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# T Cell–Derived IL-22 Amplifies IL-1 $\beta$ –Driven Inflammation in Human Adipose Tissue: Relevance to Obesity and Type 2 Diabetes

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Proinflammatory cytokines are critically involved in the alteration of adipose tissue biology leading to deterioration of glucose homeostasis in obesity. Here we show a pronounced proinflammatory signature of adipose tissue macrophages in type 2 diabetic obese patients, mainly driven by increased NLRP3-dependent interleukin (IL)-1 $\beta$  production. IL-1 $\beta$  release increased with glycemic deterioration and decreased after gastric bypass surgery. A specific enrichment of IL-17- and IL-22-producing CD4<sup>+</sup> T cells was found in adipose tissue of type 2 diabetic obese patients. Coculture experiments identified the effect of macrophage-derived IL-1 $\beta$  to promote IL-22 and IL-17 production by human adipose tissue CD4<sup>+</sup> T cells. Reciprocally, adipose tissue macrophages express IL-17 and IL-22 receptors, making them sensitive to IL-17 and IL-22. IL-22 increased IL-1 $\beta$  release by inducing *pro-IL-1 $\beta$*  transcription through activation of C-Jun pathways in macrophages. In sum, these human data identified IL-1 $\beta$  and the T-cell cytokine IL-22 as key players of a paracrine inflammatory pathway previously unidentified in adipose tissue, with a pathological relevance to obesity-induced type 2 diabetes. These results provide an additional rationale for targeting IL-1 $\beta$  in obesity-linked type 2 diabetes and may have important implications for the conception of novel

combined anti-IL-1 $\beta$  and anti-IL-22 immunotherapy in human obesity.

A causal relationship between macrophage accumulation in adipose tissue and systemic insulin resistance has been clearly established in mouse studies, in which macrophage abundance can be manipulated through diet, genetic, or pharmacological intervention (1–3). In humans, however, the amount of adipose tissue macrophages is not consistently associated with variables reflecting alteration of glycemic status (4–6). Beside macrophages, growing emphasis is laid on T-cell populations, which vary in proportion and phenotype with obesity. Th2 and CD4<sup>+</sup> regulatory T cells with anti-inflammatory properties predominate in lean mice, while proinflammatory CD4<sup>+</sup> Th1 and CD8<sup>+</sup> T cells accumulate in adipose tissue of obese mice (reviewed elsewhere [7]). In human adipose tissue, the amount of proinflammatory T lymphocytes producing interferon (IFN)- $\gamma$  (8,9) or interleukin (IL)-17 (10) increased with the degree of adiposity. Moreover, IL-17- and IL-22-producing T cells accumulate in the adipose tissue of insulin-resistant obese subjects (11).

Recently, adipose tissue macrophages were found capable of processing antigens, thereby promoting the

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expansion of antigen-specific Th1 skewed CD4<sup>+</sup> T cells in diet-induced obese mice (12). Surprisingly, treatment of obese mice with major histocompatibility complex II-neutralizing antibodies did not improve whole-body glucose homeostasis or reduce inflammatory gene expression in the adipose tissue (12). Thus, once established, chronic adipose tissue inflammation may be fostered by local proinflammatory interplays independent of antigen presentation.

In the current study, we identified IL-1 $\beta$  as the main macrophage-derived cytokine increased in type 2 diabetic obese subjects, enhancing the release of IL-17 and IL-22 by adipose tissue CD4<sup>+</sup> T cells. Reciprocally, IL-22 upregulated *pro-IL-1 $\beta$*  transcription, leading to enhanced IL-1 $\beta$  production by adipose tissue macrophages. High levels of cytokine production in cells obtained from type 2 diabetic obese patients emphasize the pathological relevance of this paracrine inflammatory pathway hitherto unidentified in visceral adipose tissue.

## RESEARCH DESIGN AND METHODS

### Human Subjects and Adipose Tissue Sampling

We enrolled three groups of participants. Groups 1 and 2 were composed of morbidly obese subjects eligible for laparoscopic Roux-en-Y gastric bypass (RYGP) surgery and included in a larger population previously described (13). Obese subjects of group 1 were segregated in nondiabetic (OB) and type 2 diabetic patients (OB/D) based on fasting glycemia over 7 mmol/L or use of antidiabetic drugs. All OB/D patients were treated with metformin, including seven patients with additional insulin. In group 2, the effect of weight loss was investigated at 3, 6, and 12 months after surgery. Four type 2 diabetic patients were treated with metformin, including one patient treated with insulin. Group 3 was composed of nonobese controls (NO) involved in programmed surgery for hernia, nissen fundoplication, or gallbladder ablation. NO participants were selected without dyslipidemia, type 2 diabetes, or chronic inflammatory or infectious diseases. In groups 1 and 3, subcutaneous and visceral adipose tissue biopsies were sampled during surgery. In group 2, subcutaneous adipose tissue biopsies were collected by periumbilical surgical incision of the skin under local anesthesia at the time of RYGP and 3, 6, and 12 months after surgery. The study was conducted in accordance with the Helsinki Declaration and was registered in a public trial registry (<http://clinicaltrials.gov/ct2/show/NCT00476658?term=poitou&rank=1>). The Ethics Committee (CPP Ile-de-France) approved the clinical investigations for both obese and nonobese individuals. All subjects provided written informed consent when included in the surgery program. Characteristics of all subjects are shown in Table 1.

### Adipose Tissue Explants

Adipose tissue biopsies (0.1 g) were minced and incubated in 1 mL of endothelial cell basal medium (Promocell,

Heidelberg, Germany) containing 1% BSA, penicillin (100 units/mL), and streptomycin (100 mg/mL) for 24 h. Tissue viability was checked using lactate dehydrogenase assay according to manufacturer's instructions (Biovision, Milpitas, CA). Conditioned media were analyzed for cytokines using 27 Bio-Plex Pro Human Cytokine (BioRad, Munich, Germany). Immunoselected cell and coculture supernatant were analyzed by specific ELISA or by the Luminex technology (Milliplex Map Kit, Human Th17 Magnetic Bead Panel, Millipore, Billerica, MA).

### Adipose Cell Isolation

Adipocytes and cells of the stroma vascular fraction were obtained by collagenase digestion of adipose tissue (14). Stroma vascular fraction cells were resuspended in endotoxin-free PBS supplemented with 2% FCS and 1 mmol/L EDTA. Isolation of CD14<sup>+</sup>, CD3<sup>+</sup>, and CD4<sup>+</sup> cells was performed using positive selection magnetic beads (Stemcell Technologies, Vancouver, Canada). The CD14<sup>-</sup>CD3<sup>-</sup> cells (Neg) were also recovered. Fraction enrichment was verified by gene expression of leptin for adipocytes, CD3 for T cells and CD68 for macrophages. Cell viability was measured by flow cytometry in CD3<sup>+</sup>, CD4<sup>+</sup>, and CD14<sup>+</sup> cells before culture experiments using the fixable Viability Dye eFluor 506 (eBioscience, San Diego, CA). Cell viability was over 80% whatever the donor's phenotype.

### DNA Microarray

An Illumina (San Diego, CA) RNA Amplification Kit (NuGEN, BiotinIL Module) was used according to the manufacturer's instructions to obtain biotin-labeled cDNA from 50 ng of total RNA extracted from adipose tissue CD14<sup>+</sup> cells. Hybridization was performed onto Illumina Human HT-12 v3.0 Expression BeadChips containing probes for 48,000 transcripts. Differential gene expression was determined with a standard analysis of microarray analysis using a false discovery rate of ~10%. We performed a functional analysis of these gene lists using the FunNet tool (15) (<http://www.funnet.info/>). Microarray experiments were performed according the Minimum Information About a Microarray Experiment guidelines. Data have been deposited in National Center for Biotechnical Information's Gene Expression Omnibus under accession number GSE54350.

### Cell Culture

CD14<sup>+</sup>, CD3<sup>+</sup>, and CD4<sup>+</sup> cell supernatants (10<sup>6</sup> cells/mL) were obtained after 48 h of culture in RPMI 1640 medium (Lonza, Berkshire, U.K.) supplemented with 10% FCS (HyClone, Thermo Fisher Scientific, San Jose, CA), plate-bound anti-CD3 (2.5  $\mu$ g/mL; UCHT1), and soluble anti-CD28 (1  $\mu$ g/mL; 37407) antibodies (R&D Systems, Minneapolis, MN). For coculture experiments, CD14<sup>+</sup> cells were cultured with autologous CD3<sup>+</sup> or CD4<sup>+</sup> for 48 h at 1/1 ratio in the same medium. When indicated, cells were cultured with 10, 50, or 100  $\mu$ mol/L glyburide (Sigma-Aldrich, St. Louis, MO), with recombinant IL (rIL)-1 $\beta$  (10 ng/mL), IL-17 (20 ng/mL), IL-22 (20 ng/mL), or

**Table 1—Bioclinical variables of three groups of study participants**

	Group 1		Group 2				Group 3
	OB	OB/D	OB-M0	OB-M3	OB-M6	OB-M12	NO
Months postsurgery	0	0	0	3	6	12	0
Number of subjects	41	31	20	20	20	9	10
Sex (female/male)	33/8	17/14	15/5	15/5	15/5	9/0	5/5
Age (years)	41.6 ± 1.9	47.5 ± 1.9†	41.9 ± 2.9	—	—	—	60.1 ± 4.6
BMI (kg/m <sup>2</sup> )	47.5 ± 1.2	48.1 ± 1.3	50.7 ± 2.2	42.0 ± 1.8*	38.4 ± 1.7*	34.8 ± 1.7*	26.1 ± 1.4
Type 2 diabetic subjects (n)	0	31	4	1	1	0	0
Glycemia (mmol/L)	5.4 ± 0.1	7.7 ± 0.7†	5.9 ± 0.2	5.1 ± 0.2*	4.7 ± 0.1*	4.8 ± 0.3*	4.9 ± 0.4
Insulinemia (μU/mL)	26.1 ± 4.0	22.4 ± 3.2	27.1 ± 3.3	10.9 ± 0.9*	10.4 ± 1.3*	10.8 ± 1.1*	—
HbA <sub>1c</sub> (%)	5.7 ± 0.1	7.5 ± 0.2†	6.1 ± 0.2	5.6 ± 0.1*	5.5 ± 0.1*	5.7 ± 0.2*	—
HbA <sub>1c</sub> (mmol/mol)	39.3 ± 0.7	56.7 ± 3.2	43.1 ± 2.1	37.4 ± 1.2	37.2 ± 0.9	38.4 ± 2.2	—
Leptin (ng/mL)	83.1 ± 5.7	65.8 ± 6.5†	49.6 ± 6.0	26.5 ± 3.3*	20.7 ± 2.3*	23.3 ± 2.9*	—
Adiponectin (μg/mL)	4.7 ± 0.4	3.4 ± 0.2†	5.1 ± 0.5	5.1 ± 0.5	5.1 ± 0.5	4.9 ± 0.5	—
CD68 <sup>+</sup> cells in SAT (n per 100 adipocytes)	11.1 ± 0.6	12.13 ± 1.3	12.1 ± 1.0	11.7 ± 1.5	8.7 ± 0.9*	11.4 ± 1.6	4.6 ± 1.7
CD68 <sup>+</sup> cells in VAT (n per 100 adipocytes)	13.5 ± 0.7	15.3 ± 1.1	13.2 ± 1.2	—	—	—	6.0 ± 2.2
Number of subjects with CLS in SAT	28	23	14	7	5	3	—

Data are shown as mean ± SEM. CLS, crown-like structure; M0, month 0; M3, month 3; M6, month 6; M12, month 12; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue. †*P* < 0.05 versus OB. \**P* < 0.05 versus OB-M0.

IFN- $\gamma$  (20 ng/mL; all from Miltenyi Biotec); anti-IL-1 $\beta$  (2.5  $\mu$ g/mL), anti-IL-17 (50 ng/mL), or anti-IL-22 (100 ng/mL; all from eBioscience); anti-IFN- $\gamma$  (100 ng/mL; R&D Systems); or Jun NH<sub>2</sub>-terminal kinase (JNK) inhibitor (SP600125; Sigma-Aldrich). The respective isotype controls were used at the same concentration. Cell viability was estimated by the release of lactate dehydrogenase. Cell cytotoxicity nonstatistically ranged from 5 to 15% of the value obtained in cell lysate positive control, whatever the culture conditions and donor's phenotype.

#### Human Monocyte-Derived Macrophages and THP1 Cell Line

Human peripheral blood mononucleated cells were isolated from enriched buffy coats of healthy volunteer donors. Monocytes were purified by negative selection using Monocyte Isolation Kit II (Miltenyi Biotec). Monocytes were differentiated for 6 days in RPMI 1640 medium supplemented with 10% FCS and 100 ng/mL of macrophage-colony stimulating factor (Miltenyi Biotec) to obtain nonpolarized monocyte-derived macrophages (MDM). THP1-defNLRP3 cells are human monocytes with reduced NLRP3 activity (InvivoGen, San Diego, CA). THP1-defNLRP3 and wild-type THP1 cells were cultured according to manufacturer's instructions.

#### Real-Time PCR

Total RNA was extracted using the RNeasy RNA Mini Kit (Invitrogen, Carlsbad, CA). cDNAs were synthesized from and prepared with Superscript II reverse transcriptase (Invitrogen). SYBR green primers were used for

quantitative real-time PCR using the 7300 real-time PCR system (Applied Biosystem, Austin, TX). 18S was used for normalization and for relative quantification of gene expression.

#### Western Blot Analysis

Samples for Western blot analyses were diluted to a concentration of 20  $\mu$ g of protein per 15  $\mu$ L and heated at 70°C for 10 min. Membranes were imaged using a LiCor Odyssey scanner. Boxes were manually placed around each band of interest, which returned near-infrared fluorescent values of raw intensity with intralane background subtracted using Odyssey 3.0 analytical software (LiCor, Lincoln, NE). The following antibodies were used: anti-NLRP3 (Cryo-2, AdipoGen, San Diego, CA) and anti-IL-17RA, anti-IL-22RA1, and anti- $\beta$ -actin (all from Abcam, Cambridge, U.K.).

#### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed as described in detail previously (16). Quantification of the precipitated DNA regions was performed by PCR. Primer sequences are listed for IL-1 $\beta$  promoter: 5'-TCCTCCCTTAGTGCCAACTATGT-3' (forward) and 5'-ACAGTCTCCACAGTTCTGCCATGT-3' (reverse) and tumor necrosis factor (TNF)- $\alpha$  5'-GCTTCCTCCAGATGAGCTT-3' (forward) and 5'-TGCTGTCCTTGCTGAGGGA-3' (reverse). The following polyclonal antibodies were used: antipolymerase 2 (H224, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-NF- $\kappa$ B p65 (acetyl K310, Abcam), anti-C-Jun (AP-1; Abcam), and normal rabbit IgG (Santa Cruz).

### Caspase-1 Assay

Caspase-1 activity was assessed using the Caspase-1 Colorimetric Assay Kit (Abcam) according to the manufacturer's instructions.

### Cell Surface Marker and Intracellular Cytokine Staining

Pacific Blue or BV421-labeled anti-CD4 (RPA-T4, BD Biosciences, San Jose, CA), PerCP-Cy5.5-labeled anti-CD8 (RPA-T8, eBioscience), phycoerythrin (PE)-labeled anti-IL-1-R1 (FAB269P, R&D Systems), and appropriate isotype controls were used to characterize freshly immunoselected adipose tissue CD3<sup>+</sup> cells. allophycocyanine-eFluor780-labeled anti-CD45 (HI30, eBiosciences), PE-Cy7-labeled anti-CD14 (M5E2, BD Biosciences), PE-labeled anti-IL-17RA (133617, R&D Systems), allophycocyanine-labeled anti-IL-22RA1 (305405, R&D Systems), and appropriate isotype controls were used to define receptor expression in CD14<sup>+</sup> cells. After 15 min incubation with Fc block (eBioscience), cells were suspended in fluorescence-activated cell sorter (FACS) buffer and stained with appropriate antibodies or isotype controls for 30 min at 4°C in the dark. Intracellular cytokine analysis of CD3<sup>+</sup> cells was performed in cells stimulated with phorbol-12-myristate-13-acetate (30 ng/mL; Sigma-Aldrich) and ionomycin (1 µg/mL; Alexis Biochemicals, San Diego, CA) at 37°C for 6 h with GolgiStop (BD Biosciences). Cells were then stained for CD4 and CD8 surface markers, permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and incubated for 40 min at room temperature in the dark with PE-labeled anti-IL-17A (ebio64CAP17, eBioscience), fluorescein-isothiocyanate-labeled anti-IFN-γ (4S.B3, BD Biosciences), Alexa647-labeled anti-IL-21 (3A3-N2.1, BD Biosciences), or PE-Cy7-labeled anti-IL-22 (22URTI, eBioscience). Cells were washed twice with FACS buffer (PBS with 0.5% BSA and 2 mmol/L EDTA) after staining and then analyzed on a FACS LSRII flow cytometer (BD Biosciences) using FACS Diva software (BD Biosciences). Data analysis was performed with Flow Jo 9.4 software (Tree Star, Ashland, OR).

### Statistical Analyses

Data are shown as mean ± SEM. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA). Differences between two groups were determined by nonparametric paired Wilcoxon or unpaired Mann-Whitney comparative tests. Correlations were assessed by Pearson's test. For visual purpose, straight lines were added based on linear regression analysis. Differences were considered significant when  $P < 0.05$ .

## RESULTS

### IL-1β Production by Adipose Tissue CD14<sup>+</sup> Macrophages

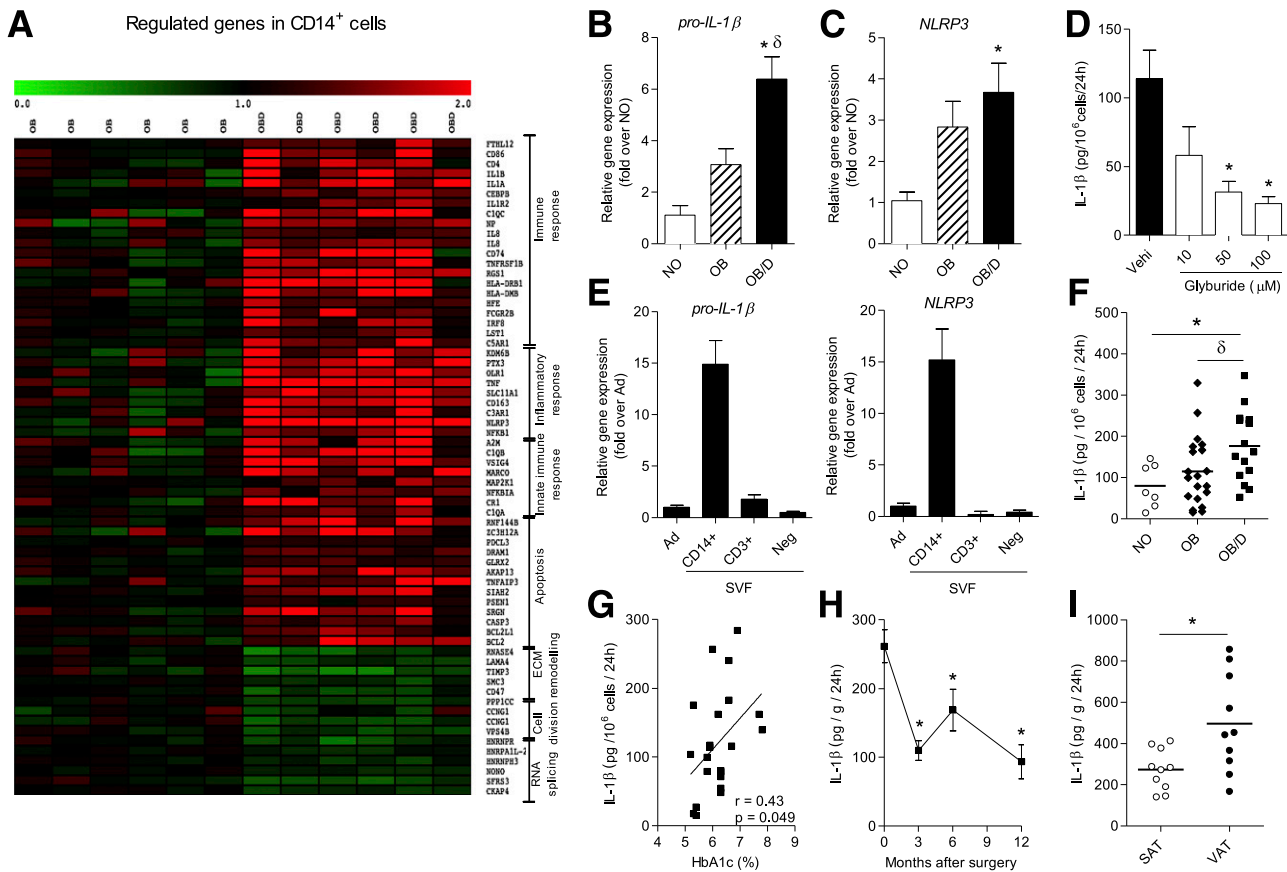
We performed genome-wide profiling of CD14<sup>+</sup> cells isolated from visceral adipose tissue of age- and sex-matched OB and OB/D subjects. In OB/D patients, 167 genes marked the expression pattern of adipose CD14<sup>+</sup> cells,

while 70 genes were characteristic of CD14<sup>+</sup> cells in non-diabetic OB individuals. Functional gene ontology annotation revealed that genes upregulated in CD14<sup>+</sup> cells from OB/D patients were mainly grouped in immune and inflammatory response pathways (Fig. 1A and Supplementary Fig. 1). *Pro-IL-1β* and *NOD-like receptor 3 (NLRP3)* were among the top 10 upregulated genes (Supplementary Fig. 1). We confirmed increased *pro-IL-1β* gene expression in adipose CD14<sup>+</sup> cells of OB/D versus OB in a larger group of subjects (Fig. 1B). *Pro-IL-1β* and *NLRP3* mRNA levels were threefold to sixfold higher in CD14<sup>+</sup> cells of OB/D than nonobese (NO) subjects (Fig. 1B and C). When adipose tissue CD14<sup>+</sup> cells were cultured in presence of the NLRP3 inhibitor glyburide, IL-1β release was dose-dependently inhibited (Fig. 1D), in line with the well-established requirement of NLRP3 for caspase-1-mediated processing of pro-IL-1β. Of note, TNF-α release was not significantly altered in the same culture experiments (Supplementary Fig. 2A). Supporting adipose tissue macrophages as the major cellular source of IL-1β, the CD14<sup>+</sup> cell-enriched fraction expressed 15-fold more *pro-IL-1β* and *NLRP3* mRNA than any other adipose tissue cellular fractions (Fig. 1E).

Several sets of observation support a relationship between adipose tissue IL-1β and altered glycemic status in human obesity. First, we showed that release of IL-1β was significantly higher in CD14<sup>+</sup> macrophages freshly isolated from adipose tissue biopsies of OB/D compared with NO or OB subjects (Fig. 1F) and was positively correlated with glycated hemoglobin (HbA<sub>1c</sub>) values of the donor (Fig. 1G). Second, in a kinetic study of cytokines released by adipose tissue biopsies obtained surgically at month 3, 6, and 12 after RYGB, IL-1β release decreased steadily (Fig. 1H), while glycemic variables improved in obese subjects (Table 1). By contrast, TNF-α production in the same explants was not significantly changed with weight loss (Supplementary Fig. 2B). Finally, we found that IL-1β release was significantly higher in visceral than in subcutaneous adipose tissue (Fig. 1I), suggesting a prominent role of visceral adipose tissue linking IL-1β release to deterioration of glycemic status.

### IL-17<sup>+</sup> and IL-22<sup>+</sup> CD4<sup>+</sup> T Cells in Adipose Tissue

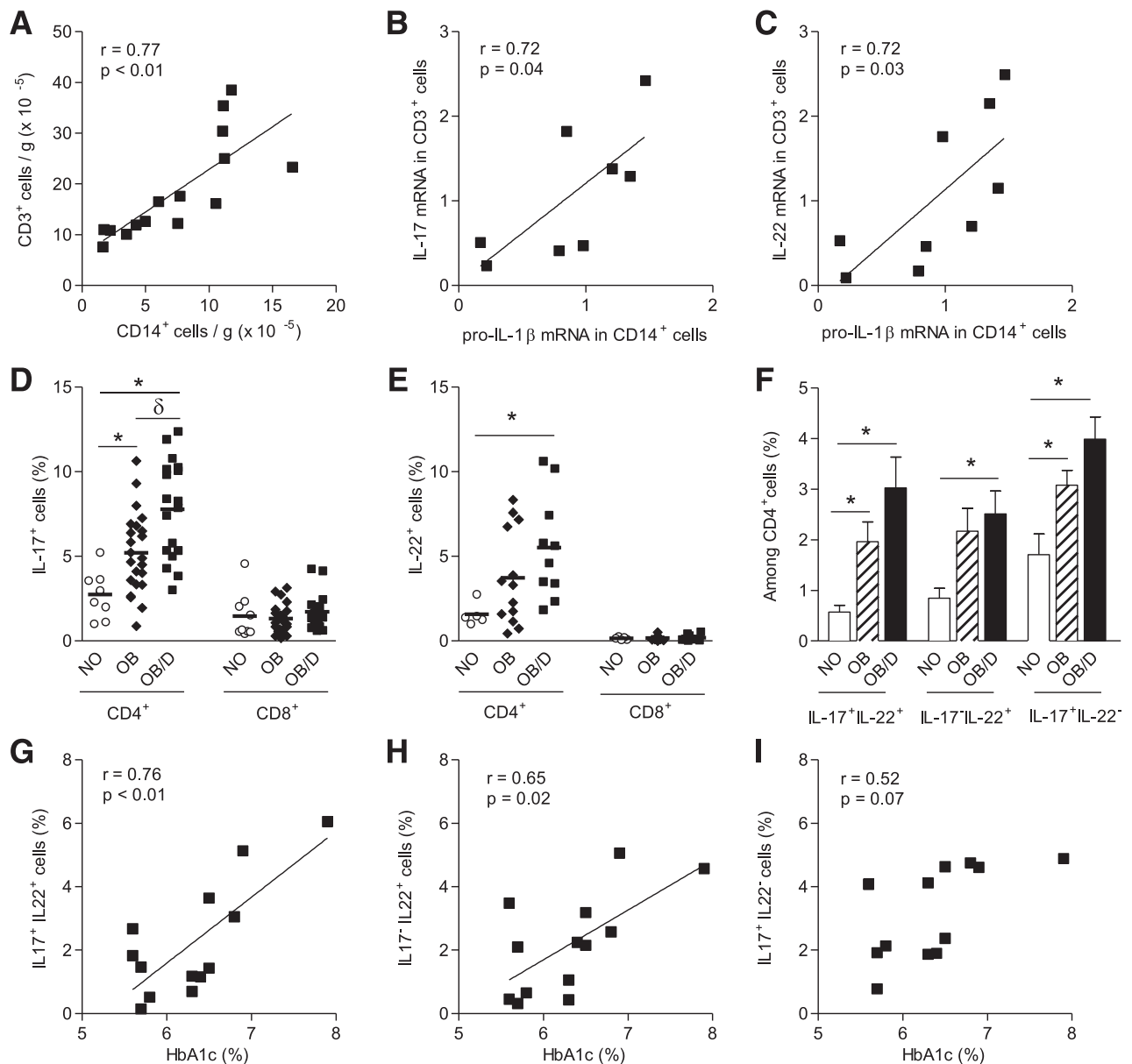
Considering that IL-1β is a master regulator of Th17 expansion in humans (17), we ought to identify adipose tissue T-cell populations potentially affected by increased IL-1β production in type 2 diabetic patients. In support of an inflammatory dialogue between macrophages and T cells in adipose tissue, the numbers of CD14<sup>+</sup> and CD3<sup>+</sup> cells isolated from the same biopsy were tightly correlated (Fig. 2A). To identify adipose tissue T-cells populations, we explored their cytokine signature, focusing on Th1 (IFN-γ) and Th17-related cytokines (IL-17 and IL-22). At gene level, the three cytokines were found to be expressed mostly in the CD3<sup>+</sup> cells enriched fraction (Supplementary Fig. 3). *Pro-IL-1β* mRNA in CD14<sup>+</sup> cells was strongly correlated with the levels of IL-17 and IL-22



**Figure 1**—Transcriptomic signature of adipose tissue CD14<sup>+</sup> cells in OB/D patients. **A**: Genome-wide mRNA expression analysis using microarrays was performed in visceral adipose tissue CD14<sup>+</sup> cells isolated from OB (*n* = 6) and OB/D (*n* = 6) subjects included in group 1. Age and BMI did not differ between the groups. Heat map representation of the genes significantly upregulated (red) or downregulated (green) in adipose tissue CD14<sup>+</sup> cells isolated from OB and OB/D subjects. Genes are ordered by gene ontology annotations. **B** and **C**: Quantitative PCR analyses of *pro-IL-1β* and *NLRP3* in CD14<sup>+</sup> cells isolated from adipose tissue of NO (*n* = 4), OB (*n* = 16), and OB/D (*n* = 12) subjects. \**P* < 0.05 versus NO; δ*P* < 0.05 versus OB. **D**: IL-1β production by CD14<sup>+</sup> cells isolated from adipose tissue of obese subjects (*n* = 5) and treated with incremental doses of NLRP3-inhibitor glyburide. \**P* < 0.05 versus vehicle using Wilcoxon matched pairs test. **E**: Quantitative PCR analyses of *pro-IL-1β* and *NLRP3* expression in cell fractions prepared from adipose tissue biopsies of obese subjects (*n* = 10). **F**: IL-1β secretion by adipose tissue CD14<sup>+</sup> cells of NO (*n* = 7), OB (*n* = 20), and OB/D (*n* = 15) subjects. Means are shown as horizontal line. δ*P* < 0.05 versus OB. **G**: Correlations between IL-1β production by adipose tissue CD14<sup>+</sup> cells isolated from obese subjects (*n* = 22) and HbA<sub>1c</sub> (percentage). The *r* correlation coefficient and *P* values obtained by Pearson’s test are indicated. **H**: Production of IL-1β by adipose tissue explants obtained from obese subjects at the time of RYGP surgery (month 0) and at months 3, 6 (*n* = 20), and 12 (*n* = 9). \**P* < 0.05 versus month 0. **I**: Comparison of IL-1β production by subcutaneous and visceral adipose tissue explants obtained from the same obese subjects (*n* = 10). \**P* < 0.05 using Wilcoxon matched pairs test. All data are shown as mean ± SEM. Ad, adipocytes; ECM, extracellular matrix; Neg, CD14<sup>-</sup>CD3<sup>-</sup> cells; SAT, subcutaneous adipose tissue; SVF, stroma vascular fraction; Vehi, vehicle; VAT, visceral adipose tissue.

gene expression in autologous T cells (Fig. 2B and C), suggesting coordinated regulation of these T-cell cytokines and IL-1β in the human adipose tissue. Further FACS analyses revealed a high prevalence of IFN-γ-expressing cells in both CD4<sup>+</sup> (83.7 ± 1.1%; *n* = 50) and CD8<sup>+</sup> (91.1 ± 0.8%; *n* = 50) subsets, whose frequencies were independent of BMI or glycemic status (data not shown). Although less abundant, IL-17- and IL-22-producing cells increased in percentage within the CD4<sup>+</sup> population in OB compared with NO subjects, with an additional effect of type 2 diabetes on IL-17<sup>+</sup> cell prevalence (Fig. 2D and E). A small proportion of CD8<sup>+</sup> cells was IL-17 or IL-22 positive, with no change according to the donor’s weight

or glycemic status. Of note, T cells producing IL-21, an additional typical Th17-like cytokine, were not detected in human adipose tissue (data not shown). Thorough analysis of adipose CD4<sup>+</sup> cell subsets showed that the percentages of single IL-17<sup>+</sup>-IL-22<sup>-</sup> and IL-17<sup>-</sup>-IL-22<sup>+</sup> cells and double IL-17<sup>+</sup>-IL-22<sup>+</sup> cells all increased with obesity (Fig. 2F) and in the relation with glycemic deterioration assessed by increased HbA<sub>1c</sub> values (Fig. 2G–I). These data identify CD4<sup>+</sup> T-cell populations characterized by IL-17<sup>+</sup> and/or IL-22<sup>+</sup> production that accumulate in visceral adipose tissue along with macrophages and are likely to contribute to altered glycemic status in obese subjects.

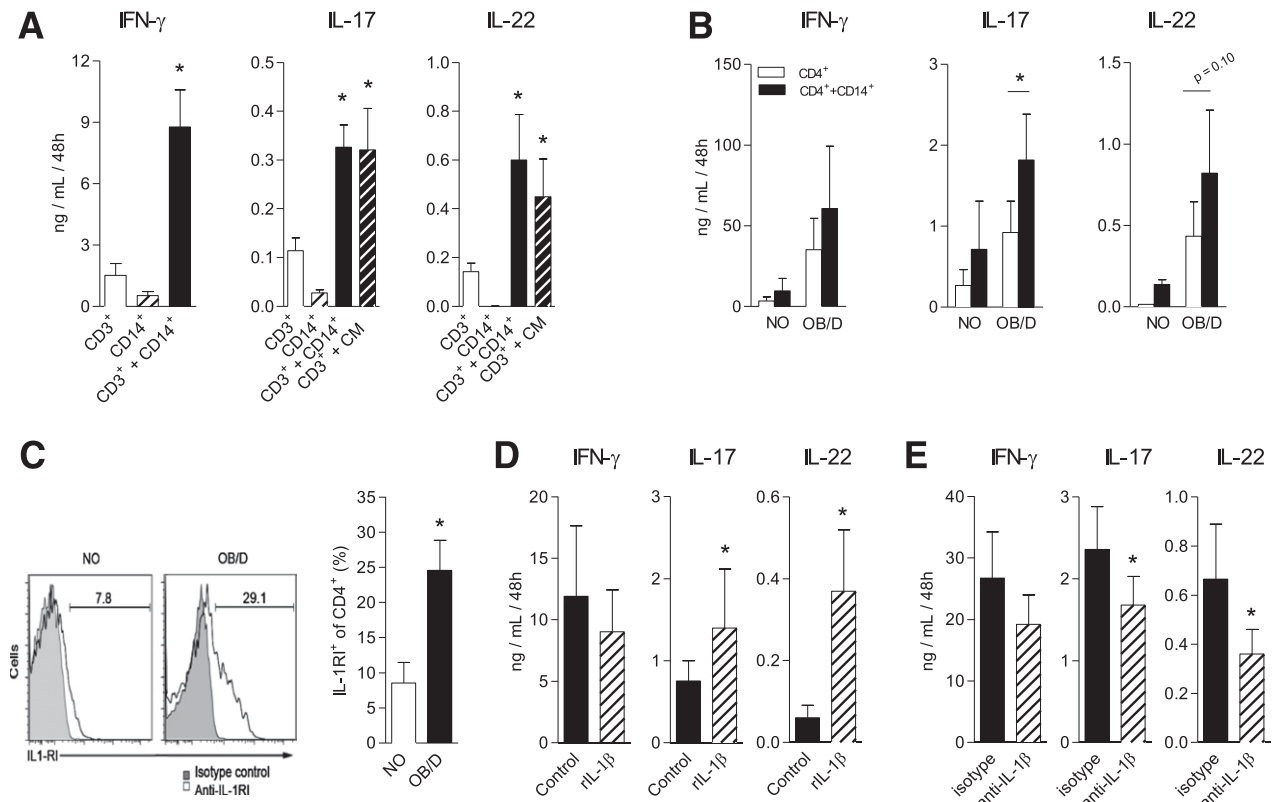


**Figure 2**—Increased prevalence of IL-17- and IL-22-producing CD4<sup>+</sup> T cell in the adipose tissue of OB/D patients. **A**: Positive correlation between the number of CD14<sup>+</sup> and CD3<sup>+</sup> cells isolated from the same visceral adipose tissue biopsy ( $n = 15$ ). Correlation between (**B**) *pro-IL-1 $\beta$*  and *IL-17* or (**C**) *IL-22* gene expression in adipose tissue of OB subjects ( $n = 8$ –9). Percentages of CD4<sup>+</sup> or CD8<sup>+</sup> T cells producing (**D**) IL-17 and (**E**) IL-22 in NO ( $n = 5$ –9), OB ( $n = 13$ –24), and OB/D ( $n = 10$ –18) subjects. Means are shown as horizontal line. \* $P < 0.05$  versus NO;  $\delta P < 0.05$  versus OB. **F**: Percentages of CD4<sup>+</sup> IL-17<sup>+</sup>IL-22<sup>+</sup>, IL-17<sup>-</sup>IL-22<sup>+</sup>, and IL-17<sup>+</sup>IL-22<sup>-</sup>-producing cells in NO ( $n = 5$ ), OB ( $n = 13$ ), and OB/D ( $n = 10$ ) subjects. Data are mean  $\pm$  SEM. \* $P < 0.05$  versus NO. Correlations between the percentages of (**G**) CD4<sup>+</sup> IL-17<sup>+</sup>IL-22<sup>+</sup>, (**H**) IL-17<sup>-</sup>IL-22<sup>+</sup>, or (**I**) IL-17<sup>+</sup>IL-22<sup>-</sup>-producing cells and HbA<sub>1c</sub> in OB subjects ( $n = 12$ –13). The  $r$  correlation coefficients and  $P$  values obtained by Pearson's test are indicated.

### Cytokine Dialogue Between Macrophages and T Lymphocytes in Adipose Tissue

We next performed coculture experiments to specify a potential cytokine dialogue between adipose CD14<sup>+</sup> and T cells. The presence of CD14<sup>+</sup> macrophages enhanced by threefold to sixfold the production of IFN- $\gamma$ , IL-17, and IL-22 by autologous CD3<sup>+</sup> T cells (Fig. 3A). This stimulatory effect on IL-17 and IL-22 release was reproduced by adipose CD14<sup>+</sup> cell conditioned media, emphasizing the

importance of paracrine over cell contact effects in our cocultures conditions. Addition of CD14<sup>+</sup> cells also increased IL-17 release by CD4<sup>+</sup> cells in OB/D subjects, with a similar trend for IL-22 release (Fig. 3B). In this experimental setting, the amounts of cytokines produced by CD4<sup>+</sup> T cells were systematically higher in OB/D than in NO subjects, although the differences did not reach statistical significance, due to a limited number of NO subjects.

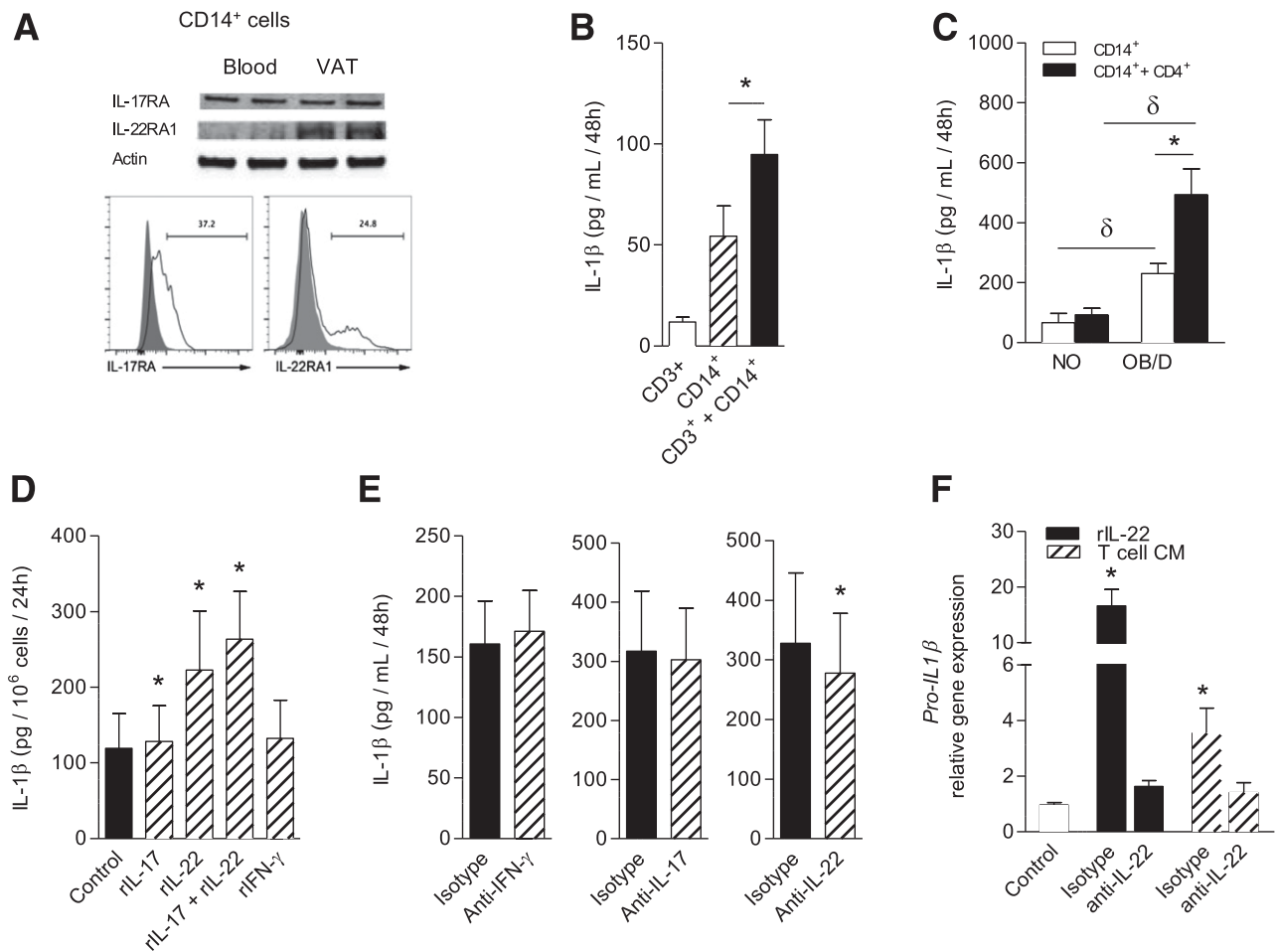


**Figure 3**—IL-1 $\beta$  selectively enhances IL-17 and IL-22 production in human adipose tissue. **A:** IFN- $\gamma$ , IL-17, and IL-22 production by adipose tissue CD3<sup>+</sup> cells cocultured with autologous adipose tissue CD14<sup>+</sup> cells or conditioned media upon anti-CD3/anti-CD28 activation in 10, 13, and 8 obese subjects, respectively. \**P* < 0.05 versus CD3<sup>+</sup> cells alone using Mann-Whitney test. **B:** IFN- $\gamma$ , IL-17, and IL-22 production by adipose tissue CD4<sup>+</sup> T cells cocultured with autologous adipose tissue CD14<sup>+</sup> cells upon anti-CD3/anti-CD28 activation in NO (*n* = 2) and OB/D (*n* = 3–5) subjects. \**P* < 0.05 versus CD4<sup>+</sup> cells alone using Wilcoxon matched pairs test. **C:** Representative flow cytometry histogram of IL-1RI expression on adipose tissue CD4<sup>+</sup> cells from one NO subject and one OB/D subject. Numbers on the histogram profiles indicate the frequency (percentage) of IL-1RI<sup>+</sup> cells in total CD4<sup>+</sup> T cells. The graph shows the percentages of CD4<sup>+</sup> T cells expressing IL-1RI in adipose tissue of NO (*n* = 3) and OB/D (*n* = 5) subjects. \**P* < 0.05 versus NO using Mann-Whitney test. **D:** IFN- $\gamma$  (*n* = 5), IL-17 (*n* = 6), and IL-22 (*n* = 6) secretion in adipose tissue CD4<sup>+</sup> cells from obese subjects treated with recombinant human IL-1 $\beta$  for 48 h. \**P* < 0.05 versus isotype control using Wilcoxon matched pairs test. **E:** IFN- $\gamma$  (*n* = 5), IL-17 (*n* = 12), and IL-22 (*n* = 12) production by adipose tissue CD4<sup>+</sup> cells cocultured with autologous adipose tissue CD14<sup>+</sup> cells of obese subjects upon anti-CD3/anti-CD28 activation and in presence of isotype control or anti-IL-1 $\beta$  antibody as indicated. \**P* < 0.05 versus control using Wilcoxon matched pairs test. All data are shown as mean  $\pm$  SEM. CM, conditioned media.

The role of IL-1 $\beta$  was first substantiated by the presence of IL-1 $\beta$ -signaling receptors (IL-1RI) on CD4<sup>+</sup> cells, which were detected in larger amounts in cells obtained from OB/D patients compared with NO (Fig. 3C). Then we determined the effects of rIL-1 $\beta$  and IL-1 $\beta$  neutralization on CD4<sup>+</sup> T-cell cytokine release. While IFN- $\gamma$  production was not significantly changed, the release of IL-17 and IL-22 was significantly increased upon rIL-1 $\beta$  treatment of CD4<sup>+</sup> T cells (Fig. 3D) and decreased by IL-1 $\beta$  neutralization in CD14<sup>+</sup> CD4<sup>+</sup> cocultures (Fig. 3E). These findings indicate that macrophage-derived IL-1 $\beta$  selectively alters the production of cytokines by adipose tissue CD4<sup>+</sup> T cells, increasing IL-17 and IL-22 release, with virtually no effect on IFN- $\gamma$ .

The receptors IL-17RA and IL-22RA1 were both detected on adipose tissue CD14<sup>+</sup> cells (Fig. 4A), suggesting that these cells are responsive to IL-17 and IL-22. Of note, the presence of IL-22RA1 on CD14<sup>+</sup> cells appears to be

adipose tissue specific, as circulating CD14<sup>+</sup> precursor monocytes did not express it (Fig. 4A). When cocultured with autologous CD3<sup>+</sup> cells, adipose tissue CD14<sup>+</sup> cells secreted twice as much IL-1 $\beta$  than when cultured alone (Fig. 4B). This stimulatory effect was observed in CD4<sup>+</sup> CD14<sup>+</sup> coculture of cells obtained from OB/D subjects, whereas it was not detected in cells from NO controls (Fig. 4C). The role of T-cell cytokines to drive IL-1 $\beta$  production was next explored in CD14<sup>+</sup> cells obtained from obese adipose tissue. rIL-22 was the most potent to promote IL-1 $\beta$  release in CD14<sup>+</sup> cells (Fig. 4D). A stimulatory effect of rIL-17, although significant, was much weaker and not synergistic with that of rIL-22. IFN- $\gamma$  did not increase IL-1 $\beta$  release in these primary cells. In CD4<sup>+</sup>-CD14<sup>+</sup> cocultures, IL-22 neutralization slightly but significantly reduced IL-1 $\beta$  secretion, while anti-IL-17 and anti-IFN- $\gamma$  antibodies were without effect (Fig. 4E). We further assessed the effect of IL-22 neutralization



**Figure 4**—IL-22 increases IL-1 $\beta$  release by adipose tissue macrophages in OB/D patients. **A:** Protein levels of IL-17RA and IL-22RA1 in CD14 $^{+}$  cells isolated from peripheral blood mononuclear cells (blood) or visceral adipose tissue of obese subjects and representative flow cytometry histogram of IL-17RA and IL-22RA1 expression on adipose tissue CD14 $^{+}$  cells from one OB/D subject. **B:** IL-1 $\beta$  production by adipose tissue CD14 $^{+}$  cells cocultured with autologous adipose tissue CD3 $^{+}$  cells upon anti-CD3/anti-CD28 activation ( $n = 13$ ). \* $P < 0.05$  versus CD14 $^{+}$  cells alone using Wilcoxon matched pairs test. **C:** IL-1 $\beta$  production by adipose tissue CD14 $^{+}$  cells cocultured with autologous adipose tissue CD4 $^{+}$  cells upon anti-CD3/anti-CD28 activation in NO ( $n = 3$ ) and OB/D ( $n = 5$ ) subjects. \* $P < 0.05$  versus CD14 $^{+}$  cells alone using Wilcoxon matched pairs test;  $\delta P < 0.05$  versus NO using Mann-Whitney test. **D:** IL-1 $\beta$  secretion from adipose tissue CD14 $^{+}$  cells stimulated with recombinant human IL-17 and IL-22 alone or combined or IFN- $\gamma$  for 24 h ( $n = 6$ ). \* $P < 0.05$  versus control using Wilcoxon matched pairs test. **E:** IL-1 $\beta$  production by adipose tissue CD14 $^{+}$  cells cocultured with autologous CD4 $^{+}$  cells upon anti-CD3/anti-CD28 stimulation and in presence of anti-IFN- $\gamma$  ( $n = 5$ ), anti-IL-17 ( $n = 9$ ), or anti-IL-22 ( $n = 7$ ) neutralizing antibodies or isotype controls. Data are shown as mean  $\pm$  SEM. \* $P < 0.05$  versus isotype control using Wilcoxon matched pairs test. **F:** *Pro-IL-1 $\beta$*  gene expression in MDM treated for 24 h with rIL-22 or T cell-conditioned media in presence of isotype control or anti-IL-22 neutralizing antibody as indicated ( $n = 5$ ). \* $P < 0.05$  versus isotype using Mann-Whitney test. All data are shown as mean  $\pm$  SEM. CM, conditioned media; VAT, visceral adipose tissue.

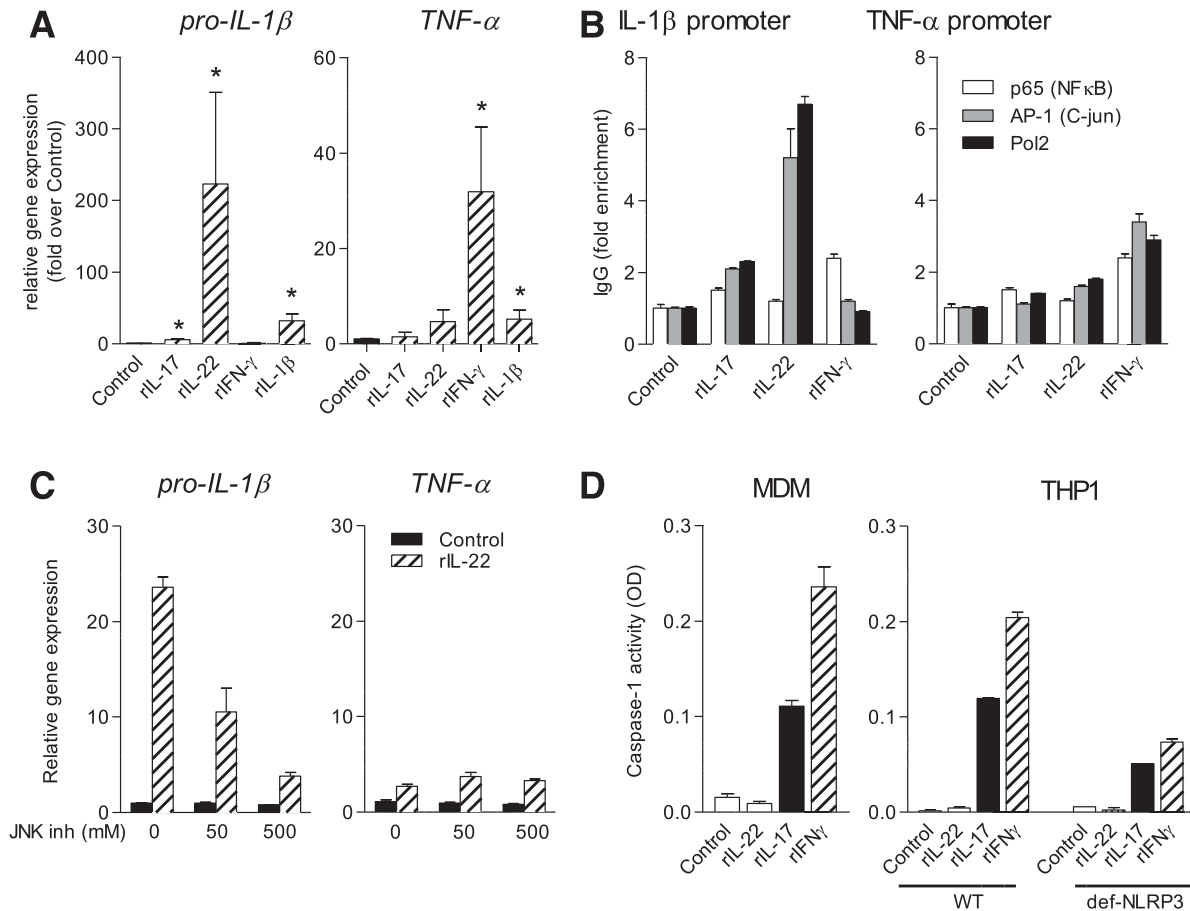
on *pro-IL-1 $\beta$*  gene expression in MDM treated with rIL-22 or CD3 $^{+}$  conditioned media. In these experimental settings, anti-IL-22 antibody abolished the stimulatory effects of rIL-22 or T cell-conditioned media (Fig. 4F). These data support a new paracrine dialogue sustained by IL-22-driven IL-1 $\beta$  production in human adipose tissue macrophages.

#### IL-17 and IL-22 Target Distinct Molecular Pathways in Macrophages

To get insights into the molecular pathways triggered by T-cell cytokines to promote IL-1 $\beta$  production, we explored

their capacity to increase *pro-IL-1 $\beta$*  gene expression and caspase-1 activity in macrophages. In line with data in Fig. 4F, rIL-22 markedly increased *pro-IL-1 $\beta$*  mRNA in MDM, while a weaker or no stimulatory effect was found for rIL-17 and recombinant IFN (rIFN)- $\gamma$ , respectively (Fig. 5A). Besides, *pro-IL-1 $\beta$*  mRNA levels were increased by rIL-1 $\beta$ , in agreement with the cytokine self-stimulatory effect (18). As a control, *TNF- $\alpha$*  gene expression was mostly promoted by rIFN- $\gamma$ . Chromatin immunoprecipitation showed that the marked stimulatory effect of rIL-22 relied on the recruitment of AP-1 (C-Jun), but not NF- $\kappa$ B (p65), onto IL-1 $\beta$  promoter with a concomitant recruitment of polymerase 2





**Figure 5**—T-cell cytokines regulate IL-1 $\beta$  production through distinct pathways. **A**: Quantitative PCR analysis of *pro-IL-1 $\beta$*  and *TNF- $\alpha$*  expression in MDM treated with rIL-17, IL-22, IFN- $\gamma$ , or IL-1 $\beta$  ( $n = 4$ ). **B**: Recruitment of AP-1 (C-Jun), NF $\kappa$ B (p65), and polymerase 2 onto the IL-1 $\beta$  or *TNF- $\alpha$*  promoter in MDM stimulated with rIL-17, IL-22, or IFN- $\gamma$ . **C**: Quantitative PCR analysis of *pro-IL-1 $\beta$*  and *TNF- $\alpha$*  expression in MDM treated for 24 h with rIL-22 in presence JNK inhibitor as indicated ( $n = 4$ ). **D**: Caspase-1 activity in MDM ( $n = 1$  in quadruplicate) and THP1 wild-type or NLRP3 deficient ( $n = 1$  in duplicate) in response to T-cell cytokines. def, deficient; inh, inhibitor; OD, optic density; POL2, polymerase 2; WT, wild-type. \* $P < 0.05$  vs. control using Mann-Whitney test.

(Fig. 5B). Stimulation by rIL-17 induced only a weak recruitment of these transcription factors onto IL-1 $\beta$  promoter. rIFN- $\gamma$  was efficient for their recruitment onto *TNF- $\alpha$*  promoter, but without effect on IL-1 $\beta$  promoter. The relevance of JNK pathways in promoting rIL-22-mediated *pro-IL-1 $\beta$*  transcription was supported by a dose-dependent inhibitory effect of SP600125 JNK inhibitor, which was observed on *pro-IL-1 $\beta$*  and not on *TNF- $\alpha$*  gene expression (Fig. 5C).

rIL-17- and rIFN- $\gamma$ -induced caspase-1 activity in MDM and THP1 macrophages, and these stimulatory effects were reduced in NLRP3-deficient cells (Fig. 5D and E). By contrast, rIL-22 was virtually without effect on caspase-1 activity. Additionally, JNK inhibitor had no effect on IL-17- and IFN- $\gamma$ -induced caspase-1 activation (data not shown). In sum, these data show that IL-22 promotes *pro-IL-1 $\beta$*  transcription through JNK pathway activation, while IL-17 or IFN- $\gamma$  may contribute to NLRP3/caspase-1 activation required for *pro-IL-1 $\beta$*  processing and IL-1 $\beta$  release in macrophages.

## DISCUSSION

Human obesity is a condition marked by changes in the immune cell composition of adipose tissue. While macrophages were initially considered major effectors, the contribution of T cells to adipose tissue inflammation is now established. The phenotyping of immune cells and their local interactions have remained largely unexplored in human adipose tissue, hampering potential translation into therapeutic strategies.

In the current study, we show here that the transcriptomic signature of adipose tissue macrophages was markedly influenced by altered glycemic status. Overexpression of genes functionally related to inflammatory and immune responses characterized macrophages of diabetic patients, where *TNF- $\alpha$*  and IL-1 $\beta$  were among the most upregulated genes. *TNF- $\alpha$*  has long been implicated in obesity-induced insulin resistance in rodent models (19). In our human population, the production of *TNF- $\alpha$*  by macrophages or adipose tissue explants was not strikingly

modulated by type 2 diabetes or RYGB-induced weight loss, whereas IL-1 $\beta$  was. Several experimental and clinical studies have stressed a pathological implication of IL-1 $\beta$  in obesity-induced alteration of glucose homeostasis (20–23). Blockade of IL-1 $\beta$  signaling improves glycemic status in mice with diet-induced obesity (24,25) and in type 2 diabetic patients (26–28). Our current data highlight the pathological relevance of increased local IL-1 $\beta$  production by adipose tissue macrophages in chronically deteriorated glycemic status, expanding the cellular targets of current anti-IL-1 $\beta$  therapies. While numerous cell types, including adipocytes, are known to process IL-1 $\beta$  (29), we show here that macrophages are the major source of IL-1 $\beta$  in human adipose tissue.

Investigating the cytokine signature of adipose tissue T lymphocytes, we found that increased frequencies of IL-17<sup>+</sup>-IL-22<sup>-</sup>, IL-17<sup>-</sup>-IL-22<sup>+</sup>, and IL-17<sup>+</sup>-IL-22<sup>+</sup> subpopulations of CD4<sup>+</sup> cells were characteristic of visceral adipose tissue of OB and OB/D subjects, in relation with deterioration of glucose homeostasis. This supports the relevance of this specific lymphocyte profile in obesity-related type 2 diabetes. In line with our observations, insulin-resistant obese patients were shown to display a greater percentage of lymphocytes producing IL-17 and IL-22 in their subcutaneous adipose tissue than metabolically normal obese or lean subjects (11).

In our study, the coincidental increase in macrophage-derived IL-1 $\beta$  and T cells producing IL-17 and IL-22 led to the hypothesis that these immune cells interact in adipose tissue. Primary cell cultures implemented with recombinant cytokine or neutralizing antibody revealed that IL-1 $\beta$  specifically promoted IL-22 and IL-17 production by adipose tissue CD4<sup>+</sup> cells. These two cytokines are established to induce inflammatory responses in various cell types (30–33). Thus the proinflammatory role of IL-1 $\beta$  in adipose tissue might be substantiated by indirect effects mediated in part through IL-17 and IL-22. Reciprocally, the presence of IL-17 and IL-22 signaling receptors, IL-17RA and IL-22RA1 (34,35), on adipose tissue CD4<sup>+</sup> cells suggest that these cells are responsive to a Th17-like environment. Of note, since IL-22RA1 is considered absent on hematopoietic cells (34), the unexpected expression of IL-22RA1 on CD4<sup>+</sup> cells might constitute a novel feature of visceral adipose tissue macrophages. The stimulatory effects of T-cell coculture or recombinant cytokines on CD4<sup>+</sup> cell-derived IL-1 $\beta$  argue for an effect of IL-22 and IL-17 to promote IL-1 $\beta$  release.

In the context of skin inflammation, both IL-17 and IL-22 stimulate NLRP3-dependent secretion of IL-1 $\beta$  from keratinocytes (36). In the human adipose tissue, our data support a prominent role for IL-22, mostly through C-Jun-mediated increase in *pro-IL-1 $\beta$*  transcription. The use of anti-IL-22 antibody was successful to abolish T cell-conditioned media-induced IL-1 $\beta$  expression in MDM, whereas the neutralizing effect was only marginal, although significant, in CD4<sup>+</sup>-CD14<sup>+</sup> coculture-mediated IL-1 $\beta$  release. A possible explanation is that an unknown

factor(s) reduced the efficiency of the antibody in our coculture conditions. IL-22 is known to mediate either proinflammatory or protective responses in different pathological conditions (reviewed elsewhere [37]). Our data suggest that IL-22 contributes to alter adipose tissue biology in type 2 diabetic patients, at least in part by increasing IL-1 $\beta$  local production. Interestingly, IL-1 $\beta$ , IL-17, and IL-22 have all been shown to promote anti-adipogenic responses and insulin resistance in adipocytes (33,38–40) likely to contribute to the development of type 2 diabetes in obese subjects. Whether or not these adipose tissue-derived cytokines also act at the systemic level remains to be defined.

In our cellular experiments, IFN- $\gamma$  release was stimulated by the presence of adipose tissue CD14<sup>+</sup> cells, but its production was not affected by rIL-1 $\beta$  or neutralizing anti-IL-1 $\beta$  antibody. Additional factors might stimulate IFN- $\gamma$  secretion in adipose tissue, including adipocyte-derived soluble mediators, such as free fatty acids and leptin (41) or the Th1-promoting cytokine IL-18 (42). We further observe that IFN- $\gamma$  increased NLRP3-dependent caspase-1 activity in adipose tissue macrophages, in line with its reported effect to increase *caspase-1* gene expression in human pancreatic islets (43). High level of adipose tissue IFN- $\gamma$  might also act locally as a negative regulator of IL-1 $\beta$  expression since it is known to inhibit IL-1 $\beta$  self-amplification at the transcription level in human peripheral blood mononuclear cells (18). Further studies are warranted to unravel the pathological relevance of IFN- $\gamma$  in human adipose tissue.

This study was performed mostly in primary human cells immunoselected from adipose tissue with some inherent limitations. First, freshly isolated macrophages might be in a highly activated state. Ex vivo caspase-1 “over-activation” could explain the limited effect of IL-17 to promote IL-1 $\beta$  secretion in these primary cells compared with MDM or THP1 macrophages. Second, we do not rule out the possibility that adipose tissue CD14<sup>+</sup> cell fraction comprises a minor subpopulation of myeloid CD14<sup>+</sup> dendritic cells (44). Third, cocultures were performed in presence of anti-CD3 and anti-CD28 antibody to minimize cell contact mediated by antigen presentation or CD80/CD86, respectively. However, this experimental setting does not preclude cell contact relying on other costimulatory molecules (e.g., CD40/CD40L). Given that CD14<sup>+</sup> cells or conditioned media produced a similar amount of stimulation of IL-17 and IL-22 release, it is likely that cell contact does not play a major role in these coculture conditions. However, we cannot rule out that cell contact mediated signaling pathways contribute to this inflammatory dialogue in vivo.

In this study exclusively performed in human samples, we cannot infer a causal relationship between obesity-induced type 2 diabetes and macrophage-derived IL-1 $\beta$  or CD4<sup>+</sup> T cell-derived IL-17/IL-22. This represents a well-known limitation of human investigation. Recent observations that cytokine production in mouse adipose tissue does not reflect the human situation, for example, with

virtually no IL-22 production (40), dampens the usefulness of mouse models in this specific context. The promising example of IL-1 $\beta$  blockade as being the most advanced immune intervention in patients with type 2 diabetes (45) strengthens our observations.

Our primary cell cultures were performed with cells isolated from visceral adipose tissue. It remains to be determined if a similar interplay between T cells and macrophages takes place at other locations. Of note, excess visceral adiposity is defined as the most proinflammatory fat depot and is widely accepted as a better correlate of metabolic abnormalities than the amount of subcutaneous adipose layer (46). In support of this idea, IL-1 $\beta$  production was higher in visceral than in subcutaneous adipose tissue of the same subject.

In summary, our data support a feed-forward proinflammatory loop between macrophages and CD4<sup>+</sup> T cells prominently mediated by IL-1 $\beta$  and Th17-related IL-22 within human visceral adipose tissue. Association of both macrophage-derived IL-1 $\beta$  and frequencies of Th17 cells with obesity-induced deterioration of glycemic variables enlightens the pathological relevance of this interplay. We provide an additional rationale for targeting IL-1 $\beta$  in obesity-linked type 2 diabetes through dampening this local proinflammatory loop. While early clinical data now describe the promising effect of IL-17 blockade in numerous autoimmune diseases (47), this study opens new avenues to the conception of innovative combinatorial anti-IL-1 $\beta$  and anti-IL-22 immunotherapy in human obesity.

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**Author Contributions.** E.D., N.V., S.A., and M.G.-M. researched the data and wrote the manuscript. C.C. and I.C. researched data and contributed to discussion. C.P. and J.A.-W. managed the human samples and databases. S.L.-D., J.B., S.V.K., and K.C. contributed to study conceptualization and participated in writing and reviewing the manuscript. M.G.-M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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