T cell-derived IL-22 amplifies IL-1β-driven inflammation in human adipose tissue: relevance to obesity and type 2 diabetes.

Short title: Th17 response in human obesity

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Abstract (197 words)

Pro-inflammatory cytokines are critically involved in the alteration of adipose tissue biology leading to deterioration of glucose homeostasis in obesity. Here, we show a pronounced pro-inflammatory signature of adipose tissue macrophages in type 2 diabetic obese patients, mainly driven by increased NLRP3-dependent IL-1β production. IL-1β release increased with glycemic deterioration and decreased after gastric bypass surgery. A specific enrichment of IL-17 and IL-22 producing $CD4^+$ T cells was found in adipose tissue of type 2 diabetic obese patients. Co-culture experiments identified the effect of macrophage-derived IL-1β to promote IL-22 and IL-17 production by human adipose tissue $CD4^+$ 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-1β release by inducing *pro-IL-1β* transcription through activation of C-Jun pathways in macrophages. In sum, these human data identified IL-1β 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-1β in obesity-linked type 2 diabetes and may have important implications for the conception of novel combined anti-IL-1β 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 $CD4^+$ regulatory T cells with anti-inflammatory properties predominate in lean mice, while pro-inflammatory $CD4^+$ Th1 and $CD8^+$ T cells accumulate in adipose tissue of obese mice (reviewed in (7)). In human adipose tissue, the amount of pro-inflammatory T lymphocytes producing IFN-γ (8; 9) or 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 expansion of antigen-specific Th1 skewed $CD4^+$ T cells in diet-induced obese mice (12). Surprisingly, treatment of obese mice with MHC 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 pro-inflammatory interplays independent of antigen presentation.

In the present study, we identified IL-1β as the main macrophage-derived cytokine increased in type 2 diabetic obese subjects and enhancing the release of IL-17 and IL-22 by adipose tissue $CD4^+$ T cells. Reciprocally, IL-22 up-regulated *pro-IL1β* transcription, leading to enhanced IL-1β 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 3 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 non diabetic (OB) and type 2 diabetic patients (OB/D) based on fasting glycemia over 7 mM or use of antidiabetic drugs. All OB/D patients were treated with metformin, including 7 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 1 patient treated with insulin. Group 3 was composed of non obese 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, 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 Ethic Committee (CPP Ile-de-France) approved the clinical investigations for both obese and non-obese 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 (0.1g) were minced and incubated in 1 mL of endothelial cell basal medium (Promocell, Heidelberg, Germany) containing 1% bovine serum albumin, penicillin (100 U/mL), and streptomycin (100 mg/mL) for 24h. Tissue viability was checked using lactate dehydrogenase (LDH) 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 (SVF) were obtained by collagenase digestion of adipose tissue (14). SVF cells were resuspended in endotoxin-free PBS supplemented with 2% FCS and 1 mM EDTA. Isolation of $CD14^+$, $CD3^+$ and $CD4^+$ cells was performed using positive selection magnetic beads (Stemcell Technologies, Vancouver, Canada). The $CD14^-CD3^-$ 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 $CD3^+$, $CD4^+$ and $CD14^+$ 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 biotinlabeled cDNA from 50 ng of total RNA extracted from adipose tissue $CD14^+$ cells. Hybridization was performed onto Illumina Human HT-12 v3.0 Expression BeadChips containing probes for 48000 transcripts. Differential gene expression was determined with a Standard Analysis of Microarray (SAM) analysis using a false discovery rate (FDR) of approximately 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 MIAME guidelines. Data have been deposited in NCBI's Gene Expression Omnibus under GEO accession number GSE54350.

Cell culture. $CD14^+$, $CD3^+$ and $CD4^+$ cell supernatants (10^6 cells/mL) were obtained after 48h of culture in RPMI 1640 medium (Lonza, Berkshire, UK) supplemented with 10% fetal calf serum (HyClone, Thermo Fisher Scientific, San Jose, CA), plate bound anti-CD3 (2.5 µg/mL; UCHT1) and soluble anti-CD28 (1 µg/mL; 37407) antibodies (R&D Systems,

Minneapolis, MN). For co-culture experiments, $CD14^+$ cells were cultured with autologous $CD3^+$ or $CD4^+$ for 48 h at 1/1 ratio in the same medium. When indicated, cells were cultured with 10, 50 or 100 μM glyburide (Sigma-Aldrich, St. Louis, MO), with recombinant IL-1β (10 ng/mL), IL-17 (20 ng/mL), IL-22 (20 ng/mL) or IFN-γ (20 ng/mL) (all from Miltenyi Biotec) or with anti-IL-1β (2.5 μg/mL), anti-IL-17 (50 ng/mL), anti-IL-22 (100 ng/mL) (all from eBioscience), anti-IFN-γ (100 ng/mL) (R&D Systems) or JNK inhibitor (SP600125) (Sigma-Aldrich). The respective isotype controls were used at the same concentration. Cell viability was estimated by the release of LDH. Cell cytotoxicity non-statistically 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 (MDM) and THP1 cell line. Human PBMC 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 M-CSF (Miltenyi Biotec) to obtain non polarized 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). Complementary DNAs were synthesized from and prepared with Supercript II reverse transcriptase (Invitrogen). SYBR green primers were used for qRT-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 μg of protein per 15 μl 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 intra-lane 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-β-actin (all from Abcam, Cambridge, UK).

Chromatin immunoprecipitation (ChIP) assay. ChIP 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-1β promoter: 5'-TCCCTCCCTTAGTGCCAACTATGT-3' (forward) and 5'-ACAGTCTCCACAGTTCTGCCATGT-3' (Reverse); and TNF-α 5 -GCTTCCTCCAGATGAGCTT-3' (Forward) and 5'-TGCTGTCCTTGCTGAGGGA-3' (Reverse). The following polyclonal antibodies were used: anti-polymerase 2 (H224, Santa Cruz Biotechnology Inc, Santa-Cruz, CA), anti-NF-κB 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), PE-labeled anti-IL1-R1 (FAB269P; R&D Systems) and appropriate isotype controls were used to characterize freshly immunoselected adipose tissue $CD3^+$ cells. APC-eFluor780-labeled anti-CD45 (HI30, eBiosciences), PE-Cy7-labeled anti-CD14 (M5E2, BD Biosciences), PE-labeled anti-IL-17RA (133617, R&D Systems), APC-labeled anti-IL-22RA1 (305405, R&D Systems) and appropriate isotype controls were used to define receptor expression in $CD14^+$ cells. After 15 min incubation with Fc block (eBioscience), cells were suspended in FACS buffer and stained with appropriate antibodies or isotype controls for

30 min at 4°C in the dark. Intracellular cytokine analysis of $CD3^+$ cells was performed in cells stimulated with phorbol-12-myristate-13-acetate (PMA) (30 ng/mL, Sigma-Aldrich) and ionomycin (1μg/mL, Alexis Biochemicals, San Diego, CA) at 37°C for 6 hours 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), FITC-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 mM 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, Asland, OR).

Statistical analyses. Data are shown as mean ± SEM. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Sotware Inc., La Jolla, CA). Differences between two groups were determined by non parametric 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 $CD14^+$ macrophages. We performed genome-wide profiling of $CD14^+$ cells isolated from visceral adipose tissue of age- and sex-matched OB and OB/D subjects. 167 genes marked the expression pattern of adipose $CD14^+$ cells in OB/D patients, while 70 genes were characteristic of $CD14^+$ cells in non-diabetic OB individuals. Functional GO annotation revealed that genes up-regulated in $CD14^+$ cells from OB/D patients were mainly grouped in immune and inflammatory response pathways (Fig. 1A and Fig S1).

Pro-IL-1β and *NOD-like receptor 3* (*NRLP3)* were among the top 10 up-regulated genes (Fig S1). We confirmed increased *pro-IL-1β* gene expression in adipose $CD14^+$ cells of OB/D *versus* OB in a larger group of subjects (Fig. 1B). *Pro-IL-β* and *NLRP3* mRNA levels were 3- to 6-fold higher in $CD14^+$ cells of OB/D than non-obese (NO) subjects (Fig. 1B, C). When adipose tissue $CD14^+$ 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 (Fig S2A). Supporting adipose tissue macrophages as the major cellular source of IL-1β, the $CD14^+$ cell-enriched fraction expressed 15-fold more *pro-IL-1β* and *NLPR3* 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 $CD14^+$ macrophages freshly isolated from adipose tissue biopsies of OB/D compared to NO or OB subjects (Fig. 1F) and was positively correlated with glycated hemoglobin (HbA1c) 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 Roux-en-Y gastric bypass (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 (Fig. S2B). 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.

$IL\text{-}17^+$ and $IL\text{-}22^+$ $CD4^+$ 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 $CD14^+$ and $CD3^+$ 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 (IFNγ) and Th17-related cytokines (IL-17 and IL-22). At gene level, the three cytokines were found to be expressed mostly in the $CD3^+$ cells enriched fraction (Fig S3). *Pro-IL-1β* mRNA in $CD14^+$ cells was strongly correlated with the levels of IL-17 and IL-22 gene expression in autologous T cells (Fig 2B, 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 $CD4^+$ (83.7 ± 1.1%, n = 50) and $CD8^+$ (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 $CD4^+$ population in OB compared to NO subjects, with an additional effect of type 2 diabetes on IL-17^+ cell prevalence (Fig. 2D, E). Small proportion of $CD8^+$ cells were 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 $CD4^+$ cell subsets showed that the percentages of single IL-17^+-IL-22^- and IL-17^--IL-22^+ cells and double IL-17^+-IL-22^+ cells all increased with obesity (Fig 2F) and in the relation with glycemic deterioration assessed by increased HbA1c values (Fig 2G-I). These data identify $CD4^+$ T cell populations characterized by IL-17^+ and/or IL-22^+ production that accumulate in visceral adipose tissue along with macrophages, and are likely to contribute to altered glycemic status in obese subjects.

Cytokine dialogue between macrophages and T lymphocytes in adipose tissue. We next performed co-culture experiments to specify a potential cytokine dialogue between adipose $CD14^+$ and T cells. The presence of $CD14^+$ macrophages enhanced by 3- to 6-fold the

production of IFN-γ, IL-17 and IL-22 by autologous $CD3^+$ T cells (Fig. 3A). This stimulatory effect on IL-17 and IL-22 release was reproduced by adipose $CD14^+$ cell conditioned media, emphasizing the importance of paracrine over cell-contact effects in our co-cultures conditions. Addition of $CD14^+$ cells also increased IL-17 release by $CD4^+$ 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 $CD4^+$ 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.

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

The receptors IL-17RA and IL-22RA1 were both detected on adipose tissue $CD14^+$ cells (Fig. 4A), suggesting that these cells are responsive to IL-17 and IL-22. Of note, the presence of IL-22RA1 on $CD14^+$ cells appears to be adipose tissue-specific, as circulating $CD14^+$ precursor monocytes did not express it (Fig. 4A). When co-cultured with autologous $CD3^+$ cells, adipose tissue $CD14^+$ cells secreted twice as much IL-1β than when cultured alone (Fig. 4B). This stimulatory effect was observed in $CD4^+$ $CD14^+$ co-culture 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-1β production was next explored in $CD14^+$ cells obtained from obese adipose tissue. Recombinant rIL-22 was the most potent to promote IL-1β release in $CD14^+$

cells (Fig 4D). A stimulatory effect of rIL-17, although significant, was much weaker and not synergistic with that of rIL-22. IFNγ did not increased IL-1β release in these primary cells. In $CD4^+$-$CD14^+$ co-cultures, IL-22 neutralization slightly but significantly reduced IL-1β secretion, while anti-IL-17 and anti-IFN-γ antibodies were without effect (Fig. 4E). We further assessed the effect of IL-22 neutralization on *pro-IL-1β* gene expression in monocyte-derived macrophages (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-1β 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-1β production, we explored their capacity to increase pro-IL-1β gene expression and caspase-1 activity in macrophages. In line with data in Fig 4F, rIL-22 markedly increased *pro-IL-1β* mRNA in MDM, while a weaker or no stimulatory effect was found for rIL-17 and rIFN-γ, respectively (Fig. 5A). Beside, *pro-IL-1β* mRNA levels were increased by rIL-1β, in agreement with the cytokine self-stimulatory effect (18). As a control, *TNF-α* gene expression was mostly promoted by rIFN-γ. Chromatin ImmunoPrecipitation (ChIP) showed that the marked stimulatory effect of rIL-22 relied on the recruitment of AP-1 (C-Jun), but not NF-κB (p65), onto IL-1β promoter with a concomitant recruitment of polymerase 2 (POL2) (Fig. 5B). Stimulation by rIL-17 induced only a weak recruitment of these transcription factors onto IL-1β promoter. rIFNγ was efficient for their recruitment onto TNF-α promoter, but without effect on IL-1β promoter. The relevance of JNK pathways in promoting rIL-22-mediated *pro-IL-1β* transcription was supported by a dose-dependent inhibitory effect of SP600125 JNK inhibitor, which was observed on *pro-IL-1β* and not on *TNF-α* gene expression (Fig 5C).

rIL-17 and rIFN-γ induced caspase-1 activity in MDM and THP1 macrophages and these

stimulatory effects were reduced in NLRP3-deficient cells (Fig. 5D, E). By contrast, rIL-22 was virtually without effect on caspase-1 activity. Additionally, JNK inhibitor had no effect on IL-17 and IFN-γ-induced caspase-1 activation (data not shown). In sum, these data show that IL-22 promotes *pro-IL-1β* transcription through JNK pathway activation, while IL-17 or IFNγ may contribute to NLRP3/caspase-1 activation required for pro-IL-1β processing and IL-1β 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 present study, we show here that the transcriptomic signature of adipose tissue macrophages was markedly influenced by altered glycemic status. Over-expression of genes functionally related to inflammatory and immune responses characterized macrophages of diabetic patients, where TNF-α and IL-1β were among the most up-regulated genes. TNF-α has long been implicated in obesity-induced insulin resistance in rodent models (19). In our human population, the production of TNF-α by macrophages or adipose tissue explants was not strikingly modulated by type 2 diabetes or RYGB-induced weight loss, whereas IL-1β was. Several experimental and clinical studies have stressed a pathological implication of IL-1β in obesity-induced alteration of glucose homeostasis (20-23). Blockade of IL-1β 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-1β production by adipose tissue macrophages in chronically deteriorated glycemic

status, expanding the cellular targets of current anti-IL-1β therapies. While numerous cell types, including adipocytes, are known to process IL-1β (29), we show here that macrophages are the major source of IL-1β in human adipose tissue.

Investigating the cytokine signature of adipose tissue T lymphocytes, we found that increased frequencies of IL-17^+-IL-22^-, IL-17^--IL-22^+ and IL-17^+-IL-22^+ subpopulations of $CD4^+$ 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-1β 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-1β specifically promoted IL-22 and IL-17 production by adipose tissue $CD4^+$ cells. These two cytokines are established to induce inflammatory responses in various cell types (30-33). Thus, the pro-inflammatory role of IL-1β 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 $CD14^+$ 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 $CD14^+$ cells might constitute a novel feature of visceral adipose tissue macrophages. The stimulatory effects of T cell co-culture or recombinant cytokines on $CD14^+$ cell-derived IL-1β argue for an effect of IL-22 and IL-17 to promote IL-1β release.

In the context of skin inflammation, both IL-17 and IL-22 stimulate NLRP3-dependent

secretion of IL-1β 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-1β* transcription. The use of anti-IL-22 antibody was successful to abolish T cell conditioned media-induced IL-1β expression in MDM, whereas the neutralizing effect was only marginal, although significant, in $CD4^+$-$CD14^+$ co-culture-mediated IL-1β release. A possible explanation is that unknown factor(s) reduced the efficiency of the antibody in our co-culture conditions. IL-22 is known to mediate either pro-inflammatory or protective responses in different pathological conditions (reviewed in (37)). Our data suggest that IL-22 contribute to alter adipose tissue biology in type 2 diabetic patients, at least in part by increasing IL-1β local production. Interestingly, IL-1β, 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-γ release was stimulated by the presence of adipose tissue $CD14^+$ cells, but its production was not affected by recombinant IL-1β or neutralizing anti-IL-1β antibody. Additional factors might stimulate IFN-γ 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-γ 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-γ might also act locally as a negative regulator of IL-1β expression since it is known to inhibit IL-1β self-amplification at the transcription level in human peripheral blood mononuclear cells (18). Further studies are warranted to unravel the pathological relevance of IFN-γ 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-1β secretion in these primary cells compared to MDM or THP1 macrophages. Second, we do not rule out the possibility that adipose tissue $CD14^+$ cell fraction comprises a minor subpopulation of myeloid $CD14^+$ dendritic cells (44). Third, co-cultures 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 co-stimulatory molecules (e.g. CD40/CD40L). Given that $CD14^+$ 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 co-culture conditions. However, we cannot rule out that cell-contact mediated signalling 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-1β or $CD4^+$ 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-1β 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 pro-inflammatory

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-1β production was higher in visceral than in subcutaneous adipose tissue of the same subject.

In summary, our data support a feed-forward pro-inflammatory loop between macrophages and $CD4^+$ T cells prominently mediated by IL-1β and Th17-related IL-22 within human visceral adipose tissue. Association of both macrophages-derived IL-1β 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-1β in obesity-linked type 2 diabetes through dampening this local pro-inflammatory 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-1β and anti-IL-22 immunotherapy in human obesity.

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 data bases. S.K, K.C, S.L-D and J.B 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.

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

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Figure legends

Fig. 1. **Transcriptomic signature of adipose tissue CD14$^+$ cells in OB/D patients**.

(A) Genome-wide mRNA expression analysis using microarrays was performed in visceral adipose tissue CD14$^+$ cells isolated from obese (OB, n = 6) and type-2 diabetic obese (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 up (red) or down-regulated (green) in adipose tissue CD14$^+$ cells isolated from OB and OB/D subjects. Genes are ordered by GO annotations. **(B, C)** Quantitative PCR analyses of *pro-IL-1β* and *NLRP3* in CD14$^+$ cells isolated from adipose tissue of non-obese (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$^+$ cells

isolated from adipose tissue of obese subjects (n = 5) and treated with incremental doses of NLRP3-inhibitor glyburide. * $p < 0.05$ *versus* vehicle (Vehi) 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). Ad: adipocytes; Neg: $CD14^-CD3^-$ cells; SVF: stroma vascular fraction. **(F)** IL-1β secretion by adipose tissue $CD14^+$ 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 $CD14^+$ cells isolated from obese subjects (n = 22) and HbA1c (%). 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 means ± SEM.

Fig. 2. **Increased prevalence of IL-17 and IL-22 producing $CD4^+$ T cell in the adipose tissue of OB/D patients. (A)** Positive correlation between the number of $CD14^+$ and $CD3^+$ cells isolated from the same visceral adipose tissue biopsy (n = 15). (**B, C**) Correlation between *pro-IL-1β* and *IL-17* (B) or *IL-22* (C) gene expression in adipose tissue of OB subjects (n=8-9). (D, E) Percentages of $CD4^+$ or $CD8^+$ T cells producing IL-17 (D) and IL-22 (E) 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; δ $p < 0.05$ *versus* OB. (**F**) Percentages of $CD4^+$ $IL\text{-}17^+\text{-}IL\text{-}22^+$, $IL\text{-}17^-\text{-}IL\text{-}22^+$ and $IL\text{-}17^+\text{-}IL\text{-}22^-$ producing cells in NO (n=5), OB (n=13) and OB/D (n=10) subjects. Data are means ± SEM. * $p < 0.05$ *versus* NO (**G-I**) Correlations between the percentages of $CD4^+$ $IL\text{-}17^+\text{-}IL\text{-}22^+$ (G), $IL\text{-}17^-\text{-}IL\text{-}22^+$ (H) or $IL\text{-}17^+\text{-}IL\text{-}22^-$ (I) producing cells and HbA1c in OB subjects (n=12-13). The r correlation coefficients and p

values obtained by Pearson's test are indicated.

Fig. 3. IL-1β selectively enhances IL-17 and IL-22 production in human adipose tissue. **(A)** IFN-γ, IL-17 and IL-22 production by adipose tissue $CD3^+$ cells co-cultured with autologous adipose tissue $CD14^+$ cells or conditioned media (CM) upon anti-CD3/anti-CD28 activation in 10, 13 and 8 obese subjects, respectively. * $p < 0.05$ *versus* $CD3^+$ cells alone using Mann Whitney test. **(B)** IFN-γ, IL-17 and IL-22 production by adipose tissue $CD4^+$ T cells co-cultured with autologous adipose tissue $CD14^+$ cells upon anti-CD3/anti CD28 activation in NO (n = 2) and OB/D (n = 3-5) subjects. * $p < 0.05$ *versus* $CD4^+$ cells alone using Wilcoxon matched-pairs test. **(C)** Representative flow cytometry histogram of IL-1RI expression on adipose tissue $CD4^+$ cells from one NO and one OB/D subjects. Numbers on the histogram profiles indicates the frequency (%) of $IL\text{-}1RI^+$ cells in total $CD4^+$ T cells. The graph shows the percentages of $CD4^+$ 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-γ (n = 5), IL-17 (n = 6) and IL-22 (n = 6) secretion in adipose tissue $CD4^+$ cells from obese subjects treated with recombinant human IL-1β for 48 hr. * $p < 0.05$ *versus* isotype control using Wilcoxon matched-pairs test. **(E)** IFN-γ (n = 5), IL-17 (n = 12) and IL-22 (n = 12) production by adipose tissue $CD4^+$ cells co-cultured with autologous adipose tissue $CD14^+$ cells of obese subjects upon anti-CD3/anti-CD28 activation and in presence of isotype control or anti-IL-1β antibody as indicated. * $p < 0.05$ *versus* control (Ctrl) using Wilcoxon matched-pairs test. All data are shown means ± SEM.

Fig. 4. IL-22 increases IL-1β 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 (VAT) 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-1β production by adipose tissue $CD14^+$

cells co-cultured 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-1β production by adipose tissue $CD14^+$ cells co-cultured 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. δ $p < 0.05$ *versus* NO using Mann-Whitney test. **(D)** IL-1β secretion from adipose tissue $CD14^+$ cells stimulated with recombinant human IL-17 and IL-22 alone or combined, or IFN-γ for 24h (n = 6). * $p < 0.05$ versus control using Wilcoxon matched-pairs test. **(E)** IL-1β production by adipose tissue $CD14^+$ cells co-cultured with autologous $CD4^+$ cells upon anti-CD3/anti-CD28 stimulation and in presence of anti-IFN-γ (n = 5), anti-IL-17 (n = 9) or anti-IL-22 (n = 7) neutralizing antibodies or isotype controls. Data are shown as means ± SEM; * $p < 0.05$ *versus* isotype control using Wilcoxon matched pairs test. **(F)** *Pro-IL-1β* gene expression in MDM treated for 24 hr with recombinant IL-22 or T cell conditioned media (T cell CM) 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 means ± SEM.

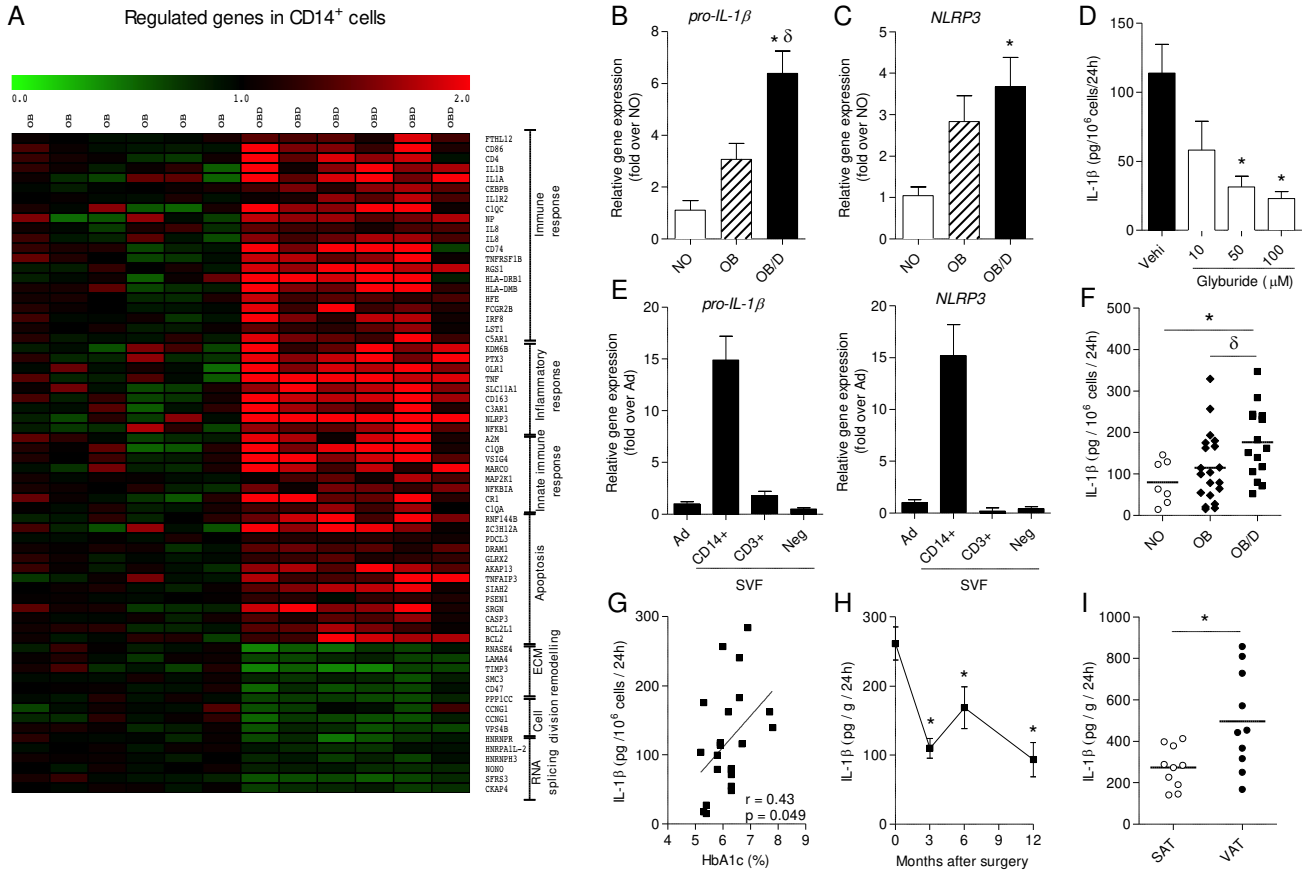
Fig. 5. T cell cytokines regulate IL-1β production through distinct pathways. **(A)** Quantitative PCR analysis of *pro-IL-1β* and *TNF-α* expression in MDM treated with recombinant IL-17, IL-22, IFN-γ or IL-1β (n = 4). **(B)** Recruitment of AP-1 (C-Jun), NFκB (p65) and POL2 onto the IL-1β or TNF-α promoter in MDM stimulated with recombinant IL-17, IL-22 or IFN-γ. **(C)** Quantitative PCR analysis of *pro-IL-1β* and *TNF-α* expression in MDM treated for 24 hr with recombinant IL-22 in presence JNK inhibitor as indicated (n = 4). (**D**) Caspase-1 activity in MDM (n = 1 in quadruplicate) and THP1 wild-type (WT) or NLRP3 deficient (def-NLRP3) (n = 1 in duplicate) in response to T cell cytokines.

Table 1: Bioclinical variables of three groups of study participants.

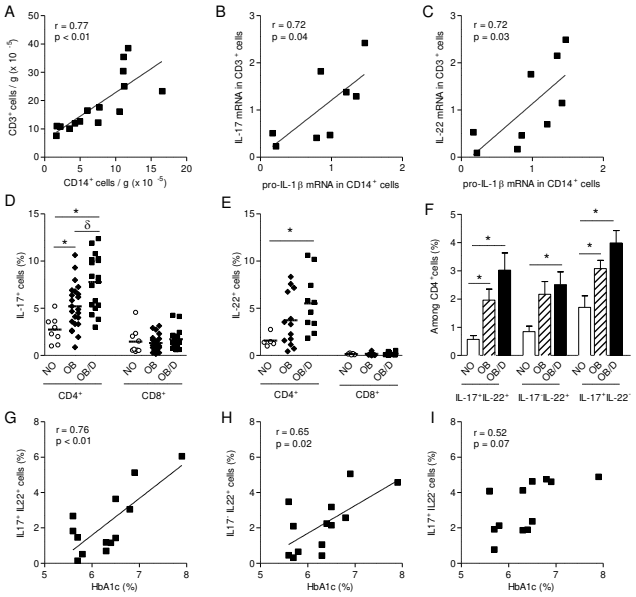
	Group 1		Group 2				Group 3
	OB	OB/D	OB-M0	OB-M3	OB-M6	OB-M12	NO
Months post-surgery	0	0	0	3	6	12	0
Number of subjects	41	31	20	20	20	9	10
Sex (F/M)	33/8	17/14	15/5	15/5	15/5	9/0	5/5
Age (years)	41.6 ± 1.9	47.5 ± 1.9$^{\$}$	41.9 ± 2.9	-	-	-	60.1 ± 4.6
BMI (kg/m^2)	47.5 ± 1.2	48.1 ± 1.3	50.7 ± 2.2	42.0 ± 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*	-
Hb1Ac (%)	5.7 ± 0.1	7.5 ± 0.2$^{\$}$	6.1 ± 0.2	5.6 ± 0.1*	5.5 ± 0.1*	5.7 ± 0.2*	-
Hb1Ac (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$^+$ 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$^+$ 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	-

BMI: body mass index; Hb1Ac: glycated hemoglobin; CLS: crown-like structure.
Data are shown as mean ± SEM. * $p < 0.05$ *versus* OB-M0; $^{\$}$ $p < 0.05$ *versus* OB.

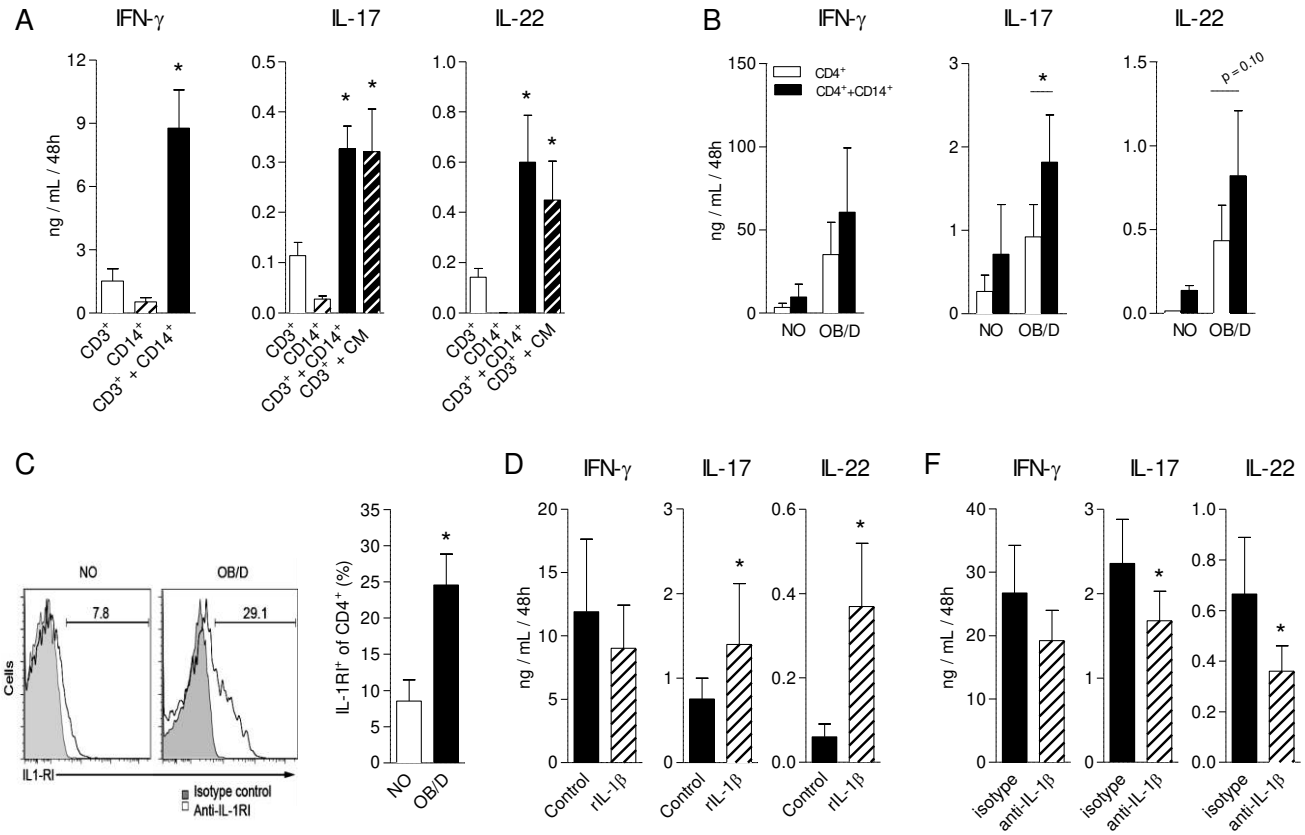
Dalmas *et al* Fig 1



Dalmas *et a*l Fig 2



Dalmas *et al* Fig 3



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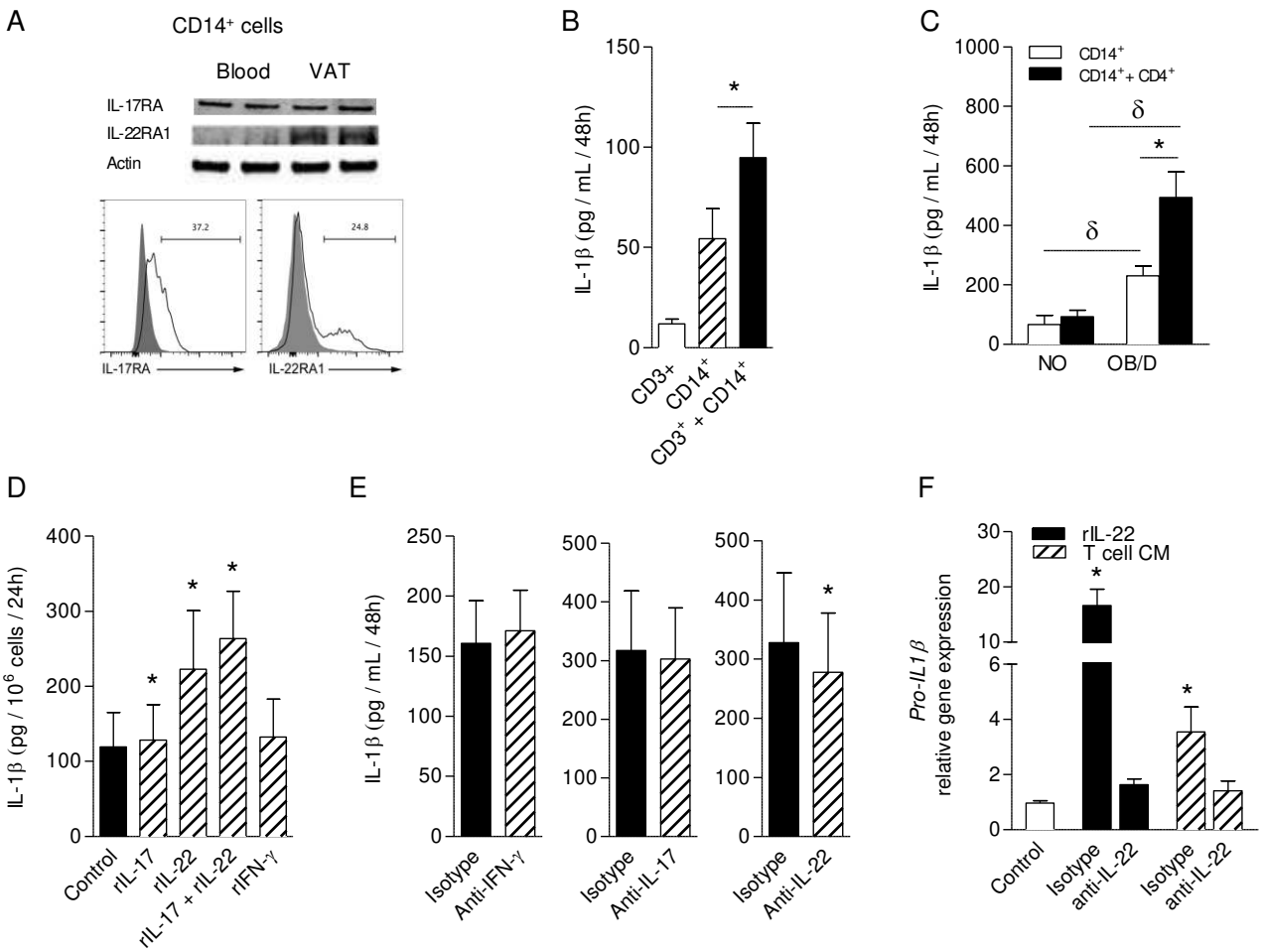
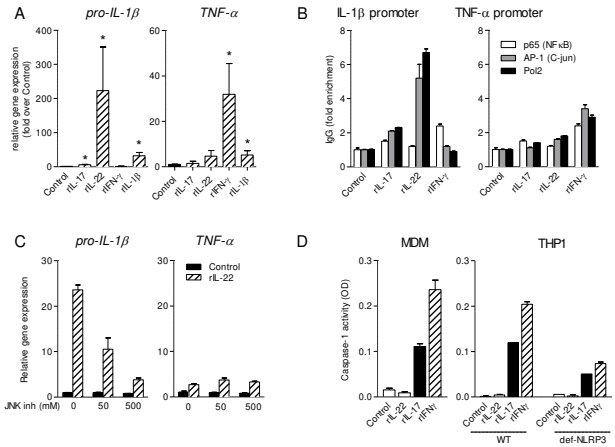
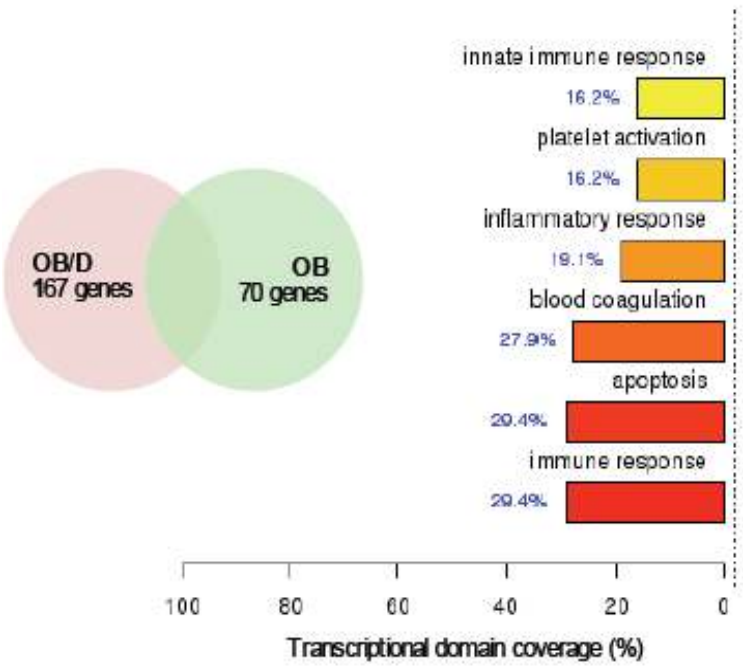


Dalmas *et a*l Fig 5



Dalmas *et al.*, Fig S1

A Transcriptomic signature of CD14+ cells from OB/D



B **Top 10 inflammatory genes in CD14+ cells**

Gene	Ratio OB/D:OB	FDR (%)
NLRP3	2.83	4.06
TNF	2.28	2.75
RGS1	2.00	0.00
PTX3	1.97	4.06
CD163	1.95	0.00
SLC11A1	1.87	4.06
IL1B	1.75	4.95
TANK	1.70	4.06
IL8	1.54	4.95
CEBPB	1.48	0.00

Figure S1: Transcriptomic signature of adipose tissue CD14+ cells from type 2 diabetic obese patients.

Genome-wide mRNA expression analysis using microarrays was performed in adipose tissue CD14+ cells isolated from obese (OB, n = 6) and type-2 diabetic obese (OB/D, n = 6) subjects. **(A)** Venn diagram depicts the number of genes specifically regulated in CD14+ cells isolated from OB and OB/D subjects. The functional themes regulated in CD14+ cells isolated from OB/D subjects represented by enriched annotating categories of Gene Ontology (GO) are shown. **(B)** Summary of the top 10 up-regulated genes in CD14+ cells isolated from OB/D compared to OB subjects. FDR represents the False Discovery Rate. Genes were considered significantly regulated when FDR is < 5 %.

Dalmas *et al.*, Fig S2

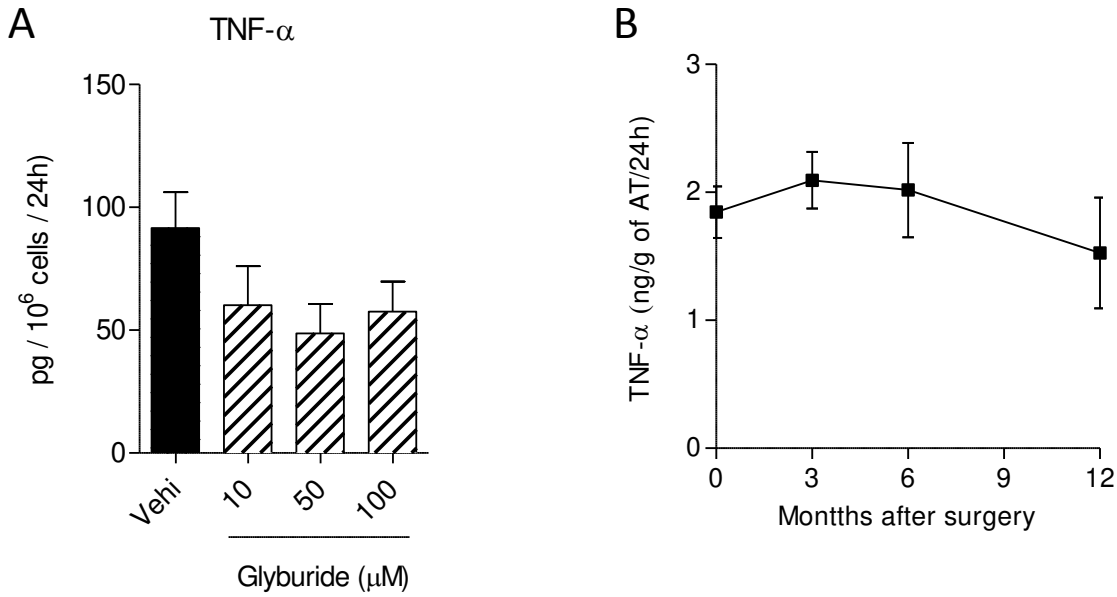


Figure S2: (A) NLRP-3-independent TNF-α production by adipose CD14⁺ cells. CD14⁺ cells were isolated from adipose tissue of obese subjects (n = 5) and treated with incremental doses of NLRP3-inhibitor glyburide. **(B) TNFα production by adipose tissue during RYGP-induced weight loss.** Adipose tissue explants were obtained from obese subjects at the time of RYGP surgery (month 0) and at months 3, 6 (n = 20) and 12 (n = 9). Data are shown as means ± SEM.

Dalmas *et al.*, Fig S3

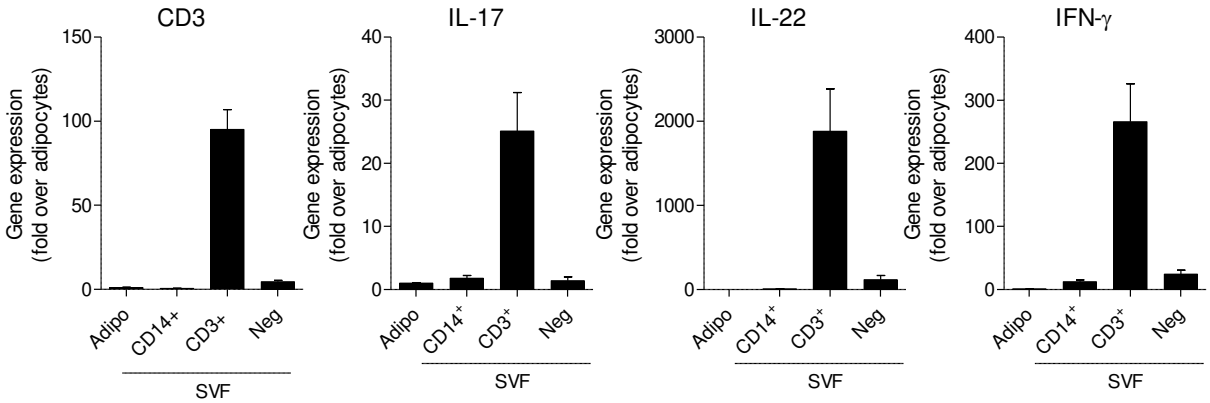


Figure S3: **Expression of T cell cytokines in adipose CD3⁺ cell enriched fraction.** Quantitative PCR analyses of *CD3* and T cell cytokine gene expression in cellular fractions prepared from adipose tissue biopsies of OB subjects (n=10). Ad: adipocytes; Neg: $CD14^{-}CD3^{-}$ cells; SVF: stroma vascular fraction.