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Increased Interleukin-32 Levels in Obesity Promote Adipose Tissue Inflammation and Extracellular Matrix Remodeling: Effect of Weight Loss

Diabetes 2016;65:3636–3648 | DOI: 10.2337/db16-0287

Interleukin (IL)-32 is a recently described cytokine involved in the regulation of inflammation. We aimed to explore whether IL-32 could function as an inflammatory and angiogenic factor in human obesity and obesity-associated type 2 diabetes. Samples obtained from 90 subjects were used in the study. Obese patients exhibited higher expression levels of IL-32 in visceral adipose tissue (AT) as well as in subcutaneous AT and peripheral blood mononuclear cells. *IL32* was mainly expressed by stromovascular fraction cells, and its expression was significantly enhanced by inflammatory stimuli and hypoxia, whereas no changes were found after the incubation with anti-inflammatory cytokines. The addition of exogenous IL-32 induced the expression of inflammation and extracellular matrix–related genes in human adipocyte cultures, and *IL32*-silenced adipocytes showed a downregulation of inflammatory genes. Furthermore, adipocyte-conditioned media obtained from obese patients increased *IL32* gene expression in human monocyte cultures, whereas the adipocyte-conditioned media from lean volunteers had no effect on *IL32* mRNA levels. These findings provide evidence, for the first time, about the inflammatory and remodeling properties of IL-32 in AT, implicating this cytokine in obesity-associated comorbidities.

Adipose tissue (AT) is now considered one of the largest endocrine organs in the body and an active tissue for cellular reactions rather than an inert tissue for energy storage (1,2). As AT expands, an increase in chronic systemic low-grade inflammation due to greater production of proinflammatory cytokines released either from adipocytes themselves or from the infiltrating macrophages takes place (3,4). Inflammation is considered as an important contributor to the development of obesity-associated metabolic complications such as insulin resistance and type 2 diabetes (T2D). Although a major function of cytokines is to initiate immune responses, their roles in the regulation of energy homeostasis and their implication in the etiology of metabolic diseases have not been clearly established (5).

The interleukin (IL) family is one of the most important groups of inflammatory-related mediators involved in AT inflammation (5). IL-32, also termed as tumor necrosis factor (TNF) α–inducing factor and natural killer cell transcript-4, is a recently described cytokine produced by T lymphocytes, natural killer cells, epithelial cells, and blood monocytes that acts as an important regulator of inflammation (6–8). In this regard, IL-32 expression has been reported in autoimmune diseases, inflammatory bowel disease, certain forms of cancer, and viral infections (8–13). The expression levels of this novel cytokine in synovial biopsies isolated from patients with rheumatoid arthritis have been correlated with the severity of inflammation and its local expression associated with the acute-phase protein CRP (8). Unexpectedly, the structure of IL-32 did not match

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Received 3 March 2016 and accepted 4 September 2016.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-0287/-/DC1.

the sequence homology seen in most of the known cytokines, and it can be expressed in six splice variants with diverse biological activity (14). The complete transcript, the IL-32γ, is the most active and potent isoform with respect to cell activation and death, and this may explain why it can be spliced into shorter and less harmful isoforms such as IL-32β or IL-32α (15–17).

IL-32 promotes inflammation by inducing other proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 via the activation of nuclear factor-κB and p38 mitogen-activated protein kinase as well as the modulation of the nucleotide-binding oligomerization domains (NOD) 1 and 2 pathways (18). IL-32 has been also described to be notably induced by interferon-γ in epithelial cells and monocytes (8,19). Importantly, IL-32 also contributes to the induction of inflammation by differentiating monocytes into macrophage-like cells (20).

The function of endogenous IL-32β in a fatty liver mouse model has been recently described (21). Lee et al. (21) showed that mice overexpressing IL-32β on a high-fat diet were protected from hepatic steatosis and inflammation. In contrast, the overexpression of IL-32γ in a streptozotocin-induced type 1 diabetic mice model contributed to initial islet β-cell injury and pancreatic inflammation (22). However, no reports are currently available on IL-32 function or expression in human obesity and AT inflammation.

Because IL-32 acts as an important regulator of inflammation and has also been proposed as an angiogenic mediator in endothelial cells (23), we hypothesized that IL-32 could also function as an inflammatory and angiogenic factor in human obesity. Therefore, the aim of the current study was to explore the potential differences in circulating IL-32 concentrations in normal weight, obesity, and obesity-associated volunteers with T2D as well as to analyze the impact of weight loss induced by bariatric surgery on its circulating levels. Furthermore, we aimed to investigate *IL32* gene expression in relevant metabolic tissues and the possible regulatory roles and mechanisms of IL-32 in inflammation and extracellular matrix (ECM) remodeling in human adipocytes. Furthermore, adipocyte-conditioned media (CM) was used to assess the effects of the secretion of adipocytes on *IL32* mRNA expression in human monocytes.

RESEARCH DESIGN AND METHODS

Patient Selection

In order to analyze the effect of obesity and T2D on the plasma, gene, and protein expression levels of IL-32, blood and AT samples from 90 subjects (22 males and 68 females) recruited from healthy volunteers and patients attending the Departments of Endocrinology & Nutrition and Surgery at the Clínica Universidad de Navarra were used. Obese (OB) patients were further subclassified into three groups (normoglycemia [NG], impaired glucose tolerance [IGT], or T2D) following the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes (24). T2D subjects were not on insulin therapy or on medication likely to influence endogenous insulin levels. It has to be stressed that the patients included in our OB T2D group did not have a long diabetes history (<2 to 3 years or even de novo diagnosis as evidenced from their anamnesis and biochemical determinations).

The tissue samples were collected from patients undergoing either Nissen fundoplication (for hiatus hernia repair in lean [LN] volunteers) or Roux-en-Y gastric bypass (RYGB) (for morbid obesity treatment in OB subjects) at the Clínica Universidad de Navarra. Both interventions were carried out via a laparoscopic approach. In addition, an intraoperative liver biopsy was performed in the OB patients during bariatric surgery to establish a histological diagnosis of the hepatic state as well as to analyze *IL32* gene expression levels. This procedure is not clinically justified in LN subjects. The diagnosis of nonalcoholic fatty liver disease (NAFLD) was established following a histopathological evaluation applying the criteria of Brunt (25). Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C for subsequent analyses.

In addition, a group of 35 OB female patients was selected to investigate the effect of weight loss on circulating IL-32 concentrations. The weight loss was achieved either by RYGB (n = 20) (evaluated 12 months after surgery) or by prescription of a conventional dietary treatment (n = 15) (evaluated after 8 months) providing a daily energy deficit of 500–1,000 kcal/d as calculated from the determination of the resting energy expenditure through indirect calorimetry (Vmax29; SensorMedics Corporation, Yorba Linda, CA) and multiplication by the physical activity level factor to obtain the individual's total energy expenditure. This hypocaloric regimen allows a safe and steady weight loss of 0.5–1.0 kg/week.

The study was approved, from an ethical and scientific standpoint, by Universidad de Navarra's Ethics Committee (Pamplona, Spain) responsible for research, and the written informed consent of participants was obtained.

Blood Assays

Plasma samples were obtained by venipuncture after an overnight fast. Glucose and lipid metabolism factors as well as hepatic and inflammatory markers were measured as previously described (26,27). IL-32α circulating levels were determined by a commercially available ELISA kit (CUSABIO, College Park, MD) following the manufacturer's guidelines, with intra- and interassay coefficients of variation being <8.0 and 10.0%, respectively.

Gene and Protein Expression Levels

AT, liver, and peripheral blood mononuclear cell (PBMC) RNA isolation was performed as previously described (26,28). Gene transcript levels were quantified by real-time PCR (7300 Real Time PCR System; Applied Biosystems, Foster City, CA) (Supplementary Table 1) (28), and protein expression was determined by Western blot (29). Blots were incubated with a rat monoclonal anti-human IL-32α antibody (R&D Systems, Minneapolis, MN) diluted 1:10,000.

Histological Analysis of IL-32

Sections (6 μm) of formalin-fixed paraffin-embedded visceral AT (VAT) were dewaxed in xylene, rehydrated in decreasing

concentrations of ethanol, and treated with 3% H_2O_2 (Sigma-Aldrich) in absolute methanol for 10 min at room temperature (RT) to quench endogenous peroxidase activity. Then, slides were blocked during 1 h with 1% BSA (Sigma-Aldrich) diluted in Tris-buffered saline (TBS) to prevent nonspecific adsorption. Sections were incubated overnight at 4°C with a rat monoclonal anti-human IL-32α antibody (R&D Systems) diluted 1:50 in TBS. To perform the immunohistochemistry, after washing with TBS, slides were incubated with Dako Real EnVision horseradish peroxidase–conjugated anti-rat (DakoCytomation, Glostrup, Denmark) for 1 h at RT. After washing in TBS, the peroxidase reaction was visualized with a 3,3′-diaminobenzidine (Amersham Biosciences, Buckinghamshire, U.K.), with chromogen and Harris hematoxylin solution (Sigma-Aldrich) as counterstaining. To accomplish the immunofluorescence, after washing with TBS, slides were incubated with biotinylated rat anti-human IgG antibody (1:100) in PBS for 1 h, washed, and reacted with fluorescein isothiocyanate conjugate (1:100; Sigma-Aldrich) for 4 h at RT. Finally, sections were dehydrated, mounted using DePeX mounting medium (Serva, Heidelberg, Germany), and observed under a Zeiss Axiovert CFL optic microscope (Zeiss, Göttingen, Germany). Negative control slides without primary antibody were included to assess nonspecific staining.

Adipocyte and Monocyte Cultures

Human stromovascular fraction cells (SVFC) were isolated from the VAT of OB NG subjects and differentiated to adipocytes as previously described (29). Differentiated adipocytes were serum-starved for 24 h and then treated with increasing concentrations of TNF-α (Sigma-Aldrich), lipopolysaccharide (LPS) (Sigma-Aldrich), IL-4 (R&D Systems), IL-13 (R&D Systems), IL-32α (R&D Systems), IL-32γ (R&D Systems), and $CoCl_2$ (Sigma-Aldrich) for 24 h.

Adipocyte CM was prepared by collecting the supernatant from differentiated adipocytes from both LN and OB volunteers. The CM was then centrifuged, diluted (40 and 60%), and frozen at −80°C. PBMC were obtained by centrifugation of whole blood over Ficoll gradients, and monocytes were isolated and cultured as previously described (20). The CM was used to assess the effects of the secretion of adipocytes on mRNA *IL32* expression in human monocytes.

Adipocyte Transfection With Small Interfering RNA

Differentiated human visceral adipocytes were serum-starved for 24 h, and then two pairs of small interfering RNAs (siRNAs) (s17656 and s17657; Ambion, Life Technologies) were annealed and transfected into adipocytes (100 pmol/L siRNA/2 × 10^5 cells/well) using 40 nmol Lipofectamine 2000 (Invitrogen). A scrambled siRNA was used as a negative control. The treatment with the two specific IL-32–siRNA resulted in 86 and 49% average knockdown of mRNA of *IL32*, respectively (Supplementary Fig. 1), leading to the selection of IL-32–siRNA s17656 for the *IL32* knocking down studies. Transfected adipocytes were cultured, and gene expression analyses were performed 24 h after siRNA transfection.

Statistical Analysis

Data are presented as mean ± SEM. Differences in the proportion of subjects within groups regarding sex were assessed using the χ^2 test. Due to their nonnormal distribution, CRP concentrations and gene expression levels were logarithmically transformed. Differences between groups were assessed by one-way ANOVA followed by Tukey post hoc tests and two-tailed unpaired Student *t* tests as appropriate. Pearson correlation coefficients (*r*) were used to analyze the association between variables. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS, Chicago, IL). A P value <0.05 was considered statistically significant.

RESULTS

Increased IL-32 Circulating Levels in Human Obesity and Obesity-Associated T2D Decrease After Weight Loss

Baseline characteristics of the subjects included in the study are shown in Table 1. No differences in age between groups were observed (P = 0.133). Mean systolic and diastolic blood pressure (BP) were significantly higher ($P < 0.01$) in the OB groups compared with the LN volunteers. Patients from both OB groups were anthropometrically similar between them, exhibiting significantly higher ($P < 0.01$) BMI, body fat percentage (BF), waist and hip circumference, as well as waist-to-hip ratio (WHR) compared with normal-weight control subjects. OB patients with T2D exhibited higher glycemia ($P < 0.01$) and a lower quantitative insulin sensitivity check index (QUICKI) ($P < 0.01$) than both LN and OB NG individuals. As expected, obesity was associated with hyperleptinemia ($P < 0.01$) and with increased concentrations of triglycerides ($P < 0.01$) accompanied by reduced circulating concentrations of HDL cholesterol ($P < 0.05$). All markers of inflammation were significantly higher ($P < 0.01$) in OB individuals. Regarding the white blood cells, no differences were found between groups.

Significant differences (P = 0.015) in circulating IL-32 concentrations among the three experimental groups were observed, being significantly increased in both OB groups as compared with LN subjects (Fig. 1*A*). No sexual dimorphism was found in plasma IL-32 concentrations (males: 11,441.37 ± 1,405.42 pg/mL; females: 10,517.31 ± 1,101.49 pg/mL; P = 0.326). Interestingly, a highly significant positive association was observed between circulating IL-32 levels and weight (r = 0.46; P = 0.003), BMI (r = 0.40; P = 0.010), waist circumference (r = 0.36; P = 0.024), and WHR (r = 0.40; P = 0.011), whereas a negative correlation was found with the QUICKI (r = −0.36; P = 0.042) and HDL cholesterol (r = −0.49; P = 0.006).

To analyze the impact of therapeutic interventions aimed at achieving weight loss in OB patients, the effect on plasma IL-32 concentrations induced by either RYGB or a conventional lifestyle intervention was examined. As expected, after an average postsurgical period of 12 months, patients submitted to RYGB experienced a significant

Table 1—Anthropometric and biochemical characteristics of subjects included in the study

	LN	OB NG	OB IGT + T2D
n (male, female)	16 (6, 10)	35 (6, 29)	39 (10, 29)
Age (years)	36 ± 3	40 ± 3	42 ± 2
BMI (kg/m^2)	22.1 ± 0.6	42.2 ± 0.8**	45.6 ± 1.2**
BF (%)	22.4 ± 1.6	52.4 ± 0.9**	52.5 ± 1.2**
Waist (cm)	75 ± 2	119 ± 2**	128 ± 2**†
Hip (cm)	94 ± 1	128 ± 2**	134 ± 2**†
WHR	0.80 ± 0.02	0.93 ± 0.02**	0.96 ± 0.01**
SBP (mmHg)	104 ± 2	120 ± 3**	133 ± 2**††
DBP (mmHg)	66 ± 2	75 ± 1**	84 ± 1**††
Fasting glucose (mg/dL)	88 ± 4	90 ± 2	115 ± 4**††
2-h OGTT glucose (mg/dL)	—	113 ± 3	185 ± 10†††
Fasting insulin (μU/mL)	6.8 ± 0.8	16.6 ± 2.7	20.2 ± 2.2*
2-h OGTT insulin (μU/mL)	—	87.8 ± 9.3	147.2 ± 17.3††
HOMA	1.5 ± 0.2	3.9 ± 0.7	5.6 ± 0.8**
QUICKI	0.371 ± 0.011	0.330 ± 0.007**	0.306 ± 0.004***†
Triglycerides (mg/dL)	67 ± 7	95 ± 6	139 ± 12**††
Cholesterol (mg/dL)	176 ± 7	190 ± 7	196 ± 6
LDL cholesterol (mg/dL)	103 ± 7	116 ± 6	118 ± 6
HDL cholesterol (mg/dL)	64 ± 4	54 ± 3	48 ± 2*
Leptin (ng/mL)	8.1 ± 1.3	56.1 ± 3.9**	48.2 ± 5.3**
Uric acid (mg/dL)	4.2 ± 0.2	5.6 ± 0.2**	5.5 ± 0.2**
Creatinine (mg/dL)	0.80 ± 0.02	0.79 ± 0.02	0.77 ± 0.02
CRP (mg/L)	1.0 ± 0.2	9.3 ± 1.4**	7.3 ± 1.2*
Fibrinogen (mg/dL)	215 ± 18	395 ± 13**	352 ± 16**
von Willebrand factor (%)	56 ± 8	128 ± 12**	131 ± 10**
Homocysteine (μmol/L)	6.8 ± 0.4	9.1 ± 0.5*	9.6 ± 0.5**
AST (UI/L)	13 ± 1	17 ± 2	15 ± 1
ALT (UI/L)	10 ± 3	22 ± 3*	24 ± 2**
γ-GT (UI/L)	11 ± 2	19 ± 2	29 ± 5*
Leukocyte (× 10^9/L)	7.35 ± 0.46	7.65 ± 0.55	7.59 ± 0.35
Neutrophils (%)	64.0 ± 1.5	61.1 ± 1.9	61.1 ± 1.6
Lymphocytes (%)	26.1 ± 1.7	29.0 ± 1.5	28.7 ± 1.3
Monocytes (%)	6.2 ± 0.8	6.3 ± 0.3	6.7 ± 0.3
Eosinophils (%)	3.5 ± 1.2	3.0 ± 0.4	2.7 ± 0.3
Basophils (%)	0.3 ± 0.1	0.6 ± 0.1	0.7 ± 0.1

Data are mean ± SEM. CRP concentrations were logarithmically transformed for statistical analysis. Differences between groups were analyzed by one-way ANOVA followed by Tukey post hoc tests or by unpaired two-tailed Student *t* tests, where appropriate. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic BP; γ-GT, γ-glutamyltransferase; OGTT, oral glucose tolerance test; SBP, systolic BP. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. LN; †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.01$ vs. OB NG.

decrease ($P < 0.0001$) in all anthropometric measurements as well as a significant improvement in the presurgical insulin resistance as evidenced by the decrease ($P < 0.0001$) in fasting glucose and insulin concentrations and the inflammatory marker CRP ($P < 0.0001$) (Supplementary Table 2). Noteworthy, a statistically significant reduction in the circulating concentrations of IL-32 was observed after bariatric surgery ($P = 0.031$) (Fig. 1*B*). In this sense, the differences in IL-32 concentrations after RYGB were positively correlated with the reduction in BF ($r = 0.45$; $P = 0.031$) as well as in waist circumference ($r = 0.57$; $P = 0.005$). After an average period of 8 months, OB patients following the conventional hypocaloric diet experienced significant decreases ($P < 0.001$) in weight, BMI, BF, waist circumference, WHR, and the lipid profile (Supplementary Table 2). However, the diet-induced weight loss was not accompanied by statistically significant changes in circulating levels of IL-32 (Fig. 1*B*).

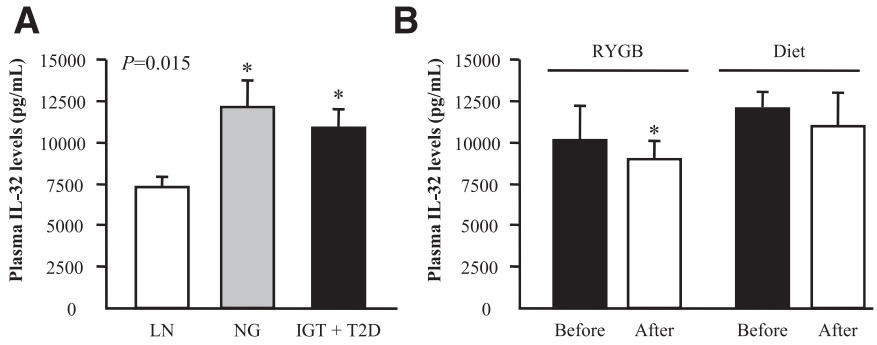

Figure 1—Circulating concentrations of IL-32 in obesity and obesity-associated T2D. Impact of weight loss. Fasting plasma concentrations of IL-32 determined by ELISA in LN volunteers (n = 16), OB NG subjects (n = 26), and OB patients with T2D (n = 28) (*A*) and comparison of plasma IL-32 concentrations in OB patients before and after weight loss achieved by either RYGB (n = 20; evaluated 12 months after surgery) or a conventional lifestyle intervention (n = 15; evaluated after 8 months) (*B*). Bars represent the mean ± SEM. Differences between groups were analyzed by one-way ANOVA followed by Tukey tests as well as by paired two-tailed Student t tests, where appropriate. *$P <$ 0.05 vs. LN subjects or presurgical values.

Obesity and Obesity-Associated T2D Upregulate IL-32 Expression Levels in Active Metabolic Tissues

Because plasma levels of IL-32 are increased in obesity and in light of the divergent contribution to obesity-associated inflammation of different tissues, we further investigated its expression in paired samples of VAT and subcutaneous AT (SAT) as well as in liver and PBMC with the higher mRNA levels for *IL32* being observed in PBMC ($P < 0.001$). We showed increased *IL32* mRNA levels ($P < 0.01$) in VAT in obesity and obesity-associated T2D (Fig. 2*A*). Protein expression levels of IL-32 were also increased in OB patients ($P < 0.05$), although no statistical significant differences were reached in patients with T2D (Fig. 2*B*). In this regard, *IL32* mRNA levels were significantly correlated with BMI (r = 0.55; P = 0.010), BF (r = 0.26; P = 0.033), and waist circumference (r = 0.31; P = 0.013). Noteworthy, a significant association with circulating levels of IL-32 (r = 0.62; P = 0.002) was also observed. OB patients with NAFLD also showed twofold increased mRNA levels of *IL32* compared with volunteers without NAFLD in VAT (OB non-NAFLD: 1.00 ± 0.13 arbitrary units; and OB NAFLD: 2.13 ± 0.20 arbitrary units; $P < 0.001$). Gene expression levels of *IL32* in SAT were significantly increased ($P <$ 0.01) in OB patients with T2D compared with LN volunteers, but no differences were detected in their protein levels (Fig. 2*C* and *D*). A marked increase ($P < 0.01$) in gene expression levels of *IL32* was shown in PBMC in both OB groups (Fig. 2*E*), whereas no changes in *IL32* transcript levels in liver were observed independently of the presence of diabetes or NAFLD (Fig. 2*F*).

Because IL-32 specifically synergizes with the NOD2 ligand for the synthesis of proinflammatory cytokines (18), we analyzed the mRNA levels of *NOD2* in VAT from LN and OB patients. Gene expression levels of *NOD2* were significantly upregulated ($P < 0.01$) in both OB groups in VAT (Fig. 2*G*). Remarkably, gene expression levels of *IL32* were positively associated with mRNA levels of *NOD2* (r = 0.33; P = 0.039).

On the basis of the relevance of VAT in obesity-associated inflammation and the fact that IL-32 exhibited higher expression levels in this depot compared with SAT (Supplementary Fig. 2*A*) as well as a positive association with their circulating concentrations, the subsequent experiments were focused on this tissue. To gain further insight into the effect of VAT excess on inflammation, the presence of IL-32 in sections of VAT was confirmed by immunohistochemistry and immunofluorescence (Supplementary Fig. 2*B* and *C*). Both adipocytes and SVFC were immunopositive for IL-32, although a strong staining in SVFC was observed. No immunoreactivity was observed when the primary antibody was omitted. To corroborate which cell type preferentially contributed to the elevated *IL32* levels previously observed, adipocytes and SVFCs were isolated from VAT samples obtained from OB patients. Although *IL32* mRNA levels were readily evident in SVFCs ($P < 0.001$), gene expression was also detected in mature adipocytes (Supplementary Fig. 2*D*).

Increased Levels of Inflammation and ECM Remodeling–Related Genes in Human VAT in OB Subjects Are Related to *IL32*

Inflammation has long been suggested as being associated with obesity and its related comorbidities. Because levels of proinflammatory ILs are elevated in patients with obesity, we evaluated the association of IL-32 with other important ILs that regulate inflammation in VAT. Both groups of OB subjects exhibited higher expression levels ($P < 0.01$) of the inflammatory genes *IL1B*, *IL6*, and *IL10* compared with LN volunteers in VAT (Table 2). Moreover, mRNA levels of *IL1B* and *IL6* were also upregulated ($P < 0.05$) in patients with T2D compared with NG subjects. No differences were detected in the mRNA levels of the anti-inflammatory *IL13*, whereas *IL4* gene expression levels were only upregulated ($P < 0.05$) in OB NG patients. In this sense, *IL32* mRNA levels were significantly associated with the proinflammatory cytokines *IL1B* (r = 0.35; P = 0.005), *IL6* (r = 0.33; P = 0.013), and *IL10* (r = 0.55; $P < 0.001$).

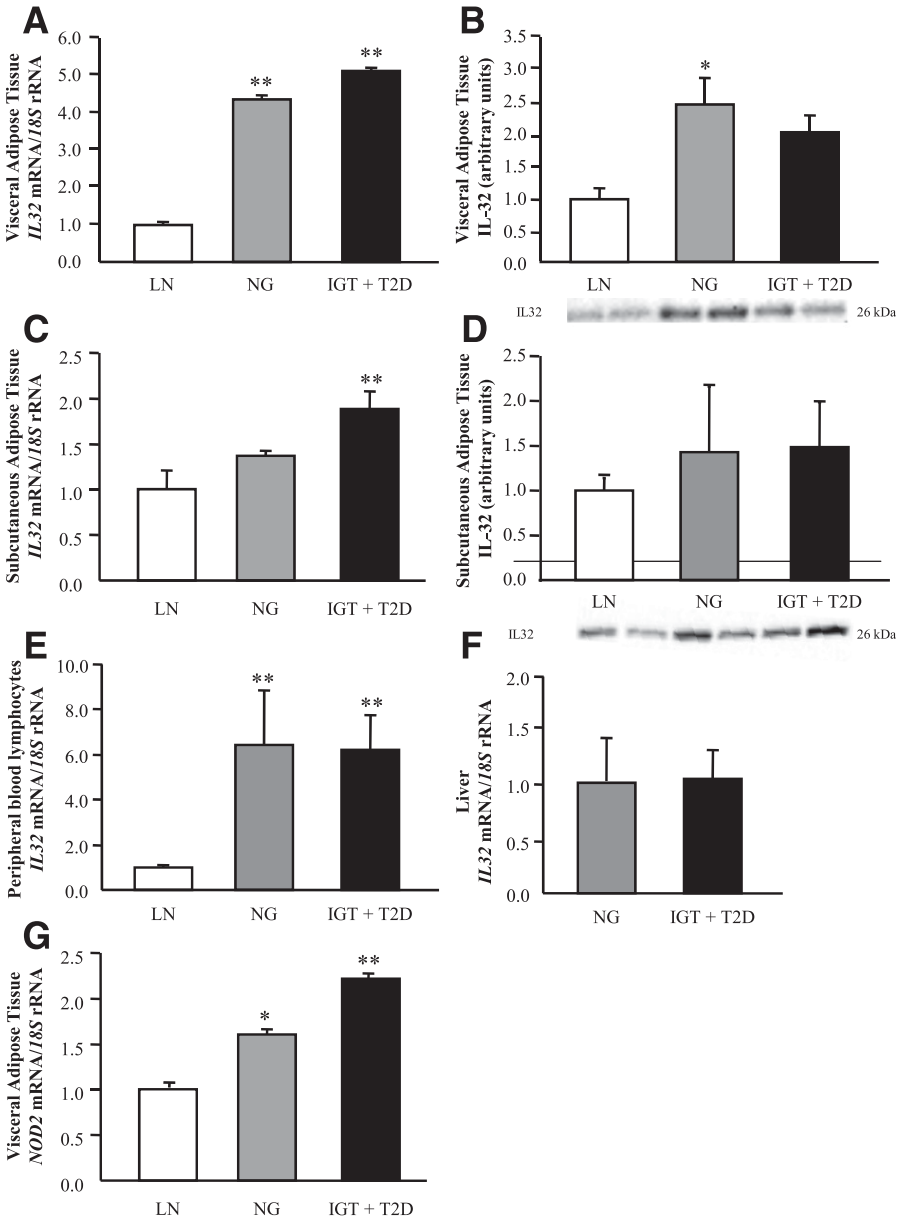

Figure 2—Impact of obesity and obesity-associated T2D on gene expression levels of IL-32 in metabolic active tissues. Bar graphs show the mRNA (LN, $n = 11$; OB NG, $n = 34$; OB T2D, $n = 31$) and protein (LN, $n = 10$; OB NG, $n = 15$; OB T2D, $n = 15$) levels of *IL32* in VAT (*A* and *B*) and SAT (*C* and *D*) as well as PBMC (*E*) and liver (*F*). *G*: Gene expression levels of *NOD2* in VAT of LN, OB NG, and OB T2D volunteers. Representative blots are shown at the bottom of the histograms. The intensity of the bands was normalized with total protein values. All assays were performed in duplicate. The gene and protein expression in LN subjects was assumed to be 1. Differences between groups were analyzed by one-way ANOVA followed by Tukey tests or unpaired two-tailed Student *t* tests, where appropriate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LN.

Because IL-32 is a versatile cytokine also involved in angiogenesis, we studied its association with important genes involved in hypoxia, angiogenesis, and ECM remodeling in VAT. OB subjects showed increased ($P < 0.05$) mRNA expression levels of the ECM remodeling genes matrix metalloproteinase 9 (*MMP9*), osteopontin (*SPP1*), Toll-like receptor 4 (*TLR4*), *TNF*, tenascin C (*TNC*), and transforming growth factor β1 (*TGFB1*), with the latter being also increased ($P < 0.01$) in OB patients with T2D compared with NG subjects (Table 2). We also found a positive correlation of *IL32* gene expression levels with hypoxia-inducible factor 1A (*HIF1A*) ($r = 0.27$; $P = 0.026$), *MMP9* ($r = 0.43$; $P < 0.001$), *SPP1* ($r = 0.79$; $P < 0.001$), *TGFB* ($r = 0.34$; $P = 0.004$), *TLR4* ($r = 0.30$; $P = 0.016$), and *TNC* ($r = 0.30$; $P = 0.014$).

Effects of Hypoxia and Inflammation-Related Factors in *IL32* Gene Expression Levels in Human Visceral Adipocytes

Because inflammation is a cardinal feature of OB VAT, we evaluated how LPS and TNF-α, well-known exogenous and endogenous inflammatory factors, respectively, influence *IL32* expression in human visceral adipocytes. Gene expression

Table 2—Analysis of gene expression levels of inflammation and ECM-related markers in VAT

Gene	LN	OB NG	OB IGT + T2D
IL1A	1.00 ± 0.34	0.85 ± 0.12	1.44 ± 0.34
IL1B	1.00 ± 0.53	2.13 ± 0.33*	6.25 ± 0.61**†
IL4	1.00 ± 0.31	1.89 ± 0.21*	1.86 ± 0.33
IL6	1.00 ± 0.40	5.09 ± 2.34**	6.19 ± 1.06***†
IL10	1.00 ± 0.51	2.79 ± 0.44***	4.92 ± 0.90***
IL13	1.00 ± 0.57	0.70 ± 0.10	1.68 ± 0.79
HIF1A	1.00 ± 0.20	1.49 ± 0.16	1.84 ± 0.23
MMP2	1.00 ± 0.15	1.36 ± 0.16	1.69 ± 0.22
MMP9	1.00 ± 0.55	2.57 ± 0.74*	3.76 ± 0.73**
SPP1	1.00 ± 0.25	7.18 ± 1.25**	5.33 ± 1.54*
TGFB	1.00 ± 0.19	1.42 ± 0.13	2.37 ± 0.25**††
TLR4	1.00 ± 0.11	1.63 ± 0.13*	1.75 ± 0.20*
TNC	1.00 ± 0.20	7.77 ± 1.77**	6.92 ± 1.27***
TNF	1.00 ± 0.39	1.30 ± 0.15*	1.41 ± 0.25

Analysis of mRNA levels in VAT of LN, OB NG, and OB T2D volunteers (LN: $n = 11$; OB NG: $n = 34$; and OB IGT + T2D: $n = 31$). Data represent the mean ± SEM of the ratio between the gene expression to *18S* rRNA. Differences between groups were analyzed by one-way ANOVA followed by Tukey post hoc tests. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. LN; †$P < 0.05$ and ††$P < 0.01$ vs. OB NG.

levels of *IL32* were strongly induced by LPS ($P < 0.001$) in visceral adipocytes (Fig. 3*A*). In the same line, as shown in Fig. 3*B*, TNF-α treatment significantly enhanced the mRNA levels of *IL32* at the highest dose. IL-4 and IL-13 are described as anti-inflammatory cytokines, so the effect of these molecules on *IL32* expression levels was also examined (Fig. 3*C* and *D*). A significant downregulation ($P < 0.05$) of gene expression levels of *IL32* after IL-4 and IL-13 treatment in human visceral adipocytes was observed.

Hypoxia plays a key role in the induction of inflammation-related adipokines in human adipocytes. Hypoxic effects can be mimicked by the divalent transition-metal ion cobalt. Therefore, the next experiments were performed in differentiated human adipocytes treated with $CoCl_2$ at concentrations of 50 and 100 nmol/L for 24 h. First, to assess whether human adipocytes respond to the $CoCl_2$, the mRNA level of vascular endothelial growth factor *A* (*VEGFA*) was examined as reference. The treatment with $CoCl_2$ induced a 5–10-fold increase ($P < 0.01$) in *VEGFA* mRNA level in the adipocytes (Fig. 3*E*). Next, we examined the effect on gene expression levels of *IL32*, which also exhibited a strong upregulation ($P < 0.01$), in the range from two- to eightfold (Fig. 3*E*).

IL-32 Induces the Expression of Inflammation and ECM Remodeling Markers in Human Visceral Adipocytes

We further explored whether IL-32α can activate the expression of genes involved in the inflammatory response and ECM remodeling in human adipocytes. Because IL-32γ has been described as the most potent isoform, we compared the response of human adipocytes to both isoforms. Cells were stimulated with increasing concentrations of IL-32α and IL-32γ for 24 h. As shown in Fig. 4, IL-32α treatment significantly enhanced ($P < 0.05$) the mRNA levels of the inflammatory markers *IL1B* and *TNF* in adipocytes. Moreover, no differences were found in the regulation of the anti-inflammatory markers *ARG1*, *MRC1*, *PPARG*, and *TRIB1*, critical for the differentiation of tissue-resident M2 macrophages (30). We also detected an increased expression ($P < 0.05$) of genes closely related to ECM remodeling such as *MMP9*, *SPP1*, and *TNC* after IL-32α treatment. In the same line, IL-32γ treatment (Fig. 5) significantly enhanced ($P < 0.05$) the mRNA levels of the inflammatory markers *IL1B*, *IL6*, *CCL2*, *COX2*, and *TNF* in adipocytes, and no differences were found in the regulation of the anti-inflammatory markers. We also detected an increased expression ($P < 0.05$) of genes closely related to ECM remodeling such as *HIF1A*, *CHI3L1*, *VEGFA*, *MMP9*, *SPP1*, and *TNC* after IL-32γ treatment.

To confirm the role of IL-32 in inflammation, we reduced the constitutive expression levels of IL-32α in human visceral adipocytes using a specific siRNA. As shown in Fig. 6, the inhibition of *IL32* expression using siRNA resulted in the downregulation ($P < 0.05$) of important inflammatory markers including *CCL2*, *TNF*, *SPP1*, and *IL1B*. We also showed an increase in mRNA levels of *TRIB1* after IL-32–siRNA treatment. No significant differences were found in the gene expression levels of *IL6*, *MMP9*, and *PPARG*.

Cross Talk Between Adipocytes and Monocytes in the Expression of IL-32

To determine whether adipocytes are able to induce the expression of IL-32 in PBMC, we studied the effects of the differentiated adipocyte CM from both LN and OB patients on human blood monocytes. A highly significant increase ($P < 0.01$) in the expression levels of *IL32* was observed in blood monocytes preincubated with the adipocyte-derived factors obtained from OB volunteers compared with blood monocytes pretreated with control media (Fig. 7*A*). Interestingly, adipocyte-CM from LN volunteers had no effect on mRNA *IL32* expression levels (Fig. 7*B*).

DISCUSSION

Inflammation in AT has been proposed as a key factor explaining the obesity-associated metabolic alterations (31). In this regard, IL-32 was initially identified as a cytokine with important roles in the amplification of inflammatory reactions (8,9). The current study suggests that the increased IL-32 expression in obesity promotes inflammation and ECM remodeling in VAT, contributing to the development of obesity-associated comorbidities. In this regard, we found that the increased circulating levels of IL-32 in human obesity and obesity-associated T2D decrease after weight loss. Consistently, we further show that the VAT

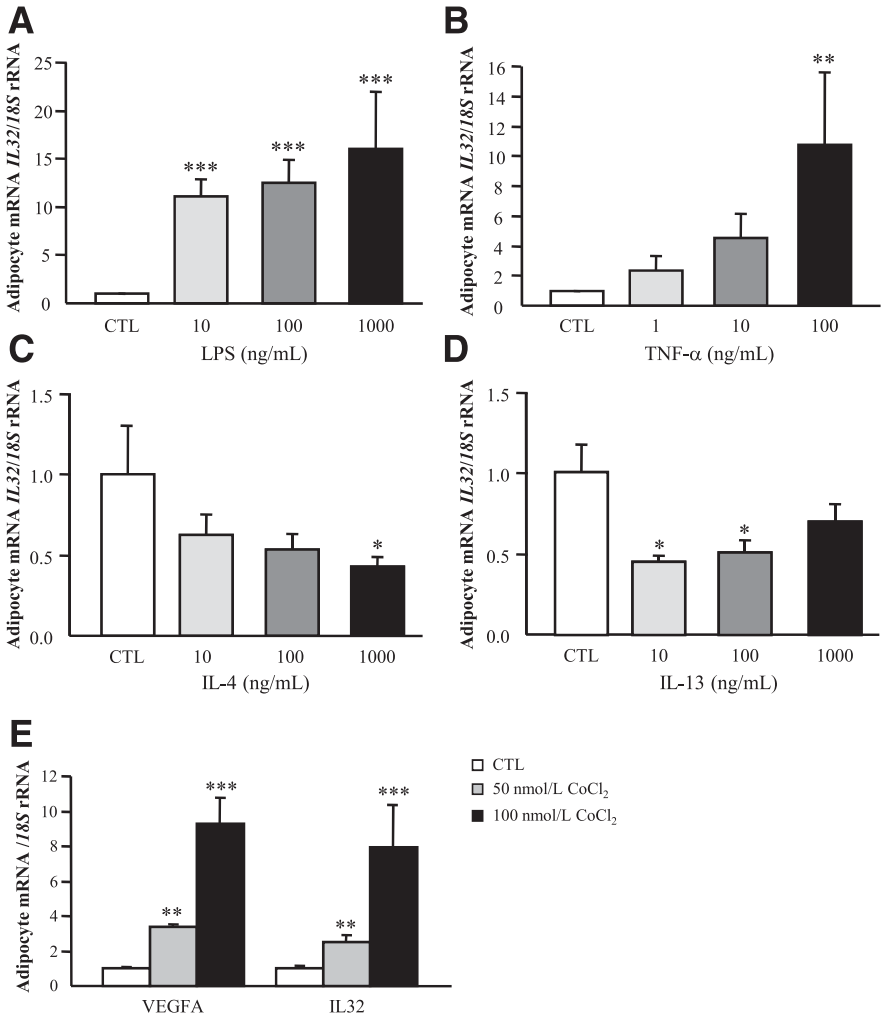

Figure 3—Effects of inflammation-related factors and hypoxia in *IL32* gene expression levels in human visceral adipocytes. Bar graphs show the effect of LPS (*A*), TNF-α (*B*), IL-4, (*C*), IL-13 (*D*), and $CoCl_2$ (*E*) incubated for 24 h on the transcript levels of *IL32* in human differentiated omental adipocytes. Gene expression levels in unstimulated cells were assumed to be 1. Values are the mean ± SEM (n = 6/group). Differences between groups were analyzed by one-way ANOVA followed by Tukey tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. unstimulated cells. CTL, control.

gene and protein expression levels of *IL32* are also upregulated in OB patients. Additionally, we reveal that IL-32 is potentially involved in enhancing AT low-grade inflammation and ECM remodeling as well as that *IL32* expression levels are regulated by hypoxia and inflammation-related factors. Moreover, we also demonstrate that adipocyte-CM stimulates the expression of *IL32* in human blood monocytes.

Increased circulating levels of IL-32 have been described in inflammation-related diseases such as autoimmune diseases, inflammatory bowel disease, and certain forms of cancer (9). The inflammatory condition associated with obesity is considered to play a major role in the pathogenesis of obesity-related morbidities. In the current study, we report for the first time that circulating concentrations of IL-32 are dramatically increased in OB patients. Moreover, circulating IL-32 was positively associated with BMI, waist circumference, and WHR. On the contrary, it has been recently reported that the body and liver weight as well as serum triglycerides of IL-32β transgenic mice were lower than that of wild-type mice on a high-fat diet and that hepatosteatosis was alleviated in this animal model (21). The different results may be due to the alternative splicing of IL-32 resulting in several isoforms with different or even opposite functions (17) or to species-specific differences. We also found that weight loss achieved by bariatric surgery was associated with a decrease in plasma IL-32. In this sense, the metabolic and hormonal changes taking place after RYGB may be influencing the IL-32 expression and/or secretion, as it occurs with other inflammatory markers such as CRP, plasminogen activator inhibitor-1, or serum amyloid A (32–35). Taken together, the decrease in circulating IL-32 concentrations after RYGB may reflect the beneficial effects not only on adiposity but also in the

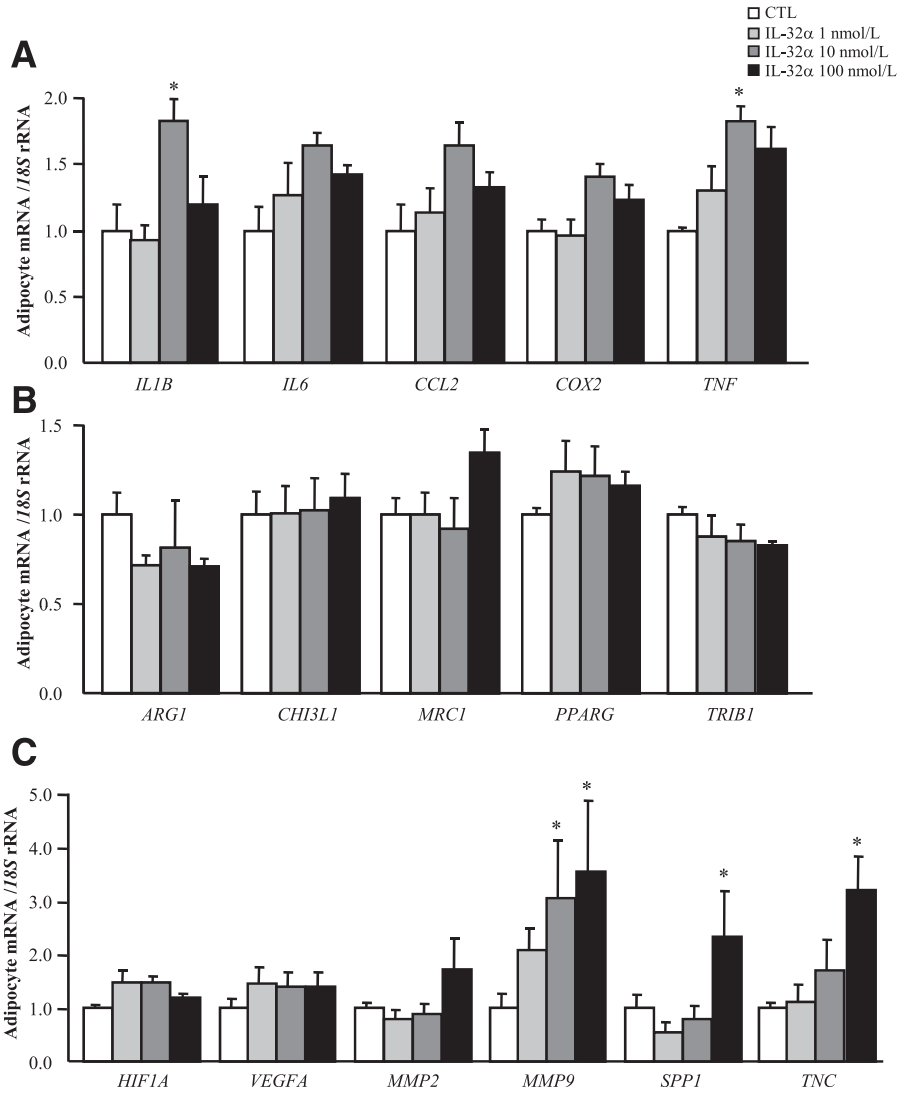

Figure 4—Effect of IL-32α treatment in human visceral adipocytes in the expression of inflammatory and ECM remodeling markers. Gene expression levels of proinflammatory (*A*) and anti-inflammatory (*B*) markers as well as ECM remodeling–related molecules (*C*) in human visceral adipocytes stimulated with recombinant IL-32α (1, 10, and 100 nmol/L) for 24 h. Gene expression levels in unstimulated cells were assumed to be 1. Values are the mean ± SEM (n = 6/group). Differences between groups were analyzed by one-way ANOVA followed by Tukey tests. *$P < 0.05$ vs. unstimulated cells. CTL, control.

amelioration of inflammation achieved by this bariatric surgery procedure (36). In this regard, the changes in IL-32 concentrations after RYGB were positively correlated with the reduction in BF and waist circumference. Diet-induced weight loss did not induce a statistically significant reduction in circulating levels of IL-32. In this line, we also showed that OB patients on the hypocaloric diet did not experience a significant decrease in CRP, another important inflammatory marker. It might be because our patients on the conventional treatment lost significantly less BF and weight compared with the patients in the RYGB group. In this sense, the potential existence of a threshold level for adiposity before any effect on the circulating concentrations of IL-32 may be put forward. It can be also hypothesized that dramatic changes in gut hormones observed after RYGB could modulate IL-32 levels via the gastro-entero-insular axis. However, the regulation of IL-32 by gut hormones has not been extensively studied, being a topic of future research. This heterogeneous response and the differences in the inflammatory response after these two ways of achieving weight loss still remain to be clarified.

OB patients exhibited increased gene and protein expression levels of *IL32* in VAT. The positive association between gene expression levels and circulating concentrations of IL-32 suggests that VAT contributes to the increased plasma IL-32 levels in obesity. We also observed higher mRNA levels of *IL32* in the SVFC of VAT compared with adipocytes, indicating that different cell types such as mononuclear or endothelial cells may produce this cytokine. In fact, *IL32* mRNA has been predominantly found

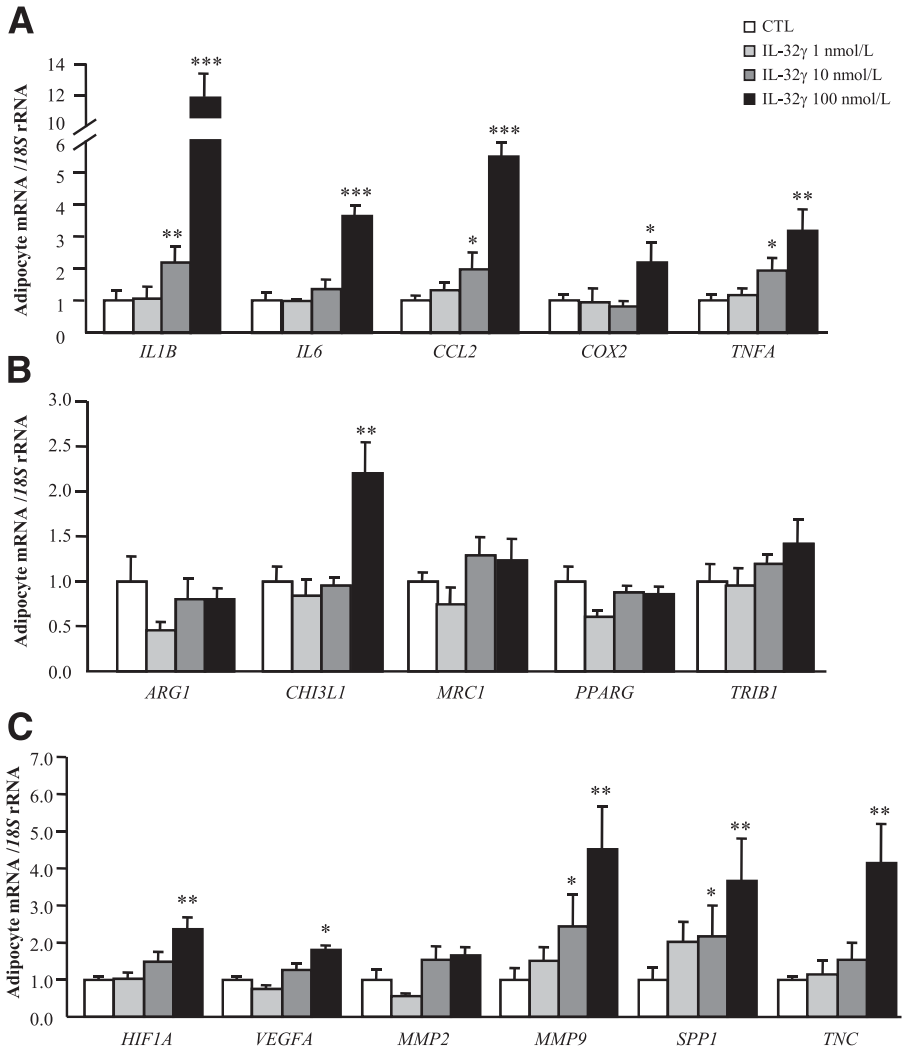

Figure 5—IL-32γ treatment induces the expression of inflammatory and ECM remodeling markers in human visceral adipocytes. Gene expression levels of proinflammatory (*A*) and anti-inflammatory (*B*) markers as well as ECM remodeling–related molecules (*C*) in human visceral adipocytes stimulated with recombinant IL-32γ (1, 10, and 100 nmol/L) for 24 h. Gene expression levels in unstimulated cells were assumed to be 1. Values are the mean ± SEM (n = 6/group). Differences between groups were analyzed by one-way ANOVA followed by Tukey tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. unstimulated cells. CTL, control.

in immune cells, but also in nonimmune cells such as epithelial cells (9). In this context, OB patients included in the study also showed increased levels of relevant inflammation (*IL1B*, *IL6*, *IL10*, and *TLR4*) and ECM remodeling–related (*MMP9*, *TGFB*, and *TNC*) genes in VAT, and their strong association with *IL32* expression levels underscores the involvement of this cytokine in AT inflammation.

The NOD family is constituted by intracellular recognition receptors for muropeptides, a component of peptidoglycans that elicit inflammation and immune reactions (18,37). NOD1 has been shown to be activated in AT of patients with metabolic syndrome, promoting an inflammatory signaling cascade and insulin resistance (38). Diet-induced obesity has been shown to increase NOD2 in hepatocytes and adipocytes, probably to counteract insulin resistance (39). We found an upregulation of mRNA levels of *NOD2* in the VAT from both groups of OB patients. In addition, we found a positive association between gene expression levels of *NOD2* and *IL32* in the VAT, which is in line with other studies demonstrating that IL-32 modulates NOD2 pathways (18).

The expression of IL-32 in monocytes, macrophages, or endothelial cells can be modulated by exposure to a plethora of stimuli including pathogen-related agents, such as LPS or different cytokines such as TNF-α or interferon-γ (9,40). In this regard, we not only found that the exposure of adipocytes to LPS and TNF-α induced the expression of *IL32*, but also that the anti-inflammatory cytokines IL-4 and IL-13 downregulated its mRNA levels. Importantly, hypoxia, a well-known inducer of inflammation in AT, also increased *IL32* gene expression levels in adipocytes. Hypoxia-induced reactive oxygen species has been reported to

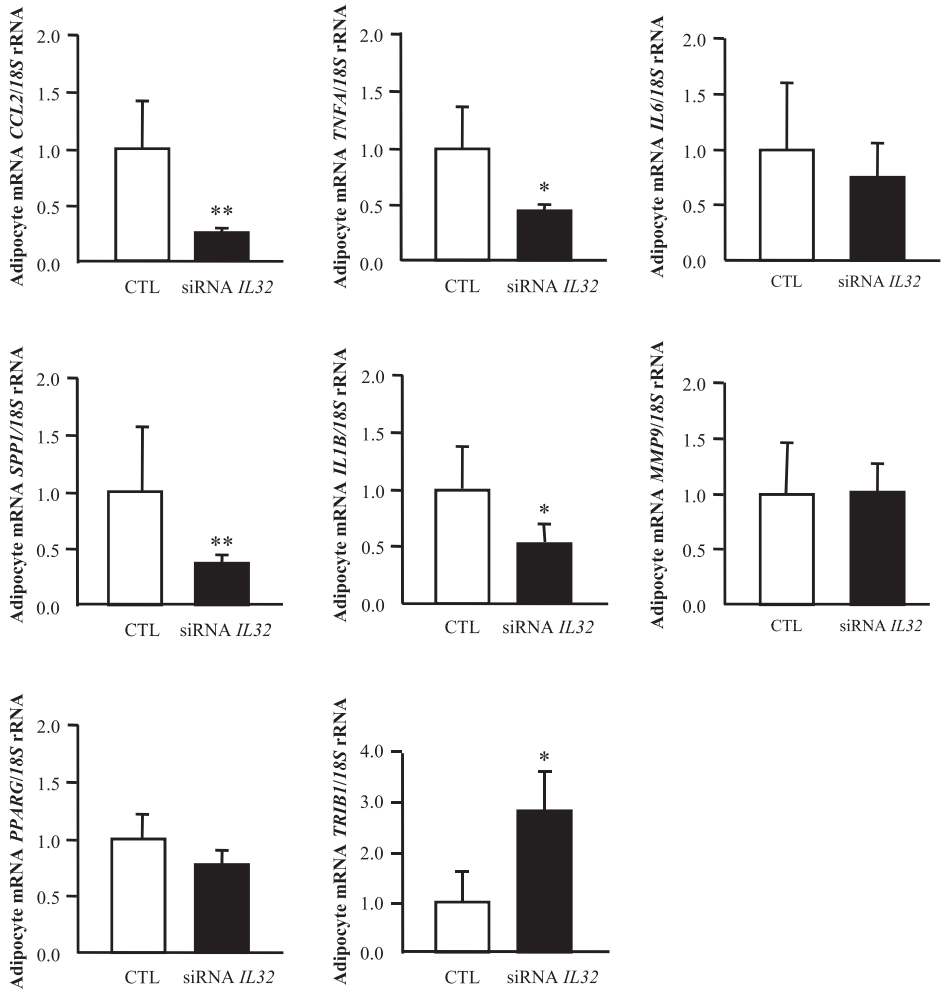

Figure 6—IL-32 silencing downregulates inflammation-related gene expression levels in human visceral adipocytes. mRNA expression levels of inflammation-related genes in human visceral adipocytes after transfection with 100 pmol/L siRNA IL-32/2 × 10^5 cells for 24 h. Gene expression levels in scrambled siRNA cells (CTL) were assumed to be 1. Values are the mean ± SEM (n = 6/group). Differences between groups were analyzed by unpaired two-tailed Student t tests. *P < 0.05, **P < 0.01 vs. scrambled siRNA. CTL, control.

increase the levels of IL-32β in breast cancer cells, resulting in enhanced glycolysis and Src activation (41). Because IL-32 is a significant driver of the inflammatory cascade, and proinflammatory stimuli also result in *IL32* overexpression, we hypothesized that IL-32 might be crucial for a positive-feedback loop in AT. We showed that both IL-32α and

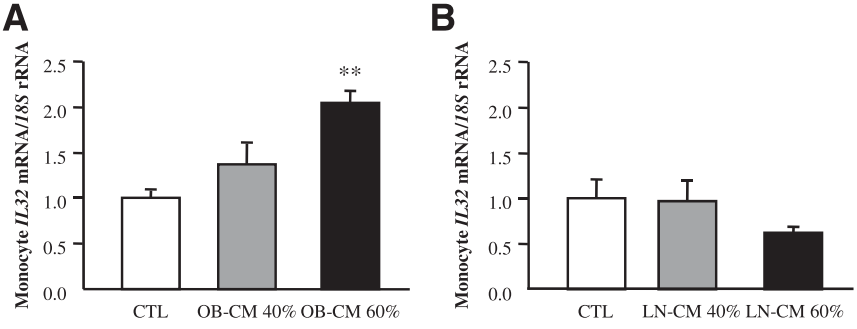

Figure 7—Adipocyte CM induces gene expression levels of *IL32* in human monocytes. Bar graphs show the effect of adipocyte CM (40 and 60%) from OB (*A*) and LN (*B*) volunteers incubated for 24 h on the transcript levels of *IL32* in human blood monocytes. Values are the mean ± SEM (n = 6/group). Differences between groups were analyzed by one-way ANOVA followed by Tukey tests. **P < 0.01 vs. unstimulated cells. CTL, control.

IL-32γ were biologically active and increased expression levels of typical inflammation markers, whereas no changes were found in mRNA levels of anti-inflammatory markers in adipocytes. In addition, higher mRNA levels of genes closely related to ECM remodeling after treatment with both isoforms were detected. We also showed that IL-32γ, the longest isoform, exhibited the highest biological activity, inducing a higher number of genes involved in inflammation and ECM remodeling. IL-32α gene silencing led to the downregulation of important inflammatory markers including *CCL2*, *TNF*, *SPP1*, and *IL1B*. Accumulating evidence indicates that IL-32 increases the expression levels of inflammatory cytokines such as TNF-α, IL-8, IL-6, and MIP-2 or VEGF in a wide range of cellular types (19,42). In this regard, a positive-feedback loop between IL-32 and other proinflammatory cytokines, leading to an increment of AT inflammation from OB patients, may be put forward.

Noteworthy, because the addition of IL-32 to primary human monocytes drives the differentiation into macrophage-type cells (20), we investigated the effect of adipocyte-CM on human monocyte cultures. Whereas a significant increase in the expression levels of *IL32* was observed with the adipocyte-derived factors obtained from OB volunteers, the adipocyte CM from LN volunteers had no effect on mRNA *IL32* expression levels, strengthening the potential involvement of IL-32 in the development of obesity-associated inflammation. Although the AT-derived IL-32 may result in a cross talk between AT and the immune system cells, suggesting a paracrine role of IL-32, the exact role of IL-32 in the polarization of AT macrophages into an M1 phenotype remains unclear.

Obesity-induced chronic inflammation occurs through complex mechanisms that are still not fully understood, and the cross talk between adipocytes and immune cells as well as their interaction with the local and systemic environment may shed light on the mechanisms by which inflammation contributes to the development of metabolic disease. In the current study, we described that the upregulated levels of IL-32 in human obesity might be implicated in its characteristic chronic proinflammatory state. In conclusion, IL-32 emerges as a nexus in AT biology at which the pathways of inflammation, ECM remodeling, and the development of obesity-associated comorbidities converge.

Acknowledgments. The authors thank all of the members of the Multidisciplinary Obesity Team, Clínica Universidad de Navarra, Pamplona, Spain, for the valuable collaboration. Centro de Investigación Biomédica en Red, Fisiopatología de la Obesidad y Nutrición, CIBEROBN, is an initiative of the Instituto de Salud Carlos III, Pamplona, Spain.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Funding. This work was supported by El Fondo de Investigación Sanitaria–Fondo Europeo de Desarrollo Regional (grants PI12/00515, PI13/00460, PI14/00950, PIE14/00045, and PI16/01217) from the Instituto de Salud Carlos III and Fundación Caja Navarra (20-2014).

Author Contributions. V.C. designed the study, collected and analyzed data, wrote the first draft of the manuscript, contributed to discussion, and reviewed the manuscript. J.G.-A. and A.R. collected and analyzed data, contributed to discussion, and reviewed the manuscript. B.R., R.M., and C.S. collected data, contributed to discussion, and reviewed the manuscript. V.V., M.F.L., and J.S. enrolled patients, collected data, contributed to discussion, and reviewed the manuscript. G.F. designed the study, enrolled patients, collected and analyzed data, wrote the first draft of the manuscript, contributed to discussion, and reviewed the manuscript. V.C. and G.F. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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