemistry. The CD40/CD40L pathway was studied using recombinant CD40L (rCD40L) in adipocyte culture and neutralising antibodies in lymphocyte/adipocyte co-culture.
Results *CD40* mRNA levels in subcutaneous adipose tissue were higher in the adipocyte than in the stromal–vascular fraction. *CD40* expression was upregulated during adipocyte differentiation. Addition of rCD40L to adipocytes induced mitogen activated protein kinase (MAPK) activation, stimulated inflammatory adipocytokine production, and decreased insulin-induced glucose transport in parallel with a downregulation of *IRS1* and *GLUT4* (also known as *SCL2A4*). rCD40L decreased the expression of lipogenic genes and increased lipolysis. *CD40* mRNA levels were significantly higher in subcutaneous adipose tissue than in visceral adipose tissue of obese patients and were positively correlated with BMI, and with *IL6* and leptin mRNA levels. Lymphocyte/adipocyte co-culture led to an upregulation of proinflammatory adipocytokines and a downregulation of leptin and adiponectin. Physical separation of the two cell types attenuated these effects, suggesting the involvement of a cell–cell contact. Blocking the CD40/CD40L interaction with neutralising antibodies reduced IL-6 secretion from adipocytes.
Conclusions/interpretation Adipocyte CD40 may contribute to obesity-related inflammation and insulin resistance. T lymphocytes regulate adipocytokine production through both the release of soluble factor(s) and heterotypic contact with adipocytes involving CD40.

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Keywords Adipocytes · CD40/CD40L · Inflammation · Lymphocytes · Obesity

Abbreviations

AF	Adipocyte fraction
CD40	CD40 molecule, TNF receptor superfamily member 5
CD40L	CD40 molecule, TNF receptor superfamily member 5 ligand
ERK	Mitogen-activated protein kinase 1
IκB	Inhibitor of NFκB
JNK	Mitogen-activated protein kinase 8
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
NFκB	Nuclear factor kappa beta
PAI-1	Plasminogen activator inhibitor 1
rCD40L	Recombinant CD40 molecule, TNF receptor superfamily member 5 ligand
SAT	Subcutaneous adipose tissue
sCD40L	Soluble CD40 molecule, TNF receptor superfamily member 5 ligand
SVF	Stromal–vascular fraction
VAT	Visceral adipose tissue

Introduction

Accumulating data link both obesity and diabetes, conditions associated with accelerated atherosclerosis, to inflammation [1]. Inflammation develops systemically and more locally in adipose tissue and may be involved in obesity-related insulin resistance and type 2 diabetes [1]. While extensive research is dedicated to the effect of an inflammatory reaction on obesity-related insulin resistance, little is known about the triggering pathway of inflammation during obesity. Considerable evidence implicates the proinflammatory CD40 molecule, TNF receptor superfamily member 5 (CD40) ligand (CD40L or CD154) in atherosclerosis [2–4], and some recent data identify CD40/CD40L as a potential contributor to inflammation associated with obesity and its metabolic complications. Soluble CD40L (sCD40L) levels are increased in obese [5, 6] and type 2 diabetic [7–9] individuals, as well as in individuals with the metabolic (insulin resistance) syndrome [10]. Variations in sCD40L plasma levels are strongly linked to differences in waist to hip ratio and to insulin sensitivity [11], with levels increased by exposure to hyperglycaemia and hyperinsulinaemia [12]. In addition, treatment with insulin-sensitising thiazolinediediones reduces serum levels of sCD40L in patients with type 2 diabetes and coronary artery disease [2, 13].

CD40L is produced by activated $CD4^+$ T cells [14]. Obesity in mice and humans has been associated recently with T cell accumulation in adipose tissue [15–18]. In obese mice, accumulation of proinflammatory T lymphocytes in visceral adipose tissue precedes the appearance of macrophages, suggesting that T lymphocytes play an important part in the initiation of adipose tissue inflammation, as well as in the development of insulin resistance [16]. Furthermore, it appears that T lymphocytes have an active role in adipose tissue biology because they strongly inhibit the differentiation of 3T3-L1 preadipocytes into adipocytes [18].

Assuming that *CD40* is expressed on adipocytes, we hypothesised that sCD40L or T lymphocytes expressing *CD40L* (also known as *CD40LG*) interact with adipocytes and trigger an inflammatory response and insulin resistance. To investigate this hypothesis we evaluated the expression of *CD40* by human adipocytes and the involvement of the CD40/CD40L pathway in adipocyte and T lymphocyte cooperation.

Methods

Sources of biological materials

Human subcutaneous adipose tissue (SAT) samples collected from overweight (BMI 25–30 kg/m^2) women during plastic surgery (n=26) were used for adipocyte culture. All participants were of white origin and did not suffer from any ongoing disease. SAT sections used for immunohistochemistry were obtained from three obese women (BMI 38.7±6.79 kg/m^2, age 28±10 years) and three lean women (BMI 22±0.72 kg/m^2, age 38±3.5 years) at the beginning of surgery for non-infectious, non-endocrine, or non-tumoural gynaecological disease. SAT and visceral adipose tissue (VAT) collected from 17 obese humans (BMI 44±1.3 kg/m^2, age 39±2.5 years) among those consecutively enrolled in the COLOMBES (Cohorte de sujets Obeses hôpital Louis Mourier: Bilan, Evaluation, Suivi) cohort, dedicated to the study and follow-up of obese individuals at the time of bariatric surgery, were used to study the correlations between mRNA levels of genes of interest. T lymphocytes were isolated from human whole blood obtained from nine lean healthy donors. All patients gave their informed consent to the study, which was approved by our local ethics committee.

Separation of mature adipocytes from stromal–vascular cells and differentiation of human preadipocytes

The adipocyte fraction (AF) and stromal–vascular fraction (SVF) were isolated from human SAT as previously described [19]. SVF cells were suspended in DMEM/F-12 Ham medium (Sigma-Aldrich, Saint-Quentin Fallavier, France)

and distributed on 12-well culture plates (Falcon, distributed by Becton Dickinson, Le Pont de Claix, France) or Lab-Tek Chamber Slides (Nunc, distributed by Dutscher, Brumath, France). After cell attachment, the medium was switched to DMEM/F-12 Ham containing 5% (wt/vol.) FCS and cells were grown for 3 days. The cells were then incubated for three more days in FCS-free medium enriched with 450 µmol/l isobutylmethylxanthine (IBMX; Sigma-Aldrich), insulin transferrin selenium (Sigma-Aldrich; 1:100), 1 µmol/l dexamethasone (Sigma-Aldrich) and 1 nmol/l triiodothyronine. IBMX was then omitted from the medium to induce differentiation (day 0). The culture medium was then changed every 72 h. After 10 days of differentiation, the percentage of macrophages assessed by Ham56 immunocytochemistry never exceeded 1% of total cultured cells. In some experiments, differentiated adipocytes (day 10–12) were exposed to 10 µg/ml of lipopolysaccharide (from *E. coli* 026:B6; Sigma-Aldrich) for 6 h or recombinant CD40L (rCD40L, 2 µg/ml; R&D Systems, Abingdon, UK) for the durations indicated on the figure legends.

Murine 3T3-L1 cell culture

3T3-L1 fibroblasts were grown and differentiated in adipocytes as previously described [20].

RNA isolation, reverse transcription and real time PCR

Total RNA was extracted using the Trizol method (Invitrogen, Cergy Pontoise, France). RT-PCR was performed as previously described [20, 21]. Primer sequences are available upon request from M. C. Alessi. The relative amounts of the different mRNAs were quantified by using the second derivative maximum method. In some cases data were expressed as Δcycle threshold (ΔC_t) values (where $\Delta C_t = C_{t\,\text{gene of interest}} - C_{t\,18S}$).

Protein extraction and Western blots

Adipocytes were washed with ice-cold buffer A (20 mmol/l Tris pH 7.4, 150 mmol/l NaCl, 10 mmol/l EDTA, 100 mmol/l NaF, 10 mmol/l pyrophosphate, 2 mmol/l sodium orthovanadate) before lysis (buffer A containing phosphatase and protease inhibitors, and 1% Triton X-100 [vol./vol.]). Cell lysates were centrifuged and proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane, which was hybridised with antibodies directed against CD40 (Abcam, Cambridge, UK), inhibitor of nuclear factor kappa B (IκB) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mitogen-activated protein kinase 1 (ERK), mitogen-activated protein kinase 8 (JNK) or p38 MAP kinase (Cell Signaling Technology, Beverly, MA, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected by enhanced chemiluminescence.

Co-culture of human adipocytes and T lymphocytes

$CD4^+$ T lymphocytes were isolated from human blood by magnetic cell sorting according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The purity of isolated cells was >90% as determined by FACS with an FITC-conjugated anti-human $CD4^+$ mAb and a phycoeritrine (PE)-conjugated anti-human $CD3^+$ mAb (data not shown).

$CD4^+$ cells were stimulated for 5 h in the presence of phorbol 12-myristate 13-acetate (10 ng/ml; Sigma-Aldrich) and phyto-hemagglutinin (5 µg/ml; Remel, Lenexa, KS, USA). Washed $CD4^+$ cells were seeded onto the human differentiated adipocyte monolayer or in the semi-permeable cell culture inserts (0.4 µm porous membrane; Falcon), which physically separate adipocytes (lower chamber) from T lymphocytes (upper chamber). As a control, adipocytes and lymphocytes were maintained alone in their respective chambers. In some experiments, neutralising CD40 (10 µg/ml; R&D Systems) and CD40L (2 µg/ml; R&D systems) antibodies or isotype-matched control antibodies (10 µg/ml; R&D Systems) were incubated for 30 min with adipocytes and $CD4^+$ cells before co-cultures. Culture medium and cells were collected 24 h later. In some experiments, adipocytes and T lymphocytes were cultured in the $CD4^+$ lymphocytes-conditioned medium or in the adipocytes-conditioned medium, respectively. Culture medium and cells were collected 24 h later.

Immunocytochemistry and immunohistochemistry

Cells cultured in Lab-Tek (Nunc) were washed, fixed in 4% paraformaldehyde (wt/vol.) and then incubated with antibodies directed against macrophage HAM56 (Dako, Trappes, France), adiponectin (HADI 773; Alexis Biochemicals, Lausen, Switzerland), adrenomedullin (provided by L'H. Ouafik, Inserm EMI 0359, Marseille, France) [22], CD3 or CD40 (M-20 and T-20, respectively; Santa Cruz Biotechnology). SAT sections were fixed in 4% paraformaldehyde (wt/vol.) and then paraffin-embedded. Deparaffinised sections were incubated with CD3 antibody. After washing, slides were incubated with a biotinylated secondary antibody and with the avidin–peroxidase complex (Vectastain ABC System, Vector Laboratories, Burlingame, CA, USA). The signal was detected using diaminobenzidine. As negative control, sections were incubated with corresponding irrelevant control isotype-matched antibody.

Assays

ELISA kits for the measurements of leptin, IL-6, adiponectin, and monocyte chemoattractant protein-1 (MCP-1)

were from R&D Systems. Plasminogen activator inhibitor-1 (PAI-1) was assayed with an in-house ELISA [23]. Glycerol and total cell proteins were assayed according to the specifications of the non-esterified glycerol reagent kit and of the bicinchoninic acid protein assay kit from Sigma-Aldrich, respectively.

Glucose uptake

Differentiated adipocytes were cultured for 72 h in DMEM with low glucose (5.5 mmol/l) and low insulin (20 pmol/l). They were then incubated in low-glucose DMEM without insulin with rCD40L, or with lymphocyte-conditioned media. After washing, cells were incubated with KRP buffer at 37°C for 20 min in the presence or absence of 100 nmol/l insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). We then added 18.5 kBq/ml of deoxy[2-^{3}H] glucose (PerkinElmer Life Sciences, Boston, MA, USA) and incubated for an additional 20 min at 37°C. The cells were washed with cold KRP buffer and solubilised in 0.1% SDS (wt/vol.). The radioactivity was determined by scintillation counting.

Statistical analysis

Results are expressed as mean±SE. Statistical analysis was performed using the Wilcoxon paired test to compare two paired groups, the Mann–Whitney *t* test to compare two unpaired groups, or ANOVA followed by Fischer's test to compare more than two groups. Pearson's correlation coefficient was used to examine the relations among studied mRNA levels in human adipose tissue. Significance was set at $p<0.05$.

Results

CD40 is expressed by human adipocytes

CD40 mRNA was detected in human adipose tissue. To address the specific cell source, *CD40* gene expression was examined in the AF and SVF isolated from SAT. The *CD40* mRNA level was 17-fold higher in the AF than in the SVF (Fig. 1a, b). By contrast, *PAI-1* was expressed predominantly in the SVF (Fig. 1c), consistent with the fact that it is mainly expressed by adipose tissue macrophages [24]; leptin was expressed 1,000-fold higher in the AF than in the SVF (Fig. 1d) and adiponectin 400-fold higher (Fig. 1e).

We next examined the changes in *CD40* mRNA levels during in vitro adipogenesis. Subcutaneous human preadipocytes were differentiated from day 0 onward. *CD40* mRNA levels, like leptin and adiponectin, increased during adipocyte differentiation (the *CD40* mRNA level increased fivefold between day 0 and day 12, when cells are fully mature) (Fig. 2a, b). The increase in *CD40* mRNA levels during in vitro adipocyte differentiation is in accordance with its mRNA abundance, which was much higher in isolated mature adipocytes than in SVF (see above). In addition, *CD40* mRNA significantly increased upon exposure of differentiated adipocytes to LPS (fold increase: 2.5±0.4; $p<0.01$).

Similarly to mRNA levels, the amount of CD40 protein increased during adipocyte differentiation (Fig. 2c). From day 3 to day 12, CD40, as well as adiponectin, appeared in cells filled with lipid droplets (Fig. 2d–f). By contrast, we detected adrenomedullin, as already reported [25], in non-fat-filled cells (Fig. 2d–f). Taken together, these results indicate that human differentiated adipocytes express the cell-surface molecule CD40.

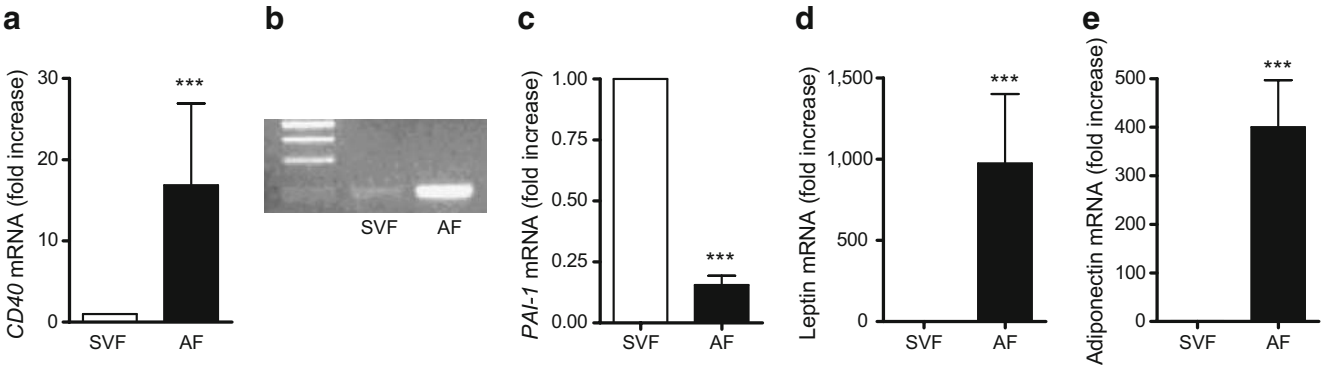

Fig. 1 mRNA levels in human SAT fractions. *CD40* (**a**) (**b** representative electrophoretic pattern of an amplified fragment of *CD40* mRNA), *PAI-1* (**c**), leptin (**d**) and adiponectin (**e**) mRNA levels in the human AF and the human SVF. Data are expressed relative to SVF and are the mean±SEM of six independent SAT from overweight women. ***$p<0.001$ (Mann–Whitney test)

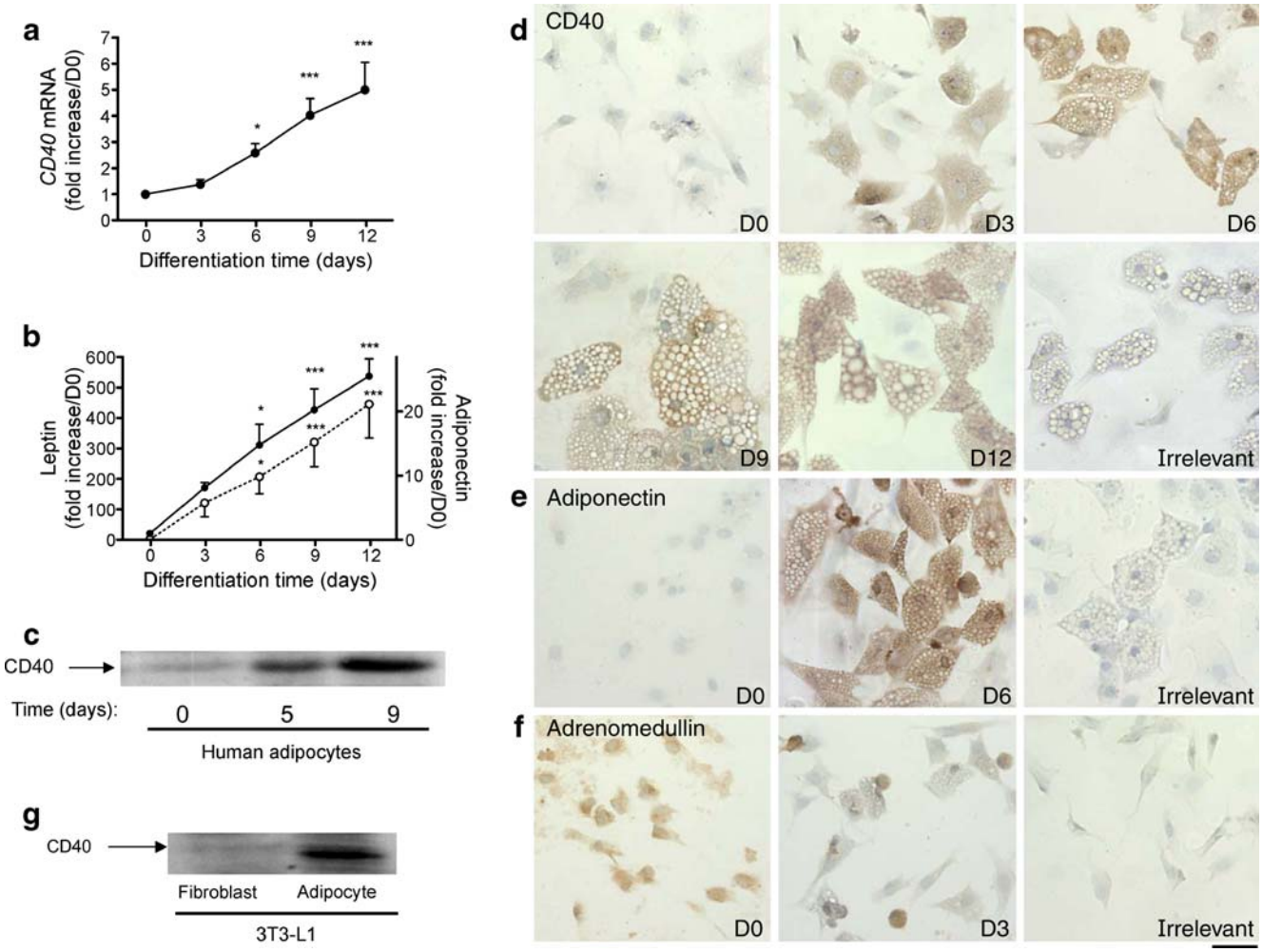

Fig. 2 *CD40* expression during human and murine adipocyte differentiation. *CD40* (**a**), and adiponectin (black circles) and leptin (white circles) (**b**) mRNA levels increased during human adipocyte differentiation. Data are expressed relative to the mRNA levels on day 0 and are the mean±SEM of six independent experiments. *$p<0.05$, ***$p<0.001$ vs day 0 (D0) (ANOVA followed by Fisher's test). CD40 protein detected by Western blot (**c**). Immunocytochemistry in human adipocyte cultures (**d**–**f**): representative brightfield micrographs of CD40 (**d**), with adiponectin used as an adipocyte-specific marker (**e**), and adrenomedullin as a stromal cell marker (**f**). Control sections were incubated with an isotype-matched control IgG (irrelevant). Slides were counterstained with Mayer's hematoxylin. Western blot of CD40 protein during differentiation of murine 3T3-L1 preadipocytes (**g**). Scale bar, 20 μm

CD40 protein is produced by the murine 3T3L1 differentiated adipocytes

Similarly, we observed an increased abundance of CD40 in 3T3-L1 murine adipocytes compared with undifferentiated fibroblasts (Fig. 2g).

rCD40L induces adipocyte changes

rCD40L activates mitogen activated protein kinase signalling pathways and reduces the level of IκB protein Resting human mature adipocytes (between day 10 and day 12) exhibited a constitutive level of phosphorylated-JNK, -p38 and -ERK, and of IκB (Fig. 3a). Addition of rCD40L transiently (within 10–30 min of the addition of rCD40L) increased the phosphorylation of p38, ERK1/2, and JNK1/2 and induced IκB degradation in a time-dependent manner, reaching maximum degradation at 30 min (Fig. 3a). These data indicate that rCD40L activates the mitogen activated protein kinase (MAPK) and IκB/nuclear factor kappa B (NF-κB) signalling in human adipocytes.

rCD40L stimulates the expression of proinflammatory adipocytokines Activation of MAPK and IκB/NF-κB upon CD40 stimulation suggests that CD40 triggers adipocyte responses. We exposed differentiated human adipocytes to rCD40L and monitored the production of IL-6, MCP-1, TNF and PAI-1, which have been shown to be produced by fat cells and non fat-cells, and leptin and adiponectin specifically produced by adipocytes. Compared with con-

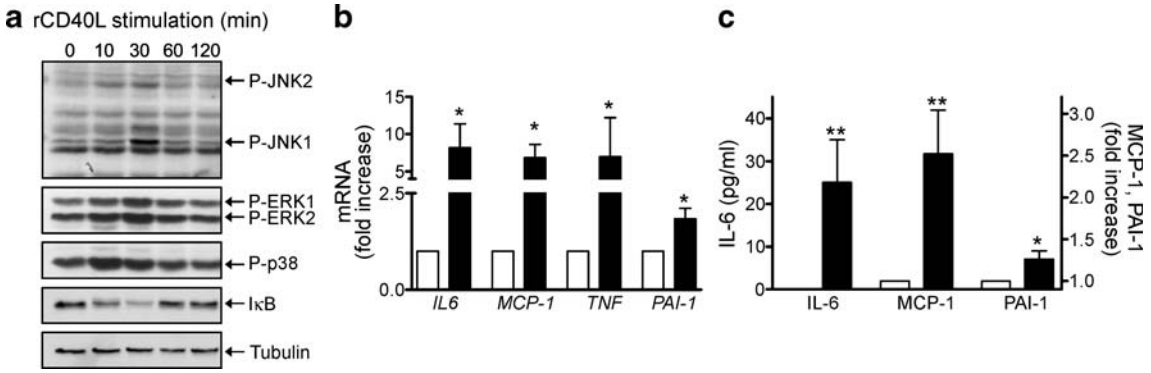

Fig. 3 Effect of rCD40L on MAPKs phosphorylation, IκB degradation and production of proinflammatory markers in human adipocytes. The amount of phosphorylated (P)-JNK, P-ERK, P-p38, IκB and tubulin was assessed by immunoblotting with appropriate antibodies after 2 μg/ml rCD40L stimulation (**a**). IL-6, MCP-1, TNF, and PAI-1 mRNA (**b**) and protein levels (**c**) were evaluated with (black bars) and without (white bars) 2 μg/ml rCD40L after 4 h (**b**) and 18 h (**c**). Results were expressed relative to control without rCD40L. Data are mean±SEM of three and six independent experiments for mRNA and protein, respectively. $^{*}p<0.05$, $^{**}p<0.01$ (Mann–Whitney test)

trols, *IL6*, *MCP-1* (also known as *CCL2*), *TNF* and *PAI-1* (also known as *SERPINE1*) mRNA levels were significantly increased 4 h after addition of rCD40L (Fig. 3b). Accordingly, rCD40L treatment resulted in increased IL-6, MCP-1 and PAI-1 protein secretion after 18 h stimulation (Fig. 3c). Neither leptin nor adiponectin expression and secretion were affected by rCD40L treatment (data not shown).

rCD40L alters insulin action and adipocyte gene expression

Several studies have demonstrated a downregulatory effect of MAPKs and proinflammatory cytokines on insulin action in adipocytes [26]. Since rCD40L induces MAPK signalling activation and pro-inflammatory cytokine production in cultured adipocytes, we investigated whether activation of the CD40 signalling pathway interferes with insulin action. rCD40L markedly reduced glucose uptake at 100 nmol/l insulin after 2 h (data not shown) and 18 h of adipocyte stimulation (Fig. 4a). We next investigated the molecular mechanism behind this inhibitory effect and found that treatment with rCD40L markedly decreased the expression of the mRNAs for *IRS1* and *GLUT4* (also known as *SLC2A4*), whereas expression of *IRS2* and *GLUT1* (also known as *SLC2A*) was not modified (Fig. 4b). Further prolonged incubation with rCD40L markedly reduced the expression of lipogenic genes and increased the release of glycerol into the medium, indicating increased lipolysis (Fig. 4c, d).

The ligand-induced MAPK and IκB/NF-κB signalling activation, the proinflammatory adipocytokine production and the alteration in insulin action presented here demonstrate that CD40L has a functional role in human adipocytes.

CD40L is a type II membrane glycoprotein produced by activated T cells. Quantitative immunohistochemical analysis revealed a significantly increased number of $CD3^{+}$ T lymphocytes in SAT from obese patients compared with lean controls (0.07±0.001 vs 0.03±0.005 number of CD3 positive cells per adipocyte; $p<0.05$). We thus investigated if T lymphocytes provide a signal to adipocytes and if *CD40L* expressed on activated T lymphocytes is involved in the crosstalk between adipocytes and T lymphocytes.

Lymphocyte/adipocyte co-culture alters adipocytokine production

Differentiated adipocytes were cultured for 24 h with resting or activated $CD4^{+}$ lymphocytes. Adipocytokine secretions were compared with stand-alone cultures of each

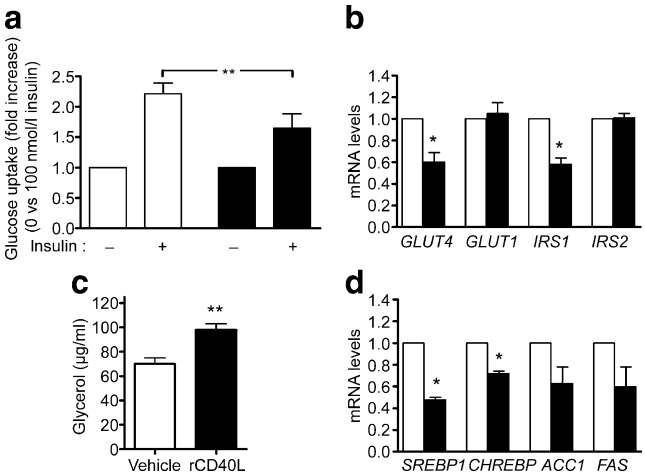

Fig. 4 Effect of rCD40L on human adipocyte biology. Adipocytes were incubated with 2 μg/ml rCD40L (black bars) or vehicle (white bars) for 18 h (**a**, **b**, **d**) or 48 h (**c**). Glucose uptake in response to 100 nmol/l insulin (**a**), mRNA levels of genes involved in insulin signalling (**b**), glycerol release (**c**) and mRNA levels of genes involved in lipogenesis (**d**). mRNA levels were expressed relative to control without rCD40L. Data are mean±SEM of three to seven independent experiments, each performed in duplicate or triplicate. $^{*}p<0.05$, $^{**}p\leq 0.01$ (Mann–Whitney test). *ACC1*, also known as *ACACA*; *CHREBP*, also known as *MLXIPL*

cell type. IL-6 was not detected in the conditioned media of either of the two cell types (Fig. 5a). Adipocytokines such as MCP-1, PAI-1, leptin and adiponectin were found in the adipocyte-conditioned media but not in the lymphocyte-conditioned media (Fig. 5b–e).

Compared with either cell type alone, co-culture of human adipocytes and T lymphocytes significantly increased the production of the proinflammatory adipocytokines IL-6, MCP-1 and PAI-1 (Fig. 5a–c, f–h), but it decreased that of leptin (Fig. 5e, j) and in a less pronounced manner that of adiponectin (Fig. 5d [p=0.057], i). Activated CD4$^+$ T lymphocytes induced more pronounced alterations than did resting CD4$^+$ T cells (Fig. 5a–e). Alterations in IL-6, MCP-1, PAI-1 and leptin secretion depended on the number of activated T lymphocytes (Fig. 6a, b). These results indicate that T lymphocytes alter adipocytokine production.

Lymphocyte-conditioned media alter adipocyte behaviour

To identify the mechanism behind the cooperation between T lymphocytes and adipocytes, we investigated whether secreted factors from resting or activated lymphocytes influenced

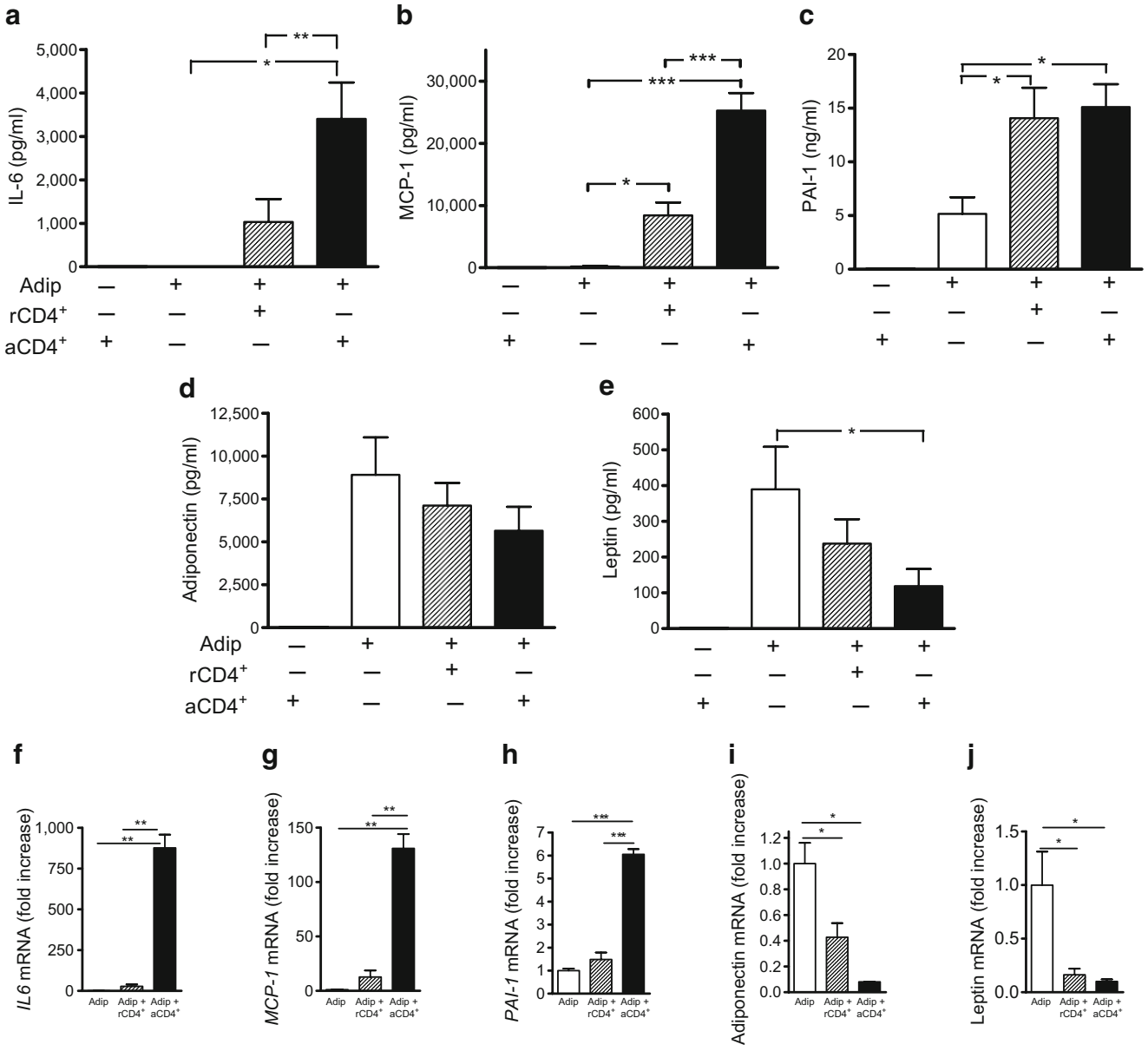

Fig. 5 Modulation of adipokine production by co-culture with T cells. Human T lymphocytes (n=500,000) were seeded onto differentiated adipocytes for 24 h. Levels of IL-6 (**a**), MCP-1 (**b**), PAI-1 (**c**), adiponectin (**d**) and leptin (**e**) secretions. Data are mean±SEM of four independent experiments, each performed in triplicate. **f**–**j** Adipocytokin mRNA levels of one representative experiment. aCD4$^+$, activated CD4$^+$; Adip, adipocytes; rCD4$^+$, resting CD4$^+$

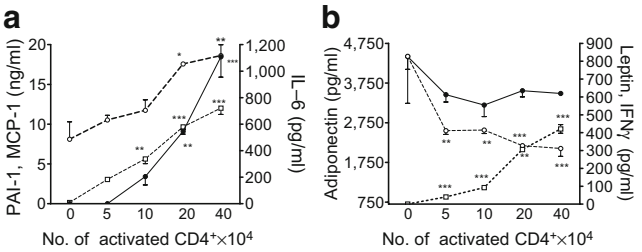

Fig. 6 PAI-1 (white circle), MCP-1 (square) IL-6 (black circle) (**a**), leptin (white circle), adiponectin (black circle) and IFNγ (square) (**b**) secretions in adipocyte/lymphocyte co-culture according to concentration of $CD4^+$ cells. Data are mean±SEM of one experiment performed in triplicate. *$p<0.05$, *$p<0.01$, ***$p<0.001$ (ANOVA followed by Fisher's test)

adipocytokine secretion. Media (conditioned for 24 h) from resting or activated human T lymphocytes were added to human adipocytes, and adipocytokine secretion was assessed. Table 1 shows that addition of medium conditioned from activated T lymphocytes significantly increased MCP-1 and IL-6 levels. By contrast, adipocytes-conditioned media did not induce the production of these cytokines by T lymphocytes (data not shown), underlining the fact that the cytokines are exclusively produced by adipocytes.

In addition, activated lymphocytes-conditioned media prevented insulin-stimulated glucose uptake (0 vs 100 nmol/l insulin: 1278±56 vs 1491±132 cpm; NS) compared with resting lymphocytes-conditioned media (0 vs 100 nmol/l insulin: 1067±85 vs 1801±156 cpm; $p=0.01$), indicating that diffusible factors from activated T lymphocytes play a role in the modification of insulin sensitivity.

Both lymphocyte-secreted factors and cell–cell contact are involved in the alteration of adipocytokine secretion

The involvement of soluble factor(s) in the crosstalk between adipocytes and lymphocytes does not rule out the possibility that direct contact between the two cell types may be involved. Such proximity, already reported in adipose tissue [15], was observed in co-culture experiments (Fig. 7a) between lymphocyte and mature but undifferentiated adipocytes, and no dissociation was seen after washing. To investigate if the contact between the two cell types actually regulates adipocytokine production, co-cultures were made with or without permeable cell culture inserts, which physically separate lymphocytes from adipocytes but allow the diffusion of secreted factors. When the cell–cell contact was impeded, T lymphocytes still stimulated adipocytokine production, confirming the involvement of diffusible factor(s) derived from T lymphocytes. Adipocytokine mRNAs were detected in the adipocytes but not in the activated lymphocytes (data not shown).

Interestingly, the physical contact between adipocytes and T lymphocytes significantly increased the rises in IL-6, MCP-1 and PAI-1 production (Fig. 7b–d), but not those of adiponectin and leptin (Fig. 7e, f), suggesting the heterotypic cell contact is involved for the former (IL-6, MCP-1 and PAI-1) but not the latter (adiponectin and leptin) secretions. Similar profiles were obtained when measuring mRNA levels (Fig. 7g, k). When lymphocytes were physically separated from adipocytes (separation condition), targeted mRNAs were only detected in the adipocyte monolayer (Fig. 7g, k). Indeed mRNA expression data demonstrated that all targeted mRNA levels were dramatically higher in adipocytes than in activated lymphocytes ($IL6^{adip}$ ΔC_t 8.6±0.6, $IL6^{lympho}$ ΔC_t 17±0.83, $p<0.001$; $MCP\text{-}1^{adip}$ ΔC_t 5.7±0.56, $MCP\text{-}1^{lympho}$ ΔC_t 16±0.21, $p<0.001$; $PAI\text{-}1^{adip}$ ΔC_t 7.3±0.61, $PAI\text{-}1^{lympho}$ ΔC_t 20±1.6, $p<0.005$; $adiponectin^{adip}$ ΔC_t 9.7±0.72, $adiponectin^{lympho}$ ΔC_t 23±1.1, $p<0.001$; $leptin^{adip}$ ΔC_t 14±1.1, $leptin^{lympho}$ ΔC_t 27±0.64, $p=0.005$).

Together, these data suggest that T lymphocytes regulate adipocytokine production through both the release of soluble factor(s) and direct heterotypic contact with adipocytes.

Table 1 Modulation of adipocytokine secretion upon stimulation by lymphocyte-conditioned media

	Adipocytes	Adipocytes + CM from resting $CD4^+$	Adipocytes + CM from activated $CD4^+$	*p* value (one-way ANOVA)
IL-6 (pg/ml)	ND	30±14 (5–53)	109±14 (80–155)*†	0.01
MCP-1 (pg/ml)	102±26 (76–155)	856±82 (738–1,014)	2,785±1,268 (1,368–5,314)*	0.04
PAI-1 (ng/ml)	3.1±0.7 (1.6–4)	4.6±1.7 (2.8–8.1)	5.7±0.8 (4.4–7.2)	0.3
Adiponectin (pg/ml)	2,708±670 (1,883–4,034)	2,158±97 (2,019–2,346)	1,787±156 (1,473–1,948)	0.3
Leptin (pg/ml)	322±48 (249–414)	197±80 (104–357)	165±85 (50–332)	0.3

Data are mean±SE (range) of four independent experiments, each performed in triplicate
Adipocytes were incubated for 24 h with 2 ml of a 24 h-conditioned media of 150,000 resting (CM resting $CD4^+$) or activated (CM activated $CD4^+$) $CD4^+$ T lymphocytes
*$p<0.05$ for control adipocytes compared with adipocytes + CM from activated $CD4^+$
†$p<0.05$ for adipocytes + CM from resting $CD4^+$ compared with adipocytes + CM from activated $CD4^+$
ND, not detected

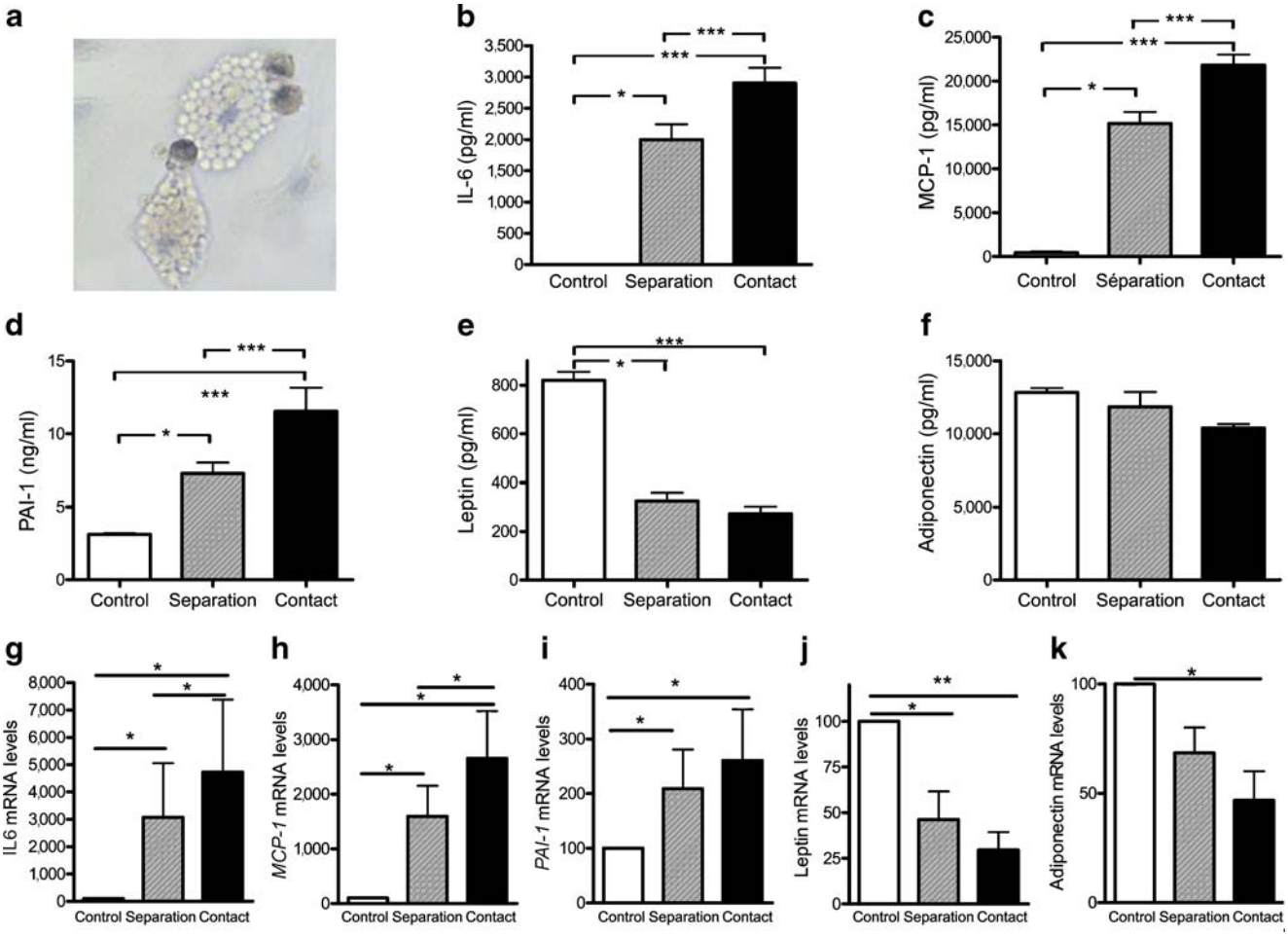

Fig. 7 Modulation of adipokine secretion by T cell diffusible factor(s) or by cell–cell contact. Human adipocytes were cultured without (white bars) or with human activated T lymphocytes ($aCD4^+$) (n= 150,000) (black bars) for 24 h. Brightfield micrograph of $CD3^+$ T lymphocyte immunocytochemistry in adipocyte/lymphocyte co-cultures showing the contact between mature adipocytes and lymphocytes after washing (scale bar, 10 μm) (**a**). $aCD4^+$ cells were seeded onto the adipocyte layer (contact) or in the cell culture insert (separation). Levels of IL-6 (**b**), MCP-1 (**c**), PAI-1 (**d**), leptin (**e**) and adiponectin (**f**) secreted in culture medium. **g**–**k** Adipokine mRNA levels. Results were expressed relative to control condition. Data are mean±SEM of four independent experiments, each performed in triplicate. *p<0.05, ***p<0.001 (ANOVA followed by Fisher's test)

Lymphocyte-induced IL-6 synthesis is partially blocked by anti-CD40/CD40L antibodies

We next investigated whether CD40L borne by activated T cells is involved in the effects mediated by cell–cell contact. For this purpose, co-cultures of adipocytes and activated T lymphocytes were established in the presence of blocking antibodies directed against CD40 and CD40L. Remarkably, addition of antibodies to co-cultures reduced the increase in IL-6 secretion (−23±4.7%, p=0.01) by the same order of magnitude than the stimulation of IL-6 release induced by the cell–cell contact (increase cell–cell contact vs separation: +30±14.6%, p=0.01) (Fig. 7b). This result indicates that the CD40 pathway is involved in lymphocyte-induced adipocyte IL-6 secretion.

CD40, leptin and *IL6* mRNA levels in adipose tissue of obese humans

SAT from obese humans had higher *CD40* and leptin mRNA levels than VAT (Fig. 8a). In both tissues, mRNA levels of *CD40* were correlated with those of *IL6* (SAT: r= 0.53, p<0.05; VAT: r=0.44, p<0.05). In SAT, mRNA levels of *CD40* were positively correlated with BMI and leptin mRNA levels (Fig. 8b, c).

Discussion

Several lines of evidence indicate that the interaction of CD40 and its counterpart CD40L plays a crucial role in the

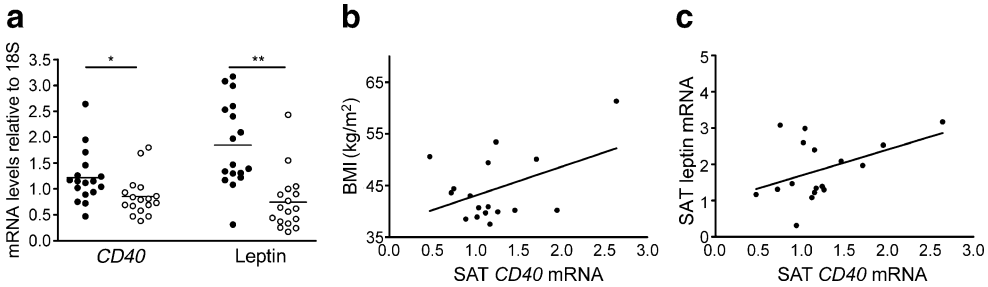

Fig. 8 CD40, leptin and IL6 mRNA levels in human AT. mRNA levels of CD40 and leptin in SAT (white circles) and VAT (black circles) from obese individuals (n=17) (**a**). **p< 0.01, ***p<0.001 (Wilcoxon paired test). Correlation (Pearson) of *CD40* mRNA with BMI and leptin mRNA in SAT (**b**, **c**)

onset and maintenance of the inflammatory reaction [14, 27]. However, to date there is no information on CD40 signalling pathways in adipocytes as potential inflammatory triggers. The present study showed that: (1) human mature adipocytes express *CD40*; (2) rCD40L activates the MAPK and IκB/NF-κB pathway, increases proinflammatory adipocytokine production, reduces insulin signalling, and modifies lipolysis and lipogenesis; and (3) T lymphocytes alter adipocyte function by both paracrine and cell–cell contact mediated mechanisms involving the CD40/CD40L pathway. These data suggest that CD40 is a new marker of adipocyte differentiation and that soluble or membrane-anchored CD40L contributes to the chronic inflammatory state and insulin resistance observed in adipose tissue during obesity.

We have shown that human adipocytes synthesise CD40 and that its expression is increased during differentiation. Interestingly, CD40 was found only in lipid-full cells, and immunoblotting analyses revealed that the CD40 content was highest at the late stages of differentiation, as was also the case for leptin. In agreement, *CD40* mRNA levels in SAT increased with BMI and leptin mRNA level in a population of obese individuals. Like leptin [28, 29], the *CD40* mRNA level was 45% higher in SAT than in VAT. This could be due to larger fat cells in SAT than VAT [30], in line with the notion that leptin production is correlated with the degree of adipocyte development [31]. Overall these data showed that *CD40* expression by the adipocyte is mainly restricted to the late stage of adipocyte differentiation.

Activation of CD40 is associated with a broad range of biological effects depending on the cell type and the nature of the stimulus. Activation of the CD40 pathway in non-immune cells leads to production of proinflammatory cytokines, chemokines and matrix metalloproteinases, and modulates apoptosis [32]. In agreement with this, addition of purified rCD40L to human adipocytes increased pro-inflammatory adipocytokine production. Whether or not stimulation of CD40 is involved in adipocyte differentiation or apoptosis deserves to be studied. Earlier studies showed that CD40 signalling results in the activation of the MAPK/NF-κB pathways in monocytes/macrophages [33–35], thereby stimulating CD40-mediated induction of inflammatory cytokine synthesis. We found that in human adipocytes rCD40L treatment activated MAPKs and induced IκB protein degradation, which is known to activate and promote nuclear translocation of NF-κB, suggesting that increases in sCD40L or T-lymphocyte-expressing CD40L may play a role in the elevated activities of these factors in obesity. CD40 lacks intrinsic kinase activity in the cytoplasmic domain but activates NF-κB transcription factor through association with TNF-receptor associated factors (TRAF), a family of adaptator proteins [36]. CD40 can bind five of the six TRAF family members (TRAF1, −2, −3, −5, −6) depending on cell type or function [37]. Interestingly, *TRAF6* is expressed in human adipocytes [38] and may participate to CD40 signalling in adipocytes.

MAPK/NF-κB activation could be involved in the inhibitory effect of CD40L on insulin-induced glucose transport, as recently demonstrated in IL-1β-stimulated 3T3-L1 adipocytes [20]. In agreement with this, we found that decreased expression of insulin signalling and lipogenesis-related genes after rCD40L stimulation was associated with a decrease in insulin-stimulated glucose uptake and an increase in lipolysis. The relevance of our in vitro studies to the clinical setting is supported by studies showing a positive relationship between sCD40L and insulin resistance [5, 11].

It is also important to determine the source of CD40L in the adipose tissue microenvironment. In accordance with recent data [17, 18], we found an increase in $CD3^+$ T cells in SAT from obese women compared with lean women.

Adipocytokine secretions were modulated by lymphocyte products. It is noteworthy that leptin secretion was downregulated. This result agrees with that of Simons et al. [39], who reported that, conversely to other proinflammatory cytokines, leptin secretion is reduced after stimulation with IFNγ, which is produced in high amounts by activated T lymphocytes. Furthermore, the conditioned media from activated lymphocytes reduced insulin responsiveness in adipocytes, probably due to altered adipocytokines and specific products of T lymphocytes.

Our results also suggest that direct cell–cell contact between T lymphocytes and adipocytes plays a role in the activation of adipocytes. We performed co-cultures by mixing $CD4^+$ T cells with mature human adipocytes. Cell contact induced higher levels of secretion of IL-6, MCP-1 and PAI-1. Any participation of an allogenic response was ruled out since undifferentiated cells isolated from adipose tissue lack the major histocompatibility complex-II [40]. In

addition we used fully activated $CD4^+$ T cells maintained in serum-free medium that is not adequate for growth of T cells. To evaluate the participation of the CD40/CD40L system we used blocking antibodies. Because CD40L may bind to integrins we used a combination of antibodies directed against both CD40 and CD40L. Blocking antibodies decreased IL6 production by the same order of magnitude as the stimulation of IL6 release induced by the cell–cell contact, indicating that this system is involved in the inflammatory response induced by T cells. Finally, the correlation of CD40 with IL-6 in SAT and VAT from obese individuals provides additional evidence for the association of CD40 and inflammation in obesity.

In conclusion, this is, to our knowledge, the first study demonstrating the presence of CD40 on human mature adipocytes, a relationship between *CD40* mRNA in SAT and BMI or leptin, and a cooperation between human T lymphocytes and adipocytes in which the CD40 pathway plays a determinant role through the regulation of adipocytokine, mainly IL-6 secretion. These data will help elucidate the mechanisms underlying the inflammatory changes in obese adipose tissue and identify therapeutic targets that may reduce obesity-related vascular comorbidities.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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