Increased Tenascin C And Toll-Like Receptor 4 Levels in Visceral Adipose Tissue as a Link between Inflammation and Extracellular Matrix Remodeling in Obesity

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Context: Obesity is associated with an altered inflammatory and extracellular matrix (ECM) profile. Tenascin C (TNC) is an ECM glycoprotein with proinflammatory effects.

Objective: We aimed to explore the expression levels of TNC in adipose tissue analyzing the contribution of adipocytes and stromovascular fraction cells (SVFC) as well as its impact on inflammation and ECM regulation. We also analyzed the effect of the stimulation with TNF-α and lipopolysaccharide (LPS) on both SVFC and adipocytes.

Patients and Methods: Samples obtained from 75 subjects were used in the study. Expression levels of *TNC*, *TLR4*, *MMP2*, and *MMP9* were analyzed in visceral adipose tissue (VAT) as well as in both adipocytes and SVFC. In addition, *Tnc* expression was measured in two mice models of obesity.

Results: We show, for the first time, that VAT expression levels of *TNC* are increased in normoglycemic and type 2 diabetic obese patients ($P < 0.01$) as well as in obese patients with nonalcoholic steatohepatitis ($P < 0.01$). Furthermore, expression levels of *Tnc* in epididymal adipose tissue from two different mice models of obesity were significantly increased ($P < 0.01$). *TNC* and *TLR4* were mainly expressed by SVFC, and its expression was significantly enhanced ($P < 0.01$) by TNF-α treatment. LPS treatment also increased mRNA levels of *TNC*. Moreover, the addition of exogenous TNC induced ($P < 0.05$) *TLR4* and *CCL2* mRNA expression in human adipocyte cultures.

Conclusions: These findings indicate that TNC is involved in the etiopathology of obesity via visceral adipose tissue inflammation representing a link with ECM remodeling. (***J Clin Endocrinol Metab* 97: E1880–E1889, 2012**)

Obesity, characterized by a prolonged positive energy balance, induces different changes in adipose tissue, including a dramatic alteration of shape and growth of adipocytes, the differentiation of preadipocytes into adipocytes, and an accumulation of inflammatory cells (1). These changes are related to the extracellular matrix (ECM) remodeling (2, 3) and give rise to functional alterations in adipose tissue and variations in its secretion profile of adipokines (4, 5). The degradation of components of the ECM and regulation of adipose tissue architecture is mediated by different systems, mainly the matrix metalloproteinase (MMP) family and the fibrinolytic plasmin-

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Abbreviations: BF, Body fat; BMI, body mass index; ECM, extracellular matrix; γ-GT, γ-glutamyltransferase; HDL, high-density lipoprotein; LN, lean; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NAFLD, nonalcoholic fatty liver disease; NG, normoglycemic; OB, obese; PUFA, polyunsaturated fatty acids; QUICKI, quantitative insulin sensitivity check index ; SAT, sc adipose tissue; SVFC, stromovascular fraction cell; T2D, type 2 diabetes; TLR, Toll-like receptor; TNC, tenascin C; VAT, visceral adipose tissue.

ogen and plasmin system (6). In this sense, the adipose tissue of obese and insulin-resistant humans shows an increase in components of the ECM, including members of the collagen family, fibrin, and thrombospondin (7, 8).

Much attention has focused on the adipose tissue changes in ECM and inflammation linked to obesity, highlighting the interrelation of both processes in the adipose transcriptomic signature of obese subjects (9). During the obesity-associated chronic inflammatory state, the ECM may act as a scaffold for cell infiltration as well as a reservoir for adipokines and growth factors (10). In addition, some ECM molecules, a class of the so-called damage-associated molecular patterns, can also directly activate the inflammatory process being rapidly released upon tissular damage (11). Tenascin C (TNC) is a large, extracellular matrix glycoprotein that belongs to the damage-associated molecular patterns family (12). The expression pattern of TNC is dynamic. Little or no TNC is found in most healthy adult tissues, being specifically induced and tightly controlled during acute inflammation and persistently expressed in chronic inflammation (13, 14). *TNC* is also reportedly overexpressed in human preadipocytes after stimulation with secreted factors from activated macrophages (15). Toll-like receptors (TLR) represent a key molecular link between tissue injury and inflammation (11, 16). TNC exhibits proinflammatory effects mediated by the activation of the TLR-4, which in turn promotes innate and adaptive immune responses, including induction of proinflammatory cytokines and the matrix metalloproteinase family (17). It has been also reported that TLR4 activation with lipopolysaccharide (LPS) induces the release of critical proinflammatory cytokines (18) as well as the expression of procollagen type 1 and integrin 1β (19), suggesting that direct activation of TLR4 has the potential to induce massive inflammation and may play a role in ECM remodeling. TNC is frequently coexpressed with MMP (20), with the inhibition of MMP suppressing the *TNC* expression (21).

Recent studies have identified close links between adipose tissue inflammation and the ECM and expression of TNC could play a key role (9, 22, 23). To our knowledge, there are no available data on the expression of TNC in human visceral (VAT) and sc (SAT) adipose tissue or its possible involvement in adipose tissue inflammation. The aim of the present study was to determine expression levels of *TNC* and *TLR4* in adipose tissue as well and to evaluate the effect of obesity and the obesity-related comorbidities type 2 diabetes (T2D) and nonalcoholic fatty liver disease (NAFLD). Furthermore, we aimed to analyze the expression levels of TNC in different models of obesity in mice during active adipose tissue expansion. The role of TNC in extracellular matrix regulation was also explored by analyzing its relation with circulating concentrations of different MMP as well as the expression levels of *MMP2* and *MMP9*. To gain insight into the molecular mechanism involved, the effect of TNF-α and LPS on the expression levels of *TNC* and *TLR4* in cultures of human adipocytes and stromovascular fraction cells (SVFC) was further explored. Finally, we also investigated whether TNC itself can activate the inflammatory response in human adipocytes.

Materials and Methods

For detailed Materials and Methods, see Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Patient selection

Adipose tissue samples from 75 subjects (18 males and 57 females) recruited from healthy volunteers and patients attending the Departments of Endocrinology and Nutrition and Surgery at the Clínica Universidad de Navarra were used. In addition, an intraoperative liver biopsy was performed in the obese patients during bariatric surgery to establish a histological diagnosis of the hepatic state as well as to analyze *TNC* gene expression levels. The samples were collected from patients undergoing either Nissen fundoplication [for hiatus hernia repair in lean (LN) volunteers] or Roux-en-Y gastric bypass [for morbid obesity treatment in obese (OB) subjects] at the Clínica Universidad de Navarra. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 C for subsequent analyses. The study was approved, from an ethical and scientific standpoint, by the Hospital's Ethical Committee responsible for research, and the written informed consent of participants was obtained. Blood assays and multiplex studies performed in the study subjects were measured as previously described (4). The transcript levels for *TNC*, *TLR4*, *MMP2*, and *MMP9* were quantified by real-time PCR (Ref. 24 and Supplemental Table 1) and protein levels were assessed by Western blot (25).

Cell culture

Human SVFC were isolated from the VAT of obese normoglycemic subjects and differentiated to adipocytes as previously described (25). Differentiated human visceral adipocytes and SVFC were serum-starved for 24 h and then treated with increasing concentrations of TNF-α (1, 10, and 100 ng/ml) (Sigma, St. Louis, MO), LPS (10, 100, and 1000 ng/ml) (Sigma), and TNC (1, 10, and 100 nmol/liter) (R&D Systems, Minneapolis, MN) for 24 h.

Study in animals

In the first animal model, 12-wk-old male C57BL/6 mice were maintained during 20 wk on a commercial high-fat diet (n = 10) to induce obesity or on a normal diet (n = 8) to serve as control. In the second murine model, 10-wk-old male wild-type (C57BL/6J) (n = 9) and obese *ob/ob* mice (C57BL/6J) (n = 8) were used to examine the effects in genetically based obesity. Both diets were isoproteic and contained a similar amount of sodium and phytates (26). Body weight was recorded on a regular basis to

monitor progression of the diet-induced and genetically obese mice. The epididymal adipose tissue depot was carefully dissected out, frozen in liquid nitrogen, and stored at −80 C. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609), and the study was approved by the Ethical Committee for Animal Experimentation of the University of Navarra.

Statistical analysis

Data are presented as mean ± SD. Differences in the proportion of subjects within groups regarding gender were assessed by using a contingency test (χ^2 test). Differences between groups were assessed by one-way ANOVA followed by Tukey's *post hoc* tests, two-tailed unpaired Student's *t* test, and Mann-Whitney *U* pairwise comparisons as appropriate. Differences between groups adjusted for age were analyzed by analysis of covariance. Pearson's correlation coefficients (r) were used to analyze the association between variables.

Results

Expression of TNC in adipose tissue is increased in obesity and obesity-associated T2D

The biochemical and hormonal characteristics of the subjects included in the study are shown in Table 1. No differences in gender distribution between groups was found ($P = 0.150$). In light of the divergent pathological consequences of adipose tissue distribution, we assessed first the gene expression levels of *TNC* and *TLR4* in paired samples of VAT and SAT. The mRNA levels of *TNC* in

TABLE 1. Anthropometric and biochemical characteristics of subjects included in the study

	Lean	Obese NG	Obese T2D
n (male, female)	13 (5, 8)	32 (7, 25)	30 (6, 24)
Age (yr)	36 ± 13	38 ± 14	42 ± 12
BMI (kg/m^2)	22.1 ± 3.0	42.2 ± 4.3[a]	45.6 ± 7.4[a]
BF (%)	22.4 ± 7.2	52.8 ± 4.8[a]	52.2 ± 7.2[a]
Waist (cm)	75.3 ± 9.7	120.3 ± 12.8[a]	127.8 ± 12.9[a,b]
Waist to hip ratio	0.80 ± 0.07	0.93 ± 0.09[a]	0.96 ± 0.08[a]
SBP (mm Hg)	105 ± 6	121 ± 16[a]	134 ± 15[a,c]
DBP (mm Hg)	66 ± 7	75 ± 7[a]	84 ± 8[a,c]
Fasting glucose (mg/dl)	87.8 ± 14.8	89.6 ± 10.9	125.4 ± 27.0[a,c]
2 h OGTT glucose (mg/dl)		114.4 ± 15.4	184.5 ± 45.4
Fasting insulin (μU/ml)	6.8 ± 2.9	17.2 ± 16.3	20.2 ± 11.7[d]
2-h OGTT insulin (μU/ml)		88.1 ± 51.1	147.2 ± 85.0
HOMA	1.5 ± 0.8	3.9 ± 2.8	5.6 ± 3.1[a]
QUICKI	0.371 ± 0.037	0.329 ± 0.038[a]	0.306 ± 0.024[a,b]
Triglycerides (mg/dl)	67 ± 25	96 ± 38	139 ± 68[a,c]
Cholesterol (mg/dl)	176 ± 24	188 ± 41	196 ± 37
LDL-cholesterol (mg/dl)	103 ± 25	115 ± 32	118 ± 32
HDL-cholesterol (mg/dl)	64 ± 12	53 ± 18	47 ± 12[d]
Leptin (ng/ml)	8.1 ± 4.5	56.6 ± 20.9[a]	48.2 ± 27.2[a]
Uric acid (mg/dl)	4.2 ± 0.7	5.7 ± 1.1[a]	5.5 ± 1.2[a]
Creatinine (mg/dl)	0.80 ± 0.06	0.80 ± 0.13	0.76 ± 0.14
CRP (mg/liter)	1.0 ± 0.7	9.9 ± 6.7[a]	7.3 ± 5.1[d]
Fibrinogen (mg/dl)	215 ± 68	398 ± 72[a]	349 ± 87[a]
von Willebrand factor (%)	56 ± 25	131 ± 58[a]	131 ± 49[a]
Homocysteine (μmol/liter)	6.8 ± 1.5	9.2 ± 2.7[d]	9.6 ± 2.6[a]
AST (UI/liter)	13 ± 4	17 ± 13	15 ± 6
ALT (UI/liter)	10 ± 7	22 ± 15[d]	24 ± 12[a]
ALP (UI/liter)	93 ± 30	96 ± 26	93 ± 30
γ-GT (UI/liter)	11 ± 6	20 ± 11	29 ± 17[d]
MMP-1 (pg/ml)	153.1 ± 102.7	241.9 ± 189.2	213.5 ± 115.5
MMP-2 (ng/ml)	21.84 ± 6.19	19.56 ± 6.36	19.89 ± 5.25
MMP-7 (ng/ml)	2.78 ± 1.53	3.36 ± 2.27	4.06 ± 1.72[d]
MMP-9 (ng/ml)	0.22 ± 0.05	3.31 ± 0.07[a]	2.27 ± 0.04[a]
MMP-10 (pg/ml)	371.3 ± 199.2	218.4 ± 176.5	236.3 ± 106.72

Data are mean ± SD. Differences between groups were analyzed by one-way ANOVA followed by Tukey's *post hoc* tests. MMP levels were logarithmically transformed for statistical analysis due to their non-normal distribution. ALP, Alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; DBP, diastolic blood pressure; HOMA, homeostatic model assessment; OGTT, oral glucose tolerance test; SBP, systolic blood pressure.

[a] $P < 0.01$ *vs.* lean.

[b] $P < 0.05$ *vs.* obese NG.

[c] $P < 0.01$ *vs.* obese NG.

[d] $P < 0.05$ *vs.* lean.

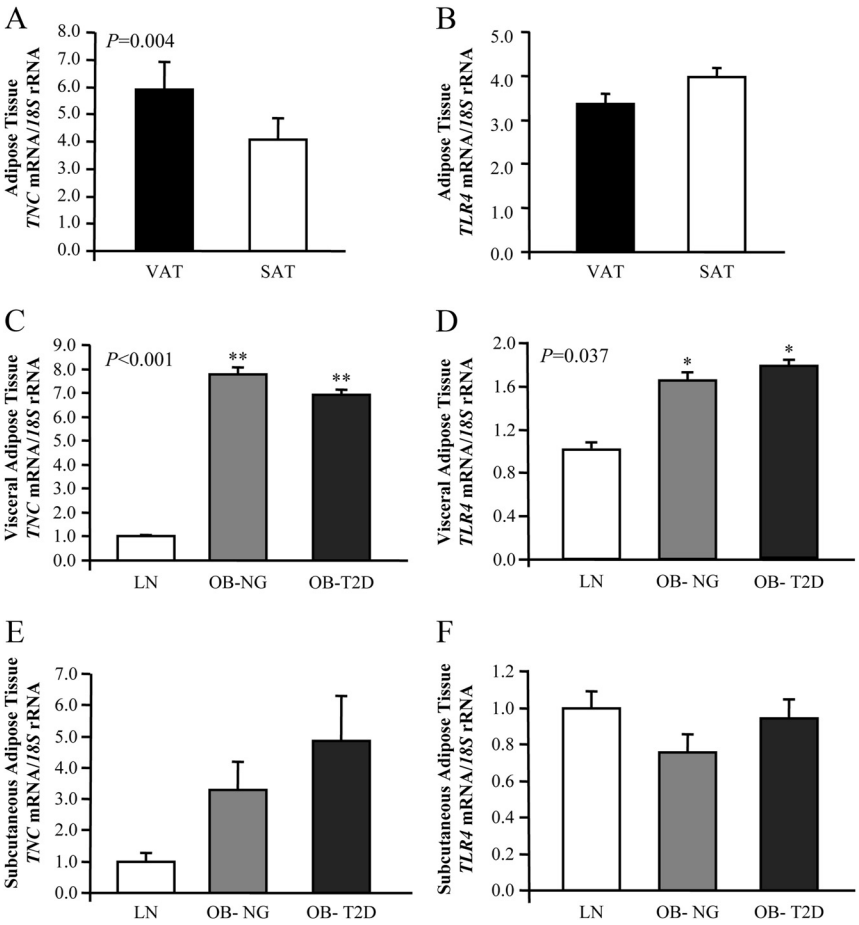

FIG. 1. Impact of obesity and obesity-associated T2D on gene expression levels of *TNC* and *TLR4* in adipose tissue. A and B, Analysis of mRNA levels of *TNC* and *TLR4* in VAT and SAT (VAT: n = 75; SAT: n = 23). *Bars* represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. Differences between groups were analyzed by a two-tailed unpaired Student's *t* test. C and D, Gene expression levels of *TNC* and *TRL4* in VAT of LN, obese NG, and obese T2D volunteers (LN: n = 13; OB-NG: n = 32; OB-T2D: n = 30). E and F, Gene expression levels of *TNC* and *TRL4* in SAT of LN, obese NG, and obese T2D volunteers (LN: n = 7; OB-NG: n = 7; OB-T2D: n = 9). *Bars* represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. The expression level in LN subjects was assumed to be 1. Differences between groups were analyzed by one-way ANOVA followed by Tukey's tests. *, $P < 0.05$, **, $P < 0.01$ *vs.* LN.

VAT were significantly increased ($P = 0.004$) compared with SAT, whereas no differences in gene expression levels of *TLR4* were found (Fig. 1, A and B).

Obesity and T2D were associated with an increased gene expression of *TNC* ($P < 0.001$) and *TLR4* ($P < 0.05$) in VAT (Fig. 1, C and D). TNC protein expression in the visceral fat depot exhibited a similar pattern to that observed in the gene expression analysis (Supplemental Fig. 1A). No changes in *TNC* and *TLR4* transcript levels were observed in SAT, although a tendency toward an increase in gene expression levels of *TNC* was shown (Fig. 1, E and F).

Although no differences between groups were detected regarding age, because the obese T2D group was on average slightly older, an analysis of covariance with age as covariable was performed to investigate the potential bias of age on mRNA levels of *TNC* and *TLR4* in VAT. Similar results were obtained, with *TNC* and *TLR4* expression being significantly increased ($P < 0.01$) in both obese normoglycemic (NG) and T2D patients compared with lean subjects. In this regard, mRNA *TNC* levels were positively associated ($P < 0.05$) with body mass index (BMI) and body fat (BF) as well as with fasting insulin concentrations and were negatively correlated with the quantitative insulin sensitivity check index (QUICKI) and high-density lipoprotein (HDL)-cholesterol concentrations (Table 2). A positive association was also found between mRNA levels of *TNC* and *TLR4* in VAT ($r = 0.26$; $P = 0.033$). No sexual dimorphism was found in the gene expression levels of both adipokines ($P = 0.547$ for *TNC* and $P = 0.296$ for *TLR4*).

Based on the fact that *TNC* exhibited the highest gene expression in VAT and given the relevance of this depot in obesity-associated metabolic disturbances, the experiments thereafter were focused on TNC in this location.

Gene expression of *Tnc* in murine models of obesity

To corroborate the human findings regarding TNC and TLR4 and to further explore the potential role of both molecules in the development of obesity, two different murine models of obesity, diet-induced obesity and genetically obese leptin-deficient (*ob/ob*) mice, were used (27). After 12 wk on a high-fat diet, mice exhibited a higher final body weight than those on a chow diet ($P < 0.001$). The same was true for *ob/ob* mice compared with their wild-type littermates ($P < 0.001$). Both murine obesity models also exhibited significantly increased epididymal fat depot weights ($P < 0.01$). *Tnc* and *Tlr4* expression was increased ($P < 0.05$) in the epididymal adipose tissue of both obesity models compared with those on a chow diet and with wild-type littermates, respectively (Supplemental Fig. 2).

TNC in adipose tissue in relation to extracellular matrix regulation and inflammation

Obese subjects with T2D exhibited higher circulating levels of MMP-7 ($P < 0.05$) and MMP-9 ($P < 0.01$) with the latter being also increased ($P < 0.01$) in obese NG patients ($P < 0.01$) compared with lean subjects (Table 1).

TABLE 2. Univariate analysis of the correlation between mRNA expression levels of *TNC* and *TLR4* in VAT with anthropometic and metabolic variables as well as with genes involved in extracellular matrix regulation

	***TNC* mRNA expression**		***TLR4* mRNA expression**	
	r	***P***	**r**	***P***
TNC mRNA expression	—	—	0.26	**0.033**
TLR4 mRNA expression	0.26	**0.033**	—	—
MMP2 mRNA expression	0.35	**0.003**	0.45	**<0.001**
MMP9 mRNA expression	0.38	**<0.001**	0.35	**0.003**
BMI	0.37	**0.002**	0.31	**0.012**
BF	0.26	**0.038**	0.22	0.085
QUICKI	−0.33	**0.011**	−0.04	0.791
HDL-cholesterol	−0.32	**0.016**	−0.06	0.651

Bold values denote statistically significant *P* values. —, The correlation of the *TNC* gene levels or *TLR4* gene expression with itself is not performed/provided.

Moreover, *MMP-9* mRNA expression levels were significantly higher ($P < 0.001$) in both obese NG and obese with T2D subjects compared with lean volunteers. Gene expression levels of *MMP2* followed a similar trend, although no statistically significant differences were detected (Supplemental Fig. 3). A positive correlation between the circulating concentrations and the expression levels in VAT of MMP-9 ($r = 0.32$; $P = 0.022$) was observed. Moreover, gene expression levels of *TNC* in VAT were positively correlated with the extracellular matrix regulatory genes *MMP9* ($P < 0.01$) and *MMP2* ($P < 0.01$) (Table 2).

We next investigated whether TNC can activate the expression of *TLR4* and genes involved in the inflammatory response in human adipocytes. Cells were stimulated with increasing concentrations of TNC for 24 h. As shown in Fig. 2, TNC treatment significantly enhanced ($P < 0.05$) the mRNA levels of *TLR4* and *CCL2* in adipocytes, whereas a slight increase of gene expression levels of *TNFA* was observed. We also detected an increased gene expression of *CD68* and *MMP9* after TNC treatment although the differences were not statistically significant.

Effect of inflammatory factors on mRNA levels of *TNC* and *TLR4* in human adipocytes and SVFC

Because VAT of obese patients exhibited an increased macrophage infiltration (especially in those individuals with a high BMI), to identify which cell type preferentially contributed to the elevated *TNC* and *TLR4* levels observed, adipocytes and SVFC were isolated from VAT samples obtained from 15 morbidly obese patients. Although both *TNC* and *TLR4* expression were readily evident in mature adipocytes, gene expression levels were mainly detected in the SVFC ($P < 0.01$) (Fig. 3, A and B). Furthermore, the presence of TNC in sections of visceral adipose tissue was confirmed by immunohistochemistry (Supplemental Fig. 1B). Both adipocytes and SVFC were immunopositive for TNC, although a marked staining in SVFC was observed.

Based on the fact that TNF-α is linked to the inflammatory response in obesity being a well-known regulator of the production of certain adipokines, the effect of TNF-α on *TNC* and *TLR4* expression in human visceral adipocytes and SVFC was examined. Cells were stimulated with increasing concentrations of TNF-α for 24 h. As shown in Fig. 3, C and E, TNF-α treatment significantly enhanced the mRNA levels of *TNC* in both SVFC and adipocytes. Although we also detected an increased gene expression of *TLR4* after TNF-α treatment in adipocytes ($P < 0.05$), the stimulation was not dose dependent, and the huge variation found between samples made it difficult to conclude that TNF-α stimulates *TLR4* expression. No statistically significant differences were found for the transcript levels of *TLR4* in SVFC (Fig. 3, D and F).

Because bacterial LPS initiates acute inflammatory responses typical of the host reaction to tissue injury or infection, we also analyzed the effect of LPS on human visceral adipocytes. Gene expression levels of *TNC* were strongly induced by LPS ($P < 0.001$), whereas gene expression of *TLR4* was increased, but the differences felt out of statistical significance (Fig. 3, G and H).

Increased expression of TNC in VAT is related to NAFLD

Because one of the best known hepatic derangements associated to obesity and diabetes is NAFLD, we aimed to investigate the regulation of *TNC* and *TLR4* as well as *MMP2* and *MMP9* in this condition. Obese patients were classified according to the presence or absence of NAFLD and were matched by age and BMI to exclude the effect of obesity in gene expression levels (Supplemental Table 2). Circulating concentrations of the hepatic enzyme γ-glutamyltransferase (γ-GT) ($P < 0.05$) were increased in NAFLD patients, who also showed elevated circulating concentrations of glucose 2 h after an oral glucose tolerance test and triglycerides ($P < 0.05$). Real-time PCR analysis indicated that mRNA expression levels of *TNC* and *TLR4* as well as those of both *MMP* in VAT were significantly higher ($P < 0.05$) in patients with NAFLD (Fig. 4). The gene expression levels of *TNC* were also assessed in hepatic biopsies in a subgroup of obese subjects, being higher in obese patients with T2D, although no statistically significant differences were reached (Supplemental Fig. 4A). We also explored the effect of NAFLD on *TNC* mRNA expression in the liver, and no differences between the groups were found (Supplemental Fig. 4B).

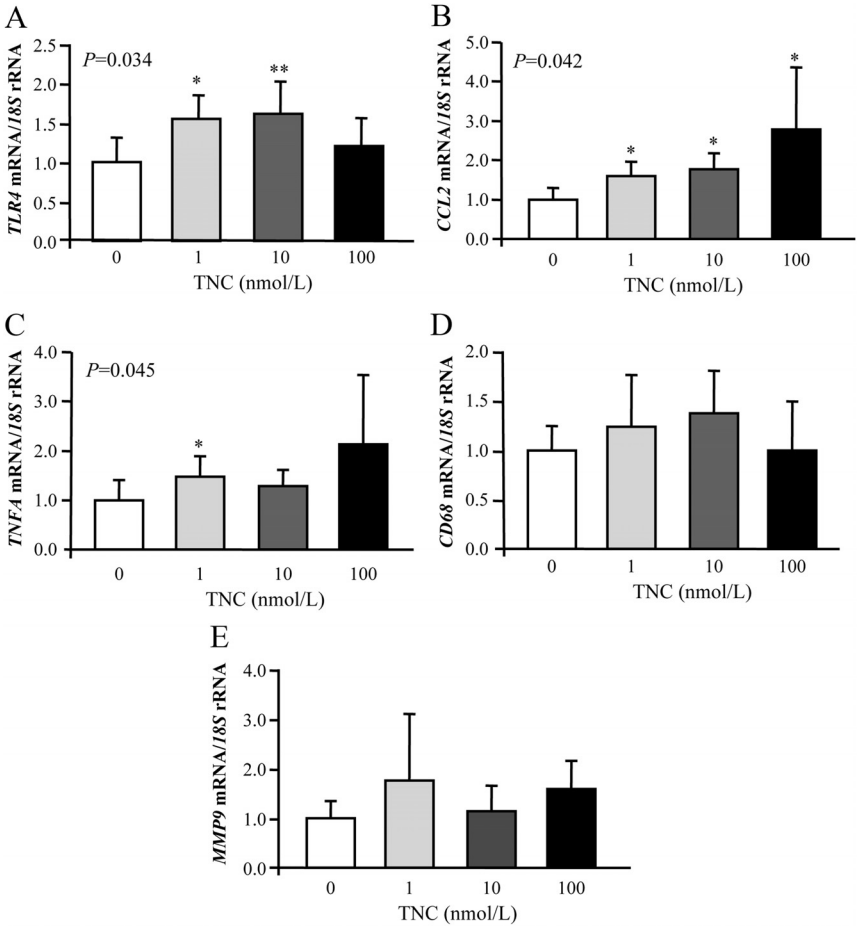

FIG. 2. Gene expression levels of *TLR4* (A), chemokine (C-C motif) ligand 2 (*CCL2*) (B), *TNFA* (C), CD68 antigen (*CD68*) (D), and *MMP9* (E) in human visceral adipocytes stimulated with recombinant TNC (1.0–100 nmol/liter) for 24 h. Gene expression levels in the unstimulated cells were assumed to be 1. Values are the mean ± SD (n = 6 per group). Differences between groups were analyzed by one-way ANOVA followed by Tukey's tests. *, $P < 0.05$ and **, $P < 0.01$ *vs.* unstimulated cells.

Discussion

Adipose tissue is a complex organ that regulates and coordinates metabolic homeostatic mechanisms. With the development of obesity, adipose tissue expands and exhibits an enhanced systemic low-grade inflammation profile involving macrophage infiltration together with an active adipokine production and release by adipocytes and other surrounding cells (2). To accommodate the changes, ECM remodeling takes place via the degradation of the existing and the production of new ECM components. The ECM is crucial for adipocyte development and function, thereby playing an important role in weight regulation, obesity and lipid metabolism (5).

A tight association between the ECM protein TNC and early inflammatory responses has been described (10). However, the clinical implications of TNC in obesity and adipose tissue remodeling remain largely unknown. Our findings provide evidence, for the first time, that TNC levels are increased in obese subjects in comparison with lean volunteers as well as in VAT compared with SAT. Consistently, we further show that the adipose tissue gene expression levels of *Tnc* are also increased in two murine models of obesity representative of the genetically based and exogenously induced disease. We also demonstrate that the increased transcript levels of *TNC* observed in obesity are further aggravated in the presence of NAFLD. Of interest, a correlation between gene expression levels of *TNC* and *TLR4* as well as *MMP2* and *MMP9* in VAT was found. Finally, we show that the proinflammatory cytokine TNF-α elevates the mRNA levels of *TNC* in cultures of human visceral adipocytes and SVFC.

VAT and SAT display different morphological and functional features with the increased visceral adiposity being responsible of the metabolic abnormalities related to obesity (28). Our study is the first to show that obese patients exhibit an 8-fold increase in gene expression levels of *TNC* in VAT compared with lean individuals, whereas no differences are evident in SAT. The significant positive correlation found between *TNC* levels and BF indicates that TNC is related to the adipose tissue amount. Interestingly, the expression of *TNC* is highly regulated and shows a highly circumscribed pattern of expression to areas of active tissue reorganization and inflammation, suggesting a link between an increase of ECM remodeling in VAT and its inflammatory profile (15). In addition to cellular adhesion receptors, cells also express receptors for ECM proteins, such as TLR, whose activation lead to the up-regulation of intracellular signaling pathways resulting in the production of inflammatory cytokines. In this regard, we detected higher expression levels of *TLR4* in both obese groups compared with lean volunteers as well as a positive association with the *TNC* expression levels. Recently it has been identified that the activation of TLR4 through TNC is required for maintaining joint inflammation but not for the initiation of inflammation (17). Moreover, *Tnc*-knockout mice have been shown to be protected from sustained and erosive joint inflammation. Induction of TNC is highly associated with a wide range of diseases related to inflammation, including diabetes, atherosclerosis, ulcerative colitis, inflammatory bowel disease, vasculitis, or lung inflammation (29). An early

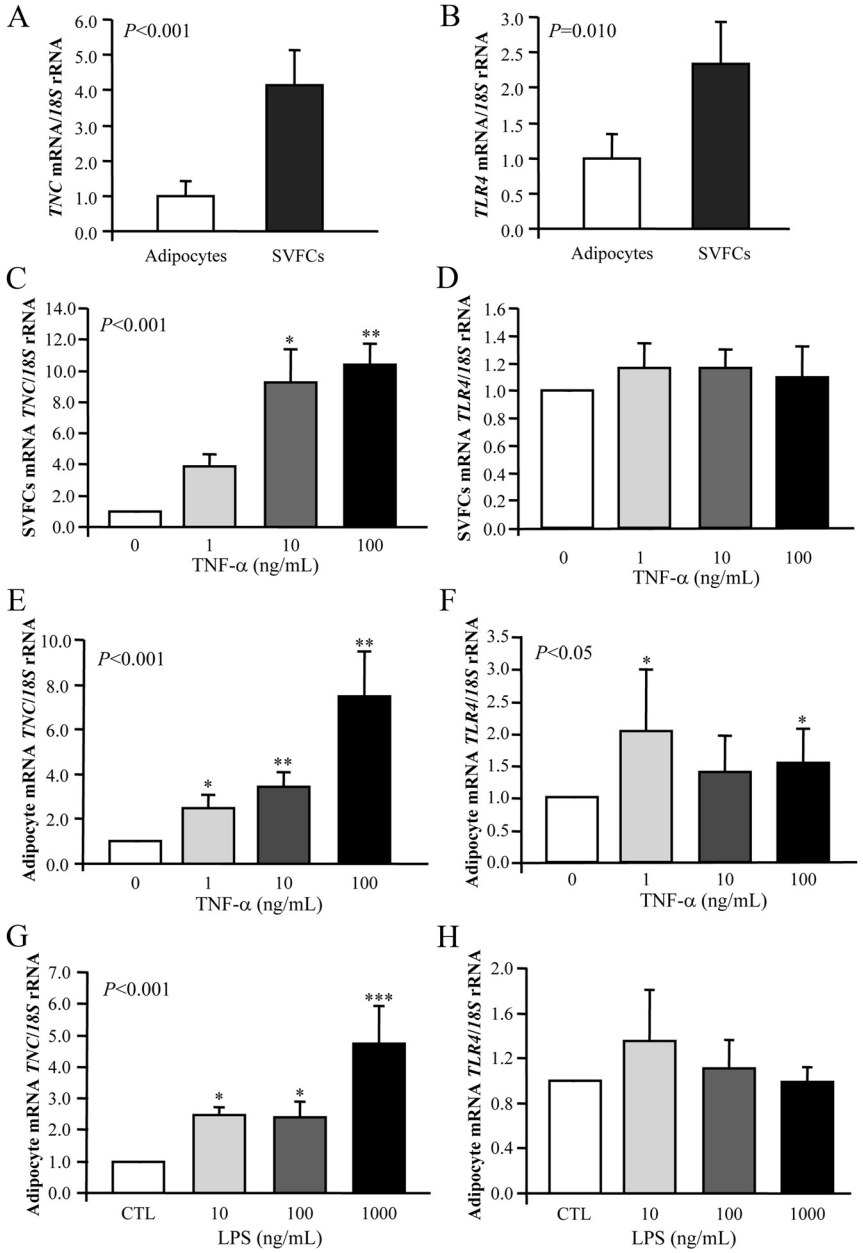

FIG. 3. Gene expression levels of *TNC* and *TLR4* in human visceral adipocytes and SVFC. A and B, Comparison of *TNC* and *TLR4* gene expression in adipocytes and SVFC isolated from VAT of obese patients. *Bars* represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. The expression level in adipocytes was assumed to be 1 (adipocytes: n = 9; SVFC: n = 11). Statistical differences were assessed by a two-tailed unpaired Student's *t* test. *, $P < 0.05$, **, $P < 0.01$ *vs.* adipocytes. C and D, Effect of TNF-α. Bar graphs show the effect of TNF-α incubated for 24 h on the transcript levels of *TNC* and *TLR4* in visceral SVFC. E and F, Transcript levels of *TNC* and *TLR4* after 24 h incubation with TNF-α in visceral adipocytes. G and H, Gene expression levels of *TNC* and *TLR4* in human visceral adipocytes after LPS treatment. The gene expression levels in the unstimulated cells were assumed to be 1. Values are the mean ± SD (n = 6 per group). Differences between groups were analyzed by one-way ANOVA followed by Tukey's tests. CTL, Control. *, $P < 0.05$; ***, $P < 0.01$ *vs.* unstimulated cells.

inflammatory response is generally associated with enhanced TNC levels both in plasma and tissue (13). In this sense, plasma levels of TNC constitute a well-known indicator for inflammatory bowel disease, chronic hepatitis C, or myocardial infarction (30, 31). It would be very interesting to evaluate circulating levels of TNC in light of its physiological consequences. The study of circulating levels of TNC may help to better understand the role of TNC in obesity and its associated comorbidities.

The higher expression levels of *TNC* and *TLR4* in the SVFC of visceral fat tissue compared with adipocytes, observed in the present study indicate that different cell types such as mononuclear cells (monocytes, macrophages, and lymphocytes) among others, may produce this glycoprotein. Noteworthy, SVFC represent a source of inflammation-related molecules that exert a local action on adipose tissue biology, particularly within the enlarged adipose mass. It has been described that macrophages that accumulate within the enlarged adipose tissue acquire particular remodeling phenotypes, releasing MMP and/or stimulating the expression of inflammatory genes in human adipocytes (32, 33). In this context, the obese patients included in the study showed increased circulating levels of MMP-7 and MMP-9. Furthermore, previous studies have demonstrated that macrophage-secreted factors induce overexpression of ECM genes in inflammatory preadipocytes increasing the deposition of TNC (15). In this sense, we have shown increased gene expression levels of *MMP9* in VAT from obese patients accompanied by a clear positive association between mRNA levels of *TNC* with *MMP9* and *MMP2*, which is in line with previous studies reporting that TNC is cleaved by MMP (34, 35). Reciprocally, TNC increases *MMP9* expression (36) and regulates vascular endothelial growth factor (37), highlighting a role in the control of angiogenesis. Recent studies have shown an alteration in the expression or activity of MMP in obesity underscoring their involvement in the pathophysiology of obesity-associated comorbidities (34).

To corroborate the human findings and to further explore the potential role of TNC in the development of obesity, gene expression levels of this protein were analyzed in two mice models of obesity differing in their underlying cause, namely genetic as opposed to diet-induced obesity. Increased expression levels of *Tnc* in epididymal fat pads were observed in both experimental

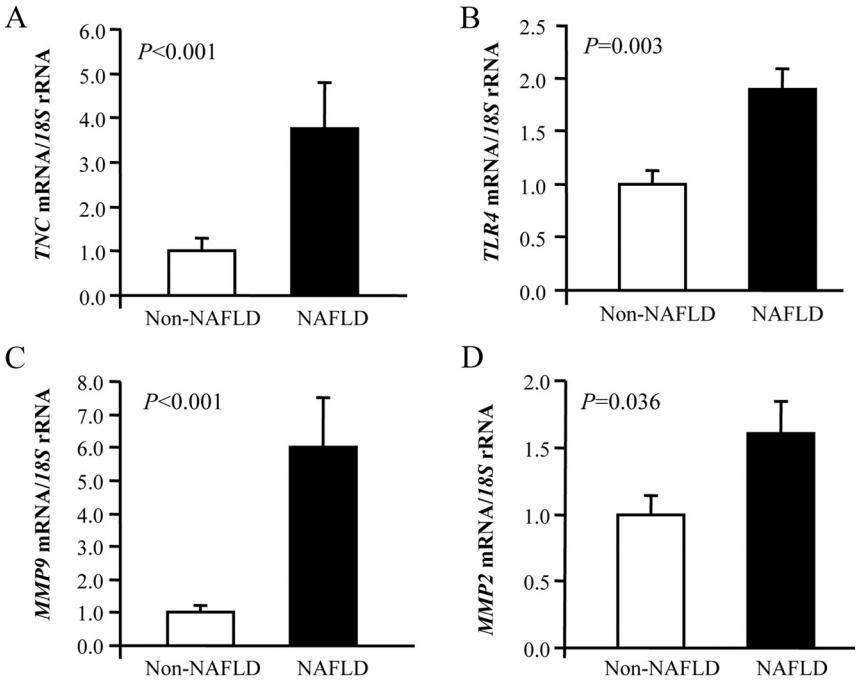

FIG. 4. Gene expression levels of *TNC* (A), *TLR4* (B), *MMP9* (C), and *MMP2* (D) in the VAT of obese patients with and without NAFLD. *Bars* represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. The expression level in non-NAFLD subjects was assumed to be 1 (non-NAFLD: n = 18; NAFLD: n = 25). Differences between groups were assessed by two-tailed unpaired Student's *t* test.

models of obesity. This is in agreement with previous studies describing that a high-fat diet increases the expression of procollagens and microfibril-forming components in gonadal adipose tissue, thereby pointing to an enhanced matrix turnover induced by high-fat feeding (38). It has been also described that n-3 polyunsaturated fatty acids (PUFA) inhibited the high-fat diet-induced up-regulation of genes involved in matrix remodeling and restored the adipocyte enlargement in adipose tissue of obese diabetic mice (38). Moreover, n-3 PUFA prevented adipose tissue inflammation induced by high-fat diet in obese diabetic mice, thereby suggesting that beneficial effects of n-3 PUFA on diabetes development could be mediated by their effect on adipose tissue inflammation (39). Furthermore, in the genetically obese *ob/ob* mice due to the lack of functional leptin, increased adipose tissue expression levels of *Tnc* have been also detected. In this regard, epididymal adipose tissue of genetically obese *db/db* mice reportedly exhibit an overexpression of collagens with collagen VI-null *ob/ob* mice showing a decrease of inflammatory markers in adipose tissue (40).

Our *in vitro* studies provide, to our knowledge, the first evidence of an induction of the *TNC* expression levels in human visceral adipocytes and SVFC upon stimulation with the proinflammatory cytokine TNF-α. Although TGF-β1 was the first growth factor reported to up-regulate the mRNA of *TNC* (41), other inflammatory and growth factors such as epidermal growth factor or basic fibroblast growth factor have been shown to increase TNC production in adipose tissue-derived stem cells and macrophages (42). Because TNC is also stimulated by antiinflammatory cytokines, it has been suggested that this ECM protein may act as a mechanism to protect tissues during inflammation that is required to resolve inflammation rather than to trigger it. In accordance with this hypothesis, TNC has been shown to alter the adhesion properties of human monocytes to fibronectin (43). Adipocytes are capable of sensing the presence of LPS, a bacterial cell wall component. Interestingly, LPS administration has been shown to induce systemic inflammation signaling via TLR-4, without changes in its expression in 3T3-L1 adipocytes (44, 45). It has been also described that in response to stimulation with LPS, human monocytes significantly increased the expression of *TNC*. We detected for the first time that LPS induced the expression of *TNC* in human visceral adipocytes with the expression of *TLR4* remaining unchanged. Moreover, we found that TNC stimulated mRNA expression levels of *TLR4* and *CCL2* in adipocytes, strengthening the role of TNC in the inflammatory response of adipose tissue.

Obesity is tightly linked to hepatic alterations, particularly NAFLD, mainly due to the state of chronic low-grade systemic inflammation. In this regard, IL-6 concentrations have been strongly associated with fatty liver (46). The expression of *TNC* detected in adipose tissue of obese patients was further enhanced by NAFLD. Moreover, gene expression levels of the remodeling metalloproteinases, *MMP2* and *MMP9*, were increased in the NAFLD patients. Noteworthy, a sequential increase in the TNC deposition and expression in the liver of the wild-type mice after concanavalin A treatment has been found (47). However, no differences were found in the *TNC* mRNA levels in liver biopsies, suggesting an important role of adipose TNC in the regulation of inflammation. Interestingly, the increasing amounts of TNC in rare tumors of Ito cells, which are fat-storing cells, which represent approximately 20% of the hepatic sinusoidal cells (48), as well as in chronic hepatitis C (49) have been described. TNC may be involved in NAFLD by enhancing the inflammatory response or by its actions on ECM synthesis and assembly during tissue repair (29, 50). Surprisingly, patients with NAFLD presented normal levels of the hepatic enzymes, alanine aminotransferase, aspartate aminotransferase,

and γ-GT, which may be due to the relatively low age of the selected patients because NAFLD worsens with advanced age (51).

In summary, our findings demonstrate that TNC levels are increased in VAT of obese subjects with or without T2D and in obese volunteers with NAFLD. The positive association with *TLR4*, *MMP2*, and *MMP9* as well as its stimulation after TNF-α treatment in both adipocytes and SVFC suggests a role for this protein in maintaining the chronic inflammatory response associated to obesity, which may be related to an increase of extracellular matrix remodeling.

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