

# Regulation of tumour necrosis factor- $\alpha$ release from human adipose tissue *in vitro*

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## Abstract

Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), secreted by cells of the macrophage-monocyte lineage, has a well established role in inflammation and host-defence. The more recent discovery that adipocytes also secrete TNF- $\alpha$  has led to a substantial body of research implicating this molecule in the insulin resistance of obesity. However, little is known about the normal regulation of TNF- $\alpha$  release from human adipose tissue. In particular, it is not known whether adipocyte production of TNF- $\alpha$  is responsive to similar or different molecular regulators than those relevant to macrophages. TNF- $\alpha$  release from cultured human adipose tissue and isolated adipocytes was examined using an ELISA. Insulin, cortisol or the thiazolidinedione, BRL 49653, did not have a significant effect on TNF- $\alpha$  release from adipose tissue or isolated adipocytes. In contrast, lipopolysaccharide (LPS), a major stimulus of TNF- $\alpha$  protein production in monocytes and macrophages, resulted in a fivefold stimulation of TNF- $\alpha$  release from human adipose tissue. Significant stimulation of TNF- $\alpha$  release was also seen from isolated adipocytes, indicating that the increase in TNF- $\alpha$  release from adipose

tissue in the presence of LPS is unlikely to be entirely attributable to contaminating monocytes or macrophages. Consistent with this observation was the finding that mRNA for CD14, a known cellular receptor for LPS, is expressed in human adipocytes. The increase in TNF- $\alpha$  protein release in response to LPS was blocked by an inhibitor of the matrix metalloproteinase responsible for the cleavage of the membrane-bound proform of TNF- $\alpha$ , indicating that this release represented regulated secretion and was not due to cell lysis.

In conclusion, the regulation of TNF- $\alpha$  protein release from human adipose tissue and isolated adipocytes appears to be similar to its regulation in cell types more traditionally implicated in host defence. The production by the adipocyte of a range of molecules involved in host defence – TNF- $\alpha$ , factors D, B and C3, interleukin-6, and macrophage colony-stimulating factor – suggest that this cell type may make a significant contribution to innate immunity.

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## Introduction

Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine secreted by several cell types such as monocytes (Waage & Bakke 1988), macrophages (Mijatovic *et al.* 1997) and adipocytes (Hotamisligil *et al.* 1995). It is produced as a 26 kDa active membrane-bound precursor that is proteolytically cleaved by a matrix metalloproteinase (TNF- $\alpha$  converting enzyme (TACE)) to release a 17 kDa soluble form (Gearing *et al.* 1994). Its expression and production in macrophages have been shown to be increased on exposure to endotoxins such as lipopolysaccharide (LPS) (Mijatovic *et al.* 1997), a component of the cell wall of Gram-negative bacteria. TNF- $\alpha$  has an important role as a mediator of the acute phase response and, in addition to an immunological function, it has been shown to have a variety of effects on lipid metabolism and adipocyte function. These include the stimulation of lipolysis through increases in hormone-

sensitive lipase expression (Sumida *et al.* 1997) and inhibition of lipoprotein lipase (Fried & Zechner 1989), induction of preadipocyte dedifferentiation (decreasing CCAAT/enhancer binding protein (Stephens & Pekala 1991) and peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) (Zhang *et al.* 1996)), promotion of adipocyte apoptosis (Prins *et al.* 1997) and induction of insulin resistance (decreasing GLUT4 (Hauner *et al.* 1995) and insulin-stimulated autophosphorylation of insulin receptor and phosphorylation of insulin receptor substrate-1 (Feinstein *et al.* 1993)). Expression of TNF- $\alpha$  in adipose tissue has been shown to be increased in rodent and human obesity and to correlate with insulin resistance (Hotamisligil *et al.* 1995, Kern *et al.* 1995). As a result of these observations, the paracrine effects of TNF- $\alpha$  released by adipocytes have been proposed to underlie the link between obesity and insulin resistance (Hotamisligil *et al.* 1993). Despite the potential pathogenic importance of adipocyte TNF- $\alpha$  in obesity remarkably little is known

about the normal role of TNF- $\alpha$  in human adipose tissue. We reasoned that a better definition of the molecular regulators of TNF- $\alpha$  in human adipose tissue might provide clues to the function of this cytokine in normal human fat cell biology.

## Materials and Methods

### *Acquisition of human tissue*

Omental and subcutaneous adipose tissue biopsies (approximately 3 g) were obtained from patients undergoing elective open surgery. None of the patients had diabetes or severe systemic illness. Cambridge Local Research Ethics Committee approval was obtained, and all patients gave their informed consent. The tissue donor group consisted of 11 women (ages  $49.2 \pm 15.2$  years; body mass index (BMI)  $25.9 \pm 4.3$  kg/m<sup>2</sup>) and five men (ages  $71.2 \pm 8.8$  years; BMI  $26 \pm 4.5$  kg/m<sup>2</sup>).

### *Adipose tissue culture*

Whole adipose tissue was separated from surrounding blood vessels, diced finely and washed twice in Hanks' balanced salt solution (HBSS) containing 0.2% BSA. Small pieces of tissue were then placed in sterile tissue culture plates containing pre-equilibrated medium 199 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 33  $\mu$ M biotin, 17  $\mu$ M pantothenic acid. Different test compounds were also added to the medium: human insulin (7  $\mu$ M), cortisol (1 mg/ml), LPS (50  $\mu$ g/ml), a thiazolidinedione BRL 49653 ( $10^{-7}$  M) (from SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK), a metalloproteinase inhibitor BB3103 (10  $\mu$ M) (from British Biotechnology, Oxford, UK) and dimethyl sulphoxide. The plates were then incubated under standard conditions (37 °C/5% CO<sub>2</sub>). After 20 h, medium was collected from each well and frozen at -80 °C before measurement of TNF- $\alpha$  protein levels by ELISA. The tissue samples were collected from each well, blotted onto filter paper to remove excess medium, and weighed. TNF- $\alpha$  levels were expressed as pg TNF $\alpha$  per mg adipose tissue per hour and fold increase or decrease relative to the control (medium only). Unless specifically indicated, all reagents were obtained from Sigma Chemical Company, Poole, Dorset, UK.

### *Adipocyte isolation and culture*

Adipose tissue biopsies were taken under sterile conditions at the time of surgery and placed in normal saline. The transport time to the laboratory was less than 1 h. The tissue was diced finely into 1–2 mm pieces. All instruments were sterile disposable or autoclaved. The diced tissue was digested in a collagenase solution containing

HBSS, 3 mg/ml collagenase (type II) and 1.5% BSA. At least three volumes more digest solution than the volume of tissue was used. The digestion was carried out in a shaking waterbath at 37 °C for approximately 1 h. After filtration through a 260  $\mu$ m steel mesh, the adipocytes were allowed to float to the surface and collected using a pasteur pipette. After two washes in HBSS, aliquots of adipocytes were dispensed into tissue culture plates containing pre-equilibrated 199 medium and test compounds (see adipose tissue culture). After 20 h, infranatants were harvested from beneath the adipocyte layer and frozen at -80 °C before measurement of TNF- $\alpha$  by ELISA. Results are expressed as pg TNF $\alpha$  per  $\mu$ l adipocyte suspension per hour or percentage increase or decrease relative to the control (medium alone).

### *TNF- $\alpha$ protein measurement*

TNF- $\alpha$  levels were determined in culture medium using a commercially available ELISA kit (Genzyme Diagnostics, Cambridge, MA, USA). The manufacturer's procedure for enhanced sensitivity was followed. The assay range was 4–512 pg/ml, with a limit of sensitivity of 500 fg/ml. The intra-assay coefficient of variation was 9.67%, and the inter-assay coefficient of variation was 12.47%.

### *Detection of CD14 mRNA by reverse transcription-polymerase chain reaction*

RT-PCR was carried out on RNA extracted from human adipocytes, preadipocytes and monocyte cells. Adipocyte and preadipocyte cells were isolated by collagenase digestion of whole adipose tissue as described above. Monocyte cells were isolated from whole blood using HISTOPAQUE-1077 from Sigma. The manufacturer's procedure was followed. RNA was extracted using a guanidium thiocyanate-phenol technique (Tri Reagent, Sigma). RNA samples were treated with RNase inhibitor and DNase and quantified by spectrophotometry. The integrity of RNA samples was assessed by agarose gel electrophoresis and ethidium bromide staining. The reverse transcription reaction was performed in a 20  $\mu$ l volume containing 5 mM MgCl<sub>2</sub>, 1  $\times$  reverse transcriptase buffer (Promega, Cambridge, UK), 1 mM dNTPs (Pharmacia Biotech, St Albans, Herts, UK), 10 ng random hexamers (Promega). The reactions were incubated at 65 °C for 5 min to remove any residual DNAase A activity and allowed to cool to room temperature. One microlitre Moloney murine leukemia virus reverse transcriptase (Promega) was added to the reactions (except the control) and incubated at 37 °C for 1 h. The control reaction, containing no reverse transcriptase, allowed for the detection of any possible genomic DNA contamination in the RNA samples after PCR. A further control containing all reagents except RNA was also included to

allow the detection of any possible DNA/RNA contamination of reagents. PCR was then performed on 200 ng first-strand cDNA in a 50  $\mu$ l volume containing 1 mM MgCl<sub>2</sub>, 0.2  $\mu$ M each of CD14 sense (5'-GGAAAGAAGCTAAAGCACTT-3') and CD14 anti-sense (5'-TTTAGAAACGGCTCTAGGTT-3') primers, 0.15 mM dNTPs, 1  $\times$  NH<sub>4</sub> reaction buffer, 2.5 units Advantage 2 Taq polymerase (Clontech, Palo Alto, CA, USA). Cycling parameters were: 94 °C for 1 min, 50 °C for 1 min, 68 °C for 1 min (30 cycles). PCR products were confirmed as CD14 by nucleotide sequencing. All sequencing was carried out using Dideoxy terminator chemistry (Perkin-Elmer, Foster City, CA, USA) and electrophoresed on an ABI 377 automated DNA sequencer.

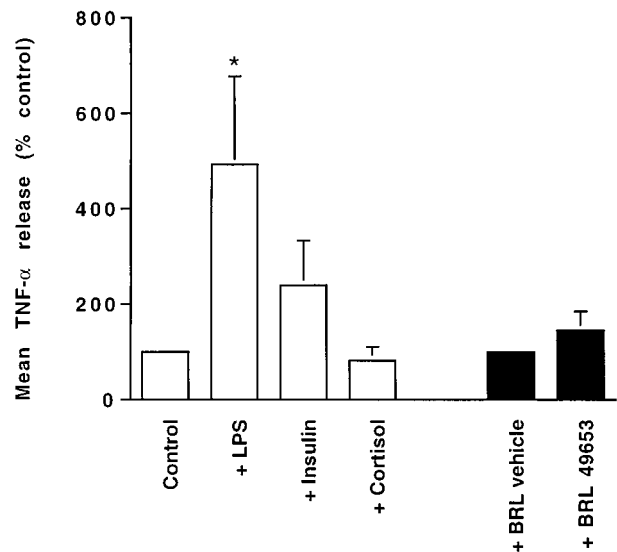
### Statistical analysis

A paired Wilcoxon non-parametric test was used for statistical analysis. Results are expressed as means  $\pm$  s.e.m.

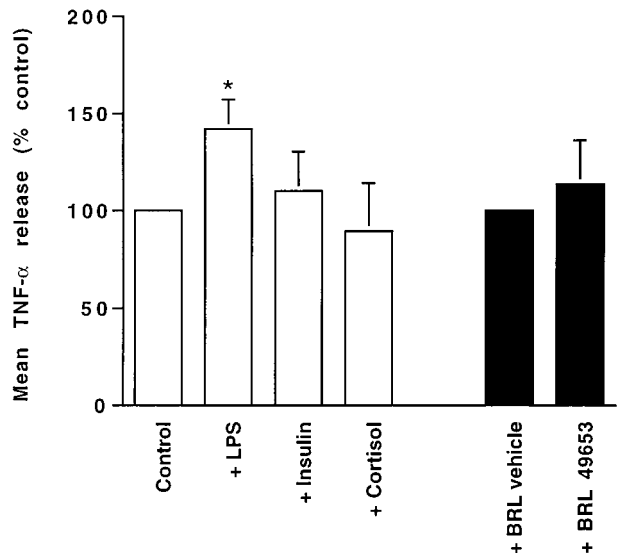
## Results

Mean release of TNF- $\alpha$  from whole adipose tissue and isolated adipocytes in culture medium was 0.068  $\pm$  0.02 pg/mg adipose tissue per hour and 0.017  $\pm$  0.01 pg/ $\mu$ l adipocyte suspension per hour respectively. Stability of TNF- $\alpha$  protein in the medium was confirmed by spiking medium containing whole adipose tissue with known amounts of TNF- $\alpha$  and quantitating TNF- $\alpha$  protein concentrations after a 20-h incubation relative to an unspiked control (data not shown). There was no correlation between TNF- $\alpha$  protein release and BMI or age and no consistent gender differences were seen.

Exposure to insulin (7  $\mu$ M), cortisol (1 mg/ml) or BRL 49653 (10<sup>-7</sup> M) had no significant effect on TNF- $\alpha$  release from either whole adipose tissue (Fig. 1) or isolated adipocytes (Fig. 2). In contrast, exposure to LPS resulted in a mean fivefold ( $\pm$  1.85 s.e.m.) increase in TNF- $\alpha$  release from adipose tissue after 20 h (relative to controls) ( $P=0.0134$ ). Figure 3 shows that BB3103 (10  $\mu$ M), a matrix metalloproteinase inhibitor, was able to inhibit LPS-stimulated TNF- $\alpha$  release from adipose tissue over the 20-h period by 90  $\pm$  3.8% ( $P=0.002$ ). BB3103 (10  $\mu$ M) inhibited basal (unstimulated) TNF- $\alpha$  release from adipose tissue by 30  $\pm$  17.4% ( $P=NS$ ). The inhibitory effect of BB3103 suggests that the increase in release of TNF- $\alpha$  from adipose tissue exposed to LPS is due to a *bona fide* effect of LPS and not due to cell lysis. LPS also had a stimulatory effect on TNF- $\alpha$  release from isolated adipocytes (Fig. 2), although to a lesser extent compared with whole adipose tissue (1.5  $\pm$  0.15-fold relative to control;  $P=0.0469$ ). In the presence of 10  $\mu$ M BB3103, this increase was inhibited by 70  $\pm$  13.75% ( $P=0.03$ )

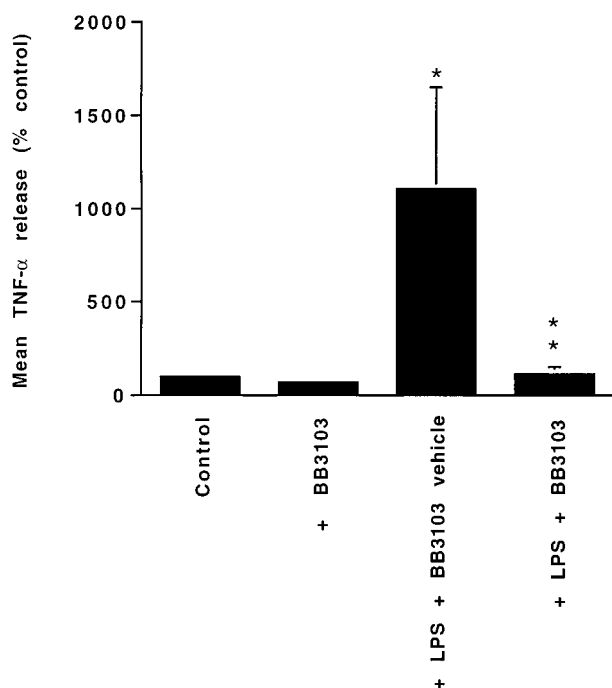


**Figure 1** TNF- $\alpha$  release into the incubation medium from human adipose tissue after a 20-h incubation period in medium alone (Control) or medium containing 50  $\mu$ g/ml LPS ( $n=14$ ), 7  $\mu$ M insulin ( $n=11$ ), 1 mg/ml cortisol ( $n=11$ ), or 10<sup>-7</sup>M BRL 49653 ( $n=11$ ). In each experiment, samples were incubated in duplicate. Data are expressed as means  $\pm$  s.e.m. \* $P=0.0314$  compared with Control.

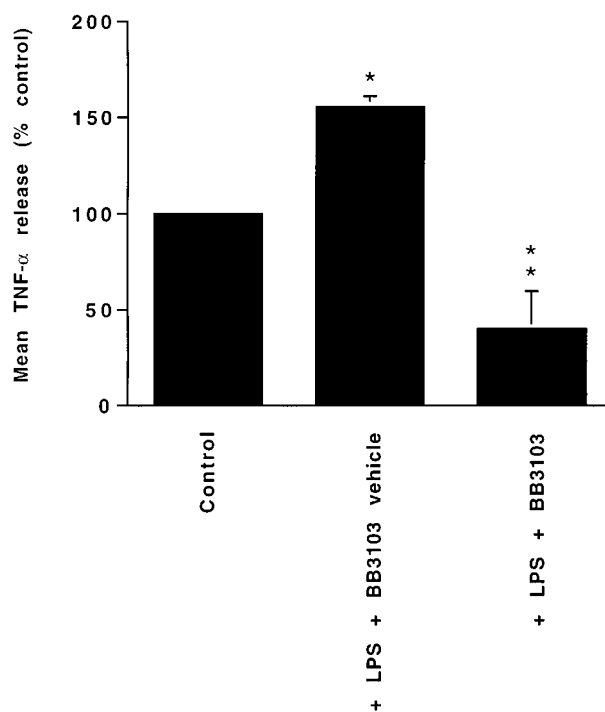


**Figure 2** TNF- $\alpha$  release into the incubation medium from isolated human adipocytes after a 20-h incubation period in medium alone (Control) or medium containing 50  $\mu$ g/ml LPS ( $n=7$ ), 7  $\mu$ M insulin ( $n=3$ ), 1 mg/ml cortisol ( $n=3$ ), or 10<sup>-7</sup>M BRL 49653 ( $n=4$ ). In each experiment, samples were incubated in duplicate. Data are expressed as means  $\pm$  s.e.m. \* $P=0.0469$  compared with Control.

(Fig. 4). Under identical conditions, exposure of human monocyte-macrophages to LPS resulted in a 50-fold increase in TNF- $\alpha$  release relative to unstimulated controls ( $P<0.0001$  data not shown).



**Figure 3** TNF- $\alpha$  release from human adipose tissue after a 20-h incubation in medium alone (Control), medium plus BB3103 (10  $\mu$ M), medium plus LPS (50  $\mu$ g/ml) plus BB3103 vehicle, or medium containing LPS (50  $\mu$ g/ml) plus 10  $\mu$ M BB3103 ( $n=10$ ). In each experiment, samples were incubated in duplicate. Data are expressed as means  $\pm$  s.e.m. \* $P=0.0078$ , LPS+BB3103 vehicle compared with Control; \*\* $P=0.002$ , LPS+BB3103 vehicle compared with LPS+BB3103.



**Figure 4** TNF- $\alpha$  release from isolated human adipocytes after a 20-h incubation in medium alone (Control), medium plus LPS (50  $\mu$ g/ml) plus BB3103 vehicle, or medium containing LPS (50  $\mu$ g/ml) plus 10  $\mu$ M BB3103 ( $n=2$ ). In each experiment, samples were incubated in duplicate. Data are expressed as means  $\pm$  s.e.m. \* $P=0.0497$ , LPS+BB3103 vehicle compared with Control; \*\* $P=0.03$ , LPS+BB3103 vehicle compared with LPS+BB3103.

Previous reports on the study of LPS induction of TNF- $\alpha$  protein production in monocytes and macrophages have shown that this is mediated by CD14, a cell-surface receptor for LPS. CD14 mRNA was detected by RT-PCR in isolated adipocytes, preadipocytes and monocytes (Fig. 5).

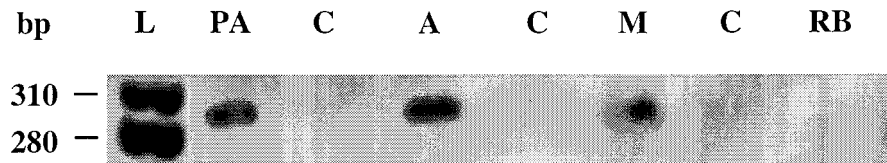
## Discussion

Although a large body of research on TNF- $\alpha$  action in metabolically important tissue such as muscle and fat has recently emerged, little information has been forthcoming on the regulation of TNF- $\alpha$  release from adipose tissue, and even less on TNF- $\alpha$  release from human adipose tissue. In this study, we have demonstrated that TNF- $\alpha$  release from human adipose tissue is responsive to LPS but not to insulin, cortisol or a thiazolidinedione. LPS also stimulated TNF- $\alpha$  release from isolated adipocytes, albeit less robustly than in whole adipose tissue. The LPS-stimulated release of TNF- $\alpha$  was prevented by an inhibitor of the metalloproteinase responsible for the cleavage of the membrane-bound form of TNF. Finally, adipocytes were

found to express mRNA encoding CD14, a known receptor for LPS.

As in unstimulated monocytes and macrophages (Crawford *et al.* 1997), TNF- $\alpha$  release from human adipose tissue and isolated adipocytes under control conditions occurred at low levels. This is consistent with our previously published data concerning TNF- $\alpha$  mRNA expression which indicated that this gene is expressed at very low abundance in human adipocytes (Montague *et al.* 1998). Interestingly, we were unable to demonstrate any correlation between adipose tissue TNF- $\alpha$  release and the BMI of the subjects studied. This is in contrast to previous studies which demonstrated a 2.5-fold increase in fat tissue TNF- $\alpha$  expression in obese (BMI=39.9  $\pm$  1.4 kg/m<sup>2</sup>) compared with lean (BMI=21.4  $\pm$  0.3 kg/m<sup>2</sup>) individuals (Hotamisilgil *et al.* 1995). The narrow range of BMIs in the present study (20–30 kg/m<sup>2</sup>) would not have allowed us to detect an effect of marked obesity. This is consistent with our previously published data concerning TNF- $\alpha$  mRNA expression in adipocytes from a study population with a similar range of BMI values (Montague *et al.* 1998).

Circulating TNF- $\alpha$  protein concentrations have been reported to correlate with hyperinsulinaemia in humans



**Figure 5** Detection of CD14 mRNA by RT-PCR in isolated adipocytes (A), monocytes (M) and preadipocytes (PA). C, RT-PCR reactions in which reverse transcriptase enzyme was omitted; L, 1 kb ladder; RB, RT-PCR amplification mixture without template as a negative control.

(Hotamisligil *et al.* 1995). In addition, insulin has been reported to have a stimulatory effect on TNF- $\alpha$  release from monocyte and macrophages (Gepner-Atlan *et al.* 1996, Hahn & Filkins 1993). In these studies, however, insulin did not have any significant effect on release of TNF- $\alpha$  protein from either adipose tissue or isolated adipocytes. Cortisol has previously been shown to markedly suppress the endotoxin-induced increase in TNF- $\alpha$  protein production in human macrophages (Beutler *et al.* 1986). No significant suppression of adipocyte TNF- $\alpha$  by cortisol was seen in this study, although there was a trend towards a reduction. The low basal levels of TNF- $\alpha$  release may have made an inhibitory effect difficult to detect, and future studies should consider the effects of cortisol on LPS-induced secretion. Thiazolidinedione compounds are agonists at the PPAR $\gamma$  receptor (Berger *et al.* 1996), a nuclear hormone receptor highly expressed in fat (Chawla *et al.* 1994). They act as insulin sensitizers (Saltiel & Olefsky 1996) and, in obese rodents treated *in vivo* have been demonstrated to reduce adipocyte TNF- $\alpha$  expression (Peraldi *et al.* 1997). In contrast, no effects of the thiazolidinedione, BRL 49653, on TNF- $\alpha$  release from fat tissue were seen in the present study. This apparent discrepancy may relate to species differences, to *in vivo* and *in vitro* differences, or to the fact that only fat from non-obese humans was studied.

LPS is a cell-wall component of Gram-negative bacteria that powerfully stimulates TNF- $\alpha$  protein production in monocytes and macrophages (Beutler *et al.* 1986). In this study, we have demonstrated that LPS also stimulates TNF- $\alpha$  release from human adipose tissue and from isolated human adipocytes. The greater stimulation of TNF- $\alpha$  from whole adipose tissue than from isolated adipocytes may suggest that cells other than adipocytes are responsible for most of the LPS response. However, precise quantitative comparisons are difficult, as the isolated adipocytes have inevitably undergone an isolation procedure that may impair their subsequent responsiveness *in vitro*. In this regard, Hotamisligil *et al.* (1993) have reported that the majority of TNF- $\alpha$  expression in fat tissue is in adipocytes, with a minor contribution from the stromovascular fraction.

We have shown that the stimulatory effect of LPS on TNF- $\alpha$  release can be blocked by BB3103, a matrix metalloproteinase inhibitor, and that this effect is seen in both whole adipose tissue and isolated adipocytes. These

findings suggest that the increase in TNF- $\alpha$  levels seen on exposure to LPS occurs via normal physiological mechanisms and is unlikely to represent a non-specific lytic effect of LPS. Our results also demonstrate, for the first time, that the release of TNF- $\alpha$  from adipocytes occurs through the same metalloproteinase-mediated mechanism as that seen in more classical TNF- $\alpha$  secreting cells.

The finding that LPS stimulates TNF- $\alpha$  production from human adipocytes suggests a role for adipocyte TNF in host defence. This adds to a growing body of evidence pointing towards a contribution of adipose tissue to innate immunity. Thus adipocytes are a major source of several components of the complement system including factors D (adipsin), B and C3 (Peake *et al.* 1997). Levine *et al.* (1998) have recently shown that adipocytes produce macrophage colony-stimulating factor, a proliferative stimulus for macrophages, and that this is upregulated in growing adipose tissue, as seen in 'creeping fat' that surrounds inflammatory intestinal lesions such as in Crohn's disease. Adipose tissue is also known to be a source of interleukin-6 (Fried *et al.* 1998), a pleiotropic cytokine that has a systemic role in infections and inflammatory disorders as part of the acute phase response. Finally, leptin, a major secretory product of adipocytes, has important effects on lymphocyte function (Lord *et al.* 1998). In addition, Gainsford and co-workers (1996) found that addition of leptin to culture medium enhanced cytokine production and phagocytosis of *Leishmania* parasites by murine peritoneal macrophages.

Thus our finding that LPS is a significant secretagogue for TNF- $\alpha$  release from human adipose tissue and adipocytes adds to this growing body of evidence for a role of the adipocyte in innate immunity. These findings do not, however, exclude additional roles for adipocyte TNF- $\alpha$  in the control of other aspects of fat cell biology, such as differentiation, apoptosis and lipid metabolism.

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