Adipose tissue inflammation in obesity and during surgery induced-weight loss : emerging paracrine dialogue between macrophages and lymphocytes

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ADIPOSE TISSUE INFLAMMATION IN OBESITY AND DURING SURGERY INDUCED-WEIGHT LOSS : EMERGING PARACRINE DIALOGUE BETWEEN MACROPHAGES AND LYMPHOCYTES

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A mon père, ce héros

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PhD sountrack: Dark Dark Dark – « Bright Bright Bright »

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ABBREVIATION LIST

A

APC – Antigen-Presenting Cell

AP-1 – Activator Protein 1

ASC – Apoptosis-associated Speck-like protein containing a Carboxy-terminal CARD

ATP – Adenosine Triphosphate

B

BCR – B-Cell Receptor

bFGF – basic Fibroblast Growth Factor

BMI - Body Mass Index

C

CARD – C-terminal Caspase-Recruitment Domain

CCL – Chemokine (C-c motif) ligand

CD – Cluster of Differentiation

C/EBP – CCAAT-Enhancer Binding Protein

Cox2 – Cyclooxygeease 2

CRP – C Reactive Protein

D

DAMP – Danger- or Damage-Associated Molecular Pattern

DC – Dendritic Cell

DXA – dual-energy X-ray absorptiometry

DIO – Diet-Induced Obesity

E

ECM – Extra-Cellular Matrix

FACS – Fluorescence-Activated Cell Sorting

FFA – Free Fatty Acids

G

GFP – Green Fluorescent Protein

(G)M-CSF – (Granulocyte)-Macrophages Colony Stimulating Factor

H

Hb1ac – Glycated hemoglobin

HDL – High Density Lipoprotein

HFD – High Fat Diet

HIF – Hypoxia-Inducible Factor

I

ICAM-1 – Intercellular Adhesion Molecule 1

IFN – Interferon

Ig – ImmunoGlobulin

IKK-β - Inhibitor of NF-κB kinase subunit beta

IL – Interleukin

IMT – Intima Media Thickness

IRF5 – Interferon Regulatory Factor 5

IRS – Insulin Receptor Substrate

J

JNK – c-JUN N-Terminal kinase

K

KLF4 – Krüppel-Like Factor 4

L

LDL – Low Density Lipoprotein

LPL – Lipoprotein Lipase

LPS – Lipopolysaccharide

M

MCP-1 – Monocyte Chemoattractant Protein-1

MHC – Major Histocompatibility Complex

MINCLE – Macrophage-Inducible C-type Lectin

MIP-1β - Macrophage Inflammatory Protein-1beta

MoDM – Monocyte-derived Macrophages

N

NAFLD – Non-Alcoholic Fatty Liver Disease

NF-κB – Nuclear Factor-kappa B

NK(T) – Natural Killer (T)

NLRP – NOD-Like Receptor family, Pyrin domain containing

NLRC – NOD-Like Receptor family, CARD domain containing

NO – Non-Obese subjects

O

ObÉpi – Enquête Epidémiologique National sur le Surpoids et l'Obésité

OB – non-diabetic Obese subjects

OB/D – Diabetic Obese subjects

P

PAMP – Pathogen-Associated Molecular Pattern

PDGF-BB – Platelet-Derived Growth Factor subunit B

PPAR – Peroxisome Proliferator-Activated Receptor

R

RANTES – Regulated upon Activation, Normal T-cell Expressed

ROS – Reactive Oxygen Species

RORγ - RAR-related Orphan Receptor gamma

RPMI – Roswell Park Memorial Institute

P

PRR – Patter Recognition Receptor

S

SAA – Serum Amyloid A

SAT – Subcutaneous Adipose Tissue

SVF – Stroma Vascular Fraction

T

T2D – Type 2 Diabetes

TCR – T-Cell Receptor

TG – Triglycerides

TGF-β - Transforming Growth Factor beta

TLR – Toll-Like Receptor

TNF – Tumor Necrosis Factor

Treg – Regulatory T cell

V

VAT – Visceral Adipose Tissue

VEGF – Vascular Endothelial Growth Factor

VWF – Von Willebrand Factor

FIGURE LIST

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BOX LIST

CLUSTERS OF DIFFERENTIATION

CD	Known or supposed function	Surface cell distribution
CD1d	Glycoprotein expressed at the surface of various antigen-presenting cells involved in the presentation of (glycol)lipid antigens to T cells, especially NKT cells	Macrophages, dendritic cells, epithelial cells
CD3	Common molecule for identifying T cells required for the surface expression of the T-cell receptor.	T cells
CD4	Glycoprotein characteristic of T helper cells (MHC class II-restricted) and a co-receptor along with T-cell receptor involved in T cell activation. It is also the major receptor for HIV.	T helper cells but also macrophages, dendritic cells
CD8	Glycoprotein characteristic of cytotoxic T cells (MHC class I-restricted) and a co-receptor along with T-cell receptor involved in T cell activation.	Cytotoxic T cells
CD11c	Integrin, alpha X or ITGAX, acting as a fibrinogen receptor and important in monocyte adhesion, chemotaxis and phagocytosis.	Monocytes, macrophages, dendritic cells, granulocytes, NK cells
CD14	Pathogen recognition receptor detecting antigens on the surface of microorganisms and acting as a co-receptor, along with TLR-4 or MD-2, to LPS.	Monocytes/macrophages, granulocytes
CD16	Fc gamma receptor III, binding various IgG molecules and mediating antibody dependent cytotoxicity of foreign cells or phagocytosis.	Monocytes/macrophages, NK cells, mastocytes
CD20	Common B cell marker, involved in early signal of B cells activation and suspected to act as a calcium channel in the cell membrane	B cells
CD28	Expressed at the surface of T cells, receptor for CD80 and CD86 that provide co-stimulatory signals required for T cell activation	T cells
CD40	Also called TNF receptor superfamily member 5, costimulatory protein on antigen-presenting cells, required for their activation	Monocytes/macrophages, dendritic cells but also endothelial cells, B cells

CD	Known or supposed function	Surface cell distribution
CD68	Also called KP1 or macrosialin, a glycoprotein specific to lysosome, binding to low density lipoprotein and playing a role in phagocytic activities	Monocytes, macrophages, but also dendritic cells and granulocytes such as mast cells and neutrophils
CD69	Also called activation inducer molecule or AIM, an early cell surface glycoprotein involved in cell activation.	T and B cells, NK cells, granulocytes, macrophages, dendritic cells
CD80/CD86	Also called B7-1and 2, costimulatory molecules expressed at the surface of antigen-presenting cells and involved in T cell activation.	Monocytes/macrophages, dendritic cells, B cells
CD163	Hemoglobin/haptoglobin scavenger receptor whose function remains unclear, although anti-inflammatory signals seems to enhance its expression	Monocytes/macrophages
CD206	C-type lectin mannose receptor acting in phagocytosis of mannose-containing solutes, mostly on the surface of pathogens.	Monocytes/macrophages, dendritic cells

FOREWORD

It is now well accepted that obesity is associated with increased circulating levels of inflammatory mediators that may contribute to the development of obesity-induced metabolic complications. Serum levels of inflammatory factors partly originate from circulating leukocytes or adipose tissue depots. Adipose tissue is infiltrated by various immune cells that all contribute to an inflammatory microenvironment, altering adipocyte physiology during obesity. Less is known about the possible cross-talk between immune cells within adipose tissue during obesity. Based on these observations, my PhD project was divided into 2 studies as illustrated below.

Study 1 focused on the regulation of circulating mediators and monocytes in morbid obese patients during bypass surgery-induced weight variations.

Study 2 aimed at unravelling the potential interactions between the two main types of immune cells in adipose tissue that are the macrophages and lymphocytes.

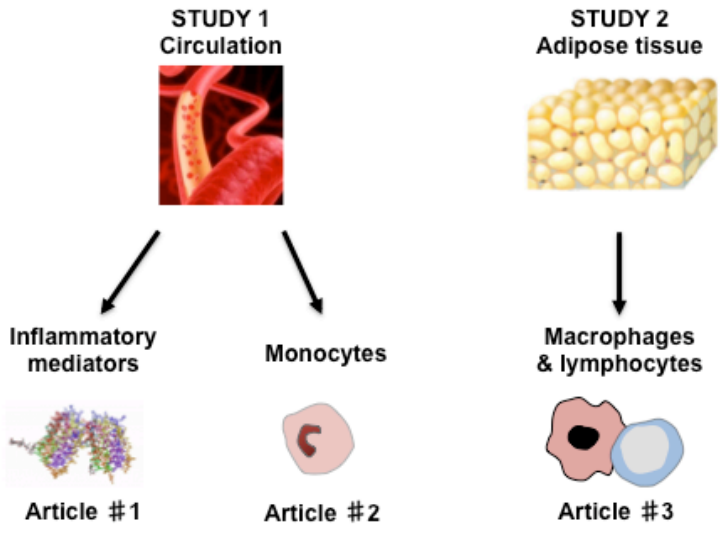

Figure 1 | Focus of studies 1 and 2 of the thesis.

INTRODUCTION

A. Introducing obesity

1. An overview of obesity in 2012

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. Body mass index or BMI (kg/m^2) is a useful index of weight-for-height that is commonly used to classify overweight and obesity in adults as shown in Figure 1. During the last decade, obesity prevalence has risen tremendously throughout the world, now affecting younger populations and low-income countries. In France, it has increased from 8,5% in 1997 to 14,5% in 2009 in the adult population with a worrying increase in severe or morbid obesity from 0,3 to 1,1% (source ObÉpi 2009 study). Obesity is a risk factor for numerous diseases from depression to type 2 diabetes (T2D) and cardiovascular events

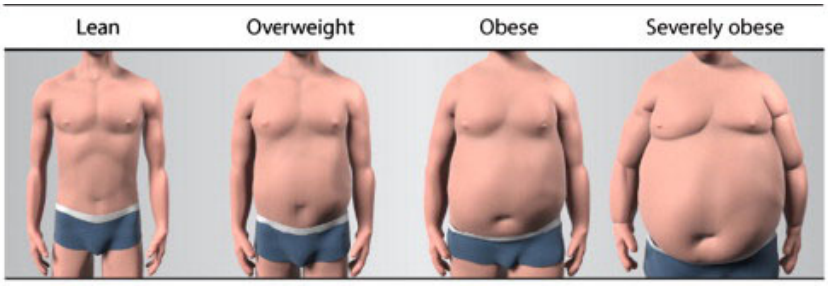

Figure 2 | Different stages of human obesity. Each stage corresponds to a range of BMI: lean, 18-25 kg/m^2; overweight, 25-30 kg/m^2; obese, 30-40 kg/m^2 and severly obese or morbid obese, >40 kg/m^2 (from (Arner and Spalding, 2010)).

One should keep in mind that the BMI value does not take into account the fat distribution throughout the body that greatly influences risk associated with obesity. Two kinds of obesity can be characterized. Increased intra-abdominal/visceral fat (central or apple-shaped

obesity) promotes a high risk of metabolic diseases, whereas increased subcutaneous fat, that is in the thighs and hips (peripheral or pear-shaped obesity) exerts less risk. To take this into account, the notion of metabolic syndrome was defined which clusters for the first time metabolic disorders and cardiovascular risk factors. One has to gather at least 3 out of 5 conditions to be diagnosed with the metabolic syndrome. The 5 criteria include: (a) increased waist circumference, for which the cutoff points are population-specific and country-specific (in France we use the following references: ≥94 cm for men and ≥80 cm for women); (b) elevated circulating triglycerides (≥1,5g/l or related treatment); (c) reduced high-density lipoprotein (HDL) cholesterol level (≤0,4g/l for men and ≤0,5g/l for women or related treatment) ; (d) elevated fasting glucose level (≥1,0g/l or related treatment); and (e) elevated blood pressure (≥ 130/85 mm Hg or related treatment). In this introduction, we will discuss the physiological origins of obesity and especially, how obesity leads to insulin resistance and, eventually to T2D (Basdevant and Ciangura, 2010).

The basic cause of obesity is an energy imbalance between calories consumed and calories expended. Modern life promotes the growing intake of energy-dense foods that are high in fat, salt and sugars, while urbanization and new technologies favor a sedentary life and decreased physical activity. Altogether, Westernized lifestyle positions obesity as a major epidemic currently challenging our society. And yet, all the population is neither overweight nor obese. Individuals submitted to the same overfeeding for a given period of time differ in their capacity to gain weight: for instance, ranging from 2 kg to more than 10kg (Basdevant and Ciangura, 2010). On the other hand, homozygote twins correlate in term of weight gain. (Bouchard et al., 1988). These observations suggest that genetic makeup partially drives individuals' susceptibility to the environment one lives in. Depending on the publications, 30 to 80% of weight variation might be explained by genetic factors (Mutch and Clement, 2006). Obesity is commonly classified into 3 subgroups depending on their etiology. Monogenic obesity is when a single gene mutation contributes to the development of extremily severe obesity. To date, 20 genes were found to be involved in monogenic forms of obesity, all having a link with the leptin action and the melanocortin pathway. Syndromic obesity, such as the Pradder-Willy syndrome, involves discrete genetic defects or chromosomal abnormalities at several genes that induce an obese phenotype associated with mental retardation, dysmorphic features, and/or organ-specific developmental abnormalities. Finally, polygenic or common obesity, that the general population experiences more and more today, arises when an individual's genome is susceptible to an environment that promotes energy intake over energy expenditure. Today, we evolve in an environment that favors weight gain mainly through food abundance and lack of physical activity. A good illustration of these gene-environment interactions is the fact that, in France, there is an « obesity gradient » from

the South (11,5% of the population is obese) to the North (20,5% of the population is obese) of France, likely to reflect differences in food traditions but also social and economical difficulties (source ObÉpi 2009 study).

Nowadays, it is well established that obesity is associated with a myriad of complications and is a risk factor for many diseases. The Word Health Organization estimates that 44% of the diabetes, 23% of the ischaemic heart disease and between 7% and 41% of certain cancer burdens are attributable to obesity.

Metabolic complications: they are the most prevalent in obese patients and include the development of hyperlypidemia, that is abnormal circulating levels of lipid species, and insulin resistance, that is, the decreased capacity of peripheral organs to response to insulin. Eventually, insulin resistance will lead to T2D. Of note, 75% of T2D patients are obese (Basdevant and Ciangura, 2010). Consersely, in the population of morbid obese subjects that we follow at the laboratory, only about 33% of subjects are type 2 diabetic. The reasons for this heterogeneity are incompletely understood, although genetics probably play a role as mentioned earlier. Obesity is also associated with hepatic complications that are the nonalcoholic fatty liver disease (NAFLD), often concomitent with the development of T2D. Compared to T2D, hepatic complications are much more frequent in obesity and concern about 84% of our obese population, ranging from mild to severe alterations. The earliest stage of NAFLD is hepatic steatosis, that is the deposition of tryglycerides as lipid droplets in the cytoplasm of hepatocytes that exceeds 5% of the liver weight. Hepatic steatosis can progress to nonalcoholic steatohepatitis (NASH) that includes hepatocyte injury, inflammation and fibrosis in addition to fat deposits. Finally, NASH, in turn, can lead to cirrhosis during which hepatocytes are replaced ObÉpi by collagen-based scar tissue and eventually, to liver cancer (Cohen et al., 2011).

Cadiovascular complications: Beside altered metabolic profile, obese subjects often develop hypertension and are predisposed to the occurrence of coronary heart disease, heart failure, and sudden death.

Respiratory complications: Obesity is mainly associated with sleep apnea, a disorder in which airways become obstructed while asleep and could concern more than 40% of obese individuals.

Others: Through mechanical contraints, excess weight leads to increased prevalence of arthritis in obese subjects. Obesity is associated with increased risks of several cancer types

such as colon and rectum, pancreas or endometrium. Finally, obesity is likely to promote depression episodes and altered social behavior

2. Adipose tissue, a complex organ at the heart of obesity

2.1. Adipose tissue depots

Adipose tissue is a complex organ, at the center of obesity physiopathology. It is a heterogenous tissue composed of different cells types, mainly the adipocytes. In physiological conditions, adipose tissue must be viewed primarily as a protective tissue that stores and prevents excessive exposure of other organs to fatty acids. Adipose tissue also has secretory and endocrine functions through the production of a myriad of factors that act locally or on peripheral organs and the brain. This puts the adipose tissue at the core of inter-organ communication networks. Adipose tissue includes different regional depots that undergo many tissular and cellular alterations during weight gain. Of note, there are two types of adipose tissue, white and brown. White adipose tissue, whose primary function is to store excess energy, is distributed throughout the body in different depots. By contrast, brown adipose tissue is composed of relatively small depots that are located at discrete sites, where it contributes to thermogenesis. Considering its ability to dissipate stored energy as heat, brown fat depots were extensively investigated in small and hibernating animals that survived in cold environments. In humans, brown adipose tissue was originaly thought to help maintain normal body temperature in newborn infants before completely regressing in adulthood. Only recently, such depots have been identified in adult humans undergoing positron emission or computed tomography technologies, tracing glucose utilization in metabolic active tissues. In adults, brown fat depots are localized in cervical-supraclavicular, perirenal and paravertebral regions (Ravussin and Galgani, 2011). These observations have opened new avenues of research in brown adipose tissue contribution to body weight regulation in humans. During my PhD, I exclusively worked on white adipose tissue and thus, only white adipose tissue will be described and discussed in this manuscript.

Total body fat mass comprises different regional adipose tissue stores, that are distributed throughout the body as illustrated in Figure 3. Obesity is virtually related to the expansion of all fat depots. Classically, visceral adipose tissue that is found in the general cavity has been distinguished from subcutaneous adipose tissue located outside. These two depots are the ones that store the most part of excess of calorie intake and have been implicated in

metabolic complications as discussed later on. Interestingly, visceral fat represents about 10-20% and 5-10% of total body fat in mean and women, respectively (reviewed in Lafontan and Girard, 2008). On the contrary, subcutaneous fat accounts for 80% of total body fat in a healthy adult. In addition, peri-organ thin layers of adipose tissue have been identified aroung the heart, kidney, muscle as illustrated in (Ouchi et al., 2011). Although, contribution of these « ectopic » depots to increased fat mass is minor, they might also play a role in obesity comorbidities.

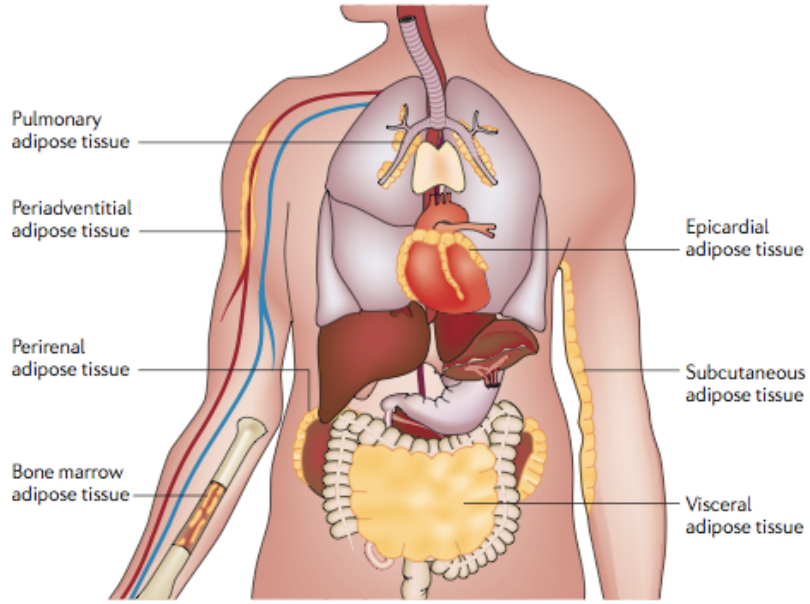

Figure 3 | Adipose tissue depots. In healthy subjects, the two primary depots are the subcutaneous and visceral adipose tissue. Additionally, smaller « ectopic » layers of adipose tissue can be found in or around organs such as the heart or the vascular epithelium (from (Ouchi et al., 2011)).

What is usually termed visceral adipose tissue can be further subdivised in different depots. It predominantly comprises the lesser and greater omentum (peritoneal membrane that is attached to the stomach and drapes over the viscera inside the abdomen) and the mesenteric fat (peritoneal membrane that mainly surrounds the jejunum and ileum sections). Besides, subcutaneous adipose tissue includes all fat layers found just beneath the skin, such as the abdominal, gluteal or femoral fat deposits. At the abdominal site, subcutaneous fat is anatomically separated by a fascial plane, termed *fascia superficialis*, into superficial and deep subcutaneous adipose tissue. It remains unclear whether this partition can also apply to non-abdominal subcutaneous depots (Tordjman et al., 2011).

Altogether, the various visceral or subcutaneous fat depots might represent distinct metabolic and/or inflammatory conditions. Beside, their regional localization is likely to determine their impact of the body physiology. For instance, visceral adipose tissue is drained by the portal

vein to the liver, which makes its potential secretions of special interest. Thus, heterogeneity in fat sampling could underlie experimental discrepancies often observed in human studies. During my PhD, I studied both visceral and subcutaneous adipose tissue biopsies. Visceral fat was sampled at the time of gastric surgery in the great omentum area, while subcutaneous fat was sampled at the time of surgery and during weight loss follow-up in the superficial subcutaneous region.

2.2. Adipose tissue cellular heterogeneity

Adipose tissue is composed of two cell fractions that can be easily separated through collagenase enzymatic digestion.

2.2.1. Adipocytes

The first fraction, that represents around 70% of the total adipose mass, includes exclusively adipocytes that are characterized by large unilocular lipid droplets. Adipocytes are a unique specialized cell type. In humans, the fat mass is the product of both adipocyte number and adipocyte volume (Arner and Spalding, 2010).

In a given fat depots, adipocyte number reflects the capacity of progenitor cells to develop into mature and functional adipocytes. This differentiation process comprises two successive phases. The first is termed « determination » during which a pluripotent progenitor commits itself to adipocyte lineage. It becomes a preadipocyte, that is, unable to differentiate into any other cell type. During the second phase, « the terminal differentiation », the preadipocyte differentiates into a mature adipocyte that acquire all the machinery required for lipid transport and synthesis, insulin sensitivity and adipokine secretion. Terminal differentiation involves crucial transcriptional cascade. Predominantly, PPARγ appears to be the master regulator of adipogenesis and is required to maintain the differentiated state. Besides, several member of the CCAAT-Enhancer Binding Protein (C/EBP) family are known to contribute to adipogenesis following a strict temporal regulation, C/EBPβ and C/EBPδ inducing C/EBPα (reviewed in Rosen and MacDougald, 2006).

Adipocyte volume reflects the specific function of adipocyte to store excess energy in the form of lipids, and thus, the cell capacity to dramatically modulate its size in response to changes in energy balance. Fat accumulation is determined by the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis) (reviewed in Rajala and Scherer, 2003 ;

Attie and Scherer, 2009). In period of caloric abundance, adipocytes store free fatty acids (FFA) in the form of triglycerides through their resterification to glycerol-3-phosphate. Insulin is a major inducer of lipogenesis. Lipogenesis includes two different pathways: *de novo* lipogenesis from non-lipid precursors such as glucose and uptake of circulating FFA to synthetize subsequent intracellular triglycerides. In humans, fatty acids mainly come from the hydrolysis of triglycerides that are transported by lipoproteins (such as very-low-density lipoproteins and dietary chylomicrons) in the circulation. Adipocytes are known to secrete the lipoprotein lipase that processes triglycerides into FFA and glycerol-3-phosphate inside the vascular luminal space. On the contrary, in time of energy shortage, adipocytes release FFA back in the circulation during the lipolysis process. Intracellular triglycerides undergo hydrolysis through the action of different lipases including the adipose triglyceride lipase. It results in the efflux of glycerol-3-phosphate and FFA that serve as a source of energy for most body tissues. Lipolysis rate depends on the functional equilibrium between the efficacity of lipolytic (such as adrenal catecholamines) and antilipolytic (such as insulin) regulators.

Regional variations in the metabolic activity of adipocytes have been observed in healthy subjects, especially in regards to regulation of lipolysis. Basal (spontaneous) lipolysis is higher in the subcutaneous than the visceral compartment. Beside, the antilipolytic effect of insulin is much more pronounced in subcutaneous adipocytes than in visceral counterparts, and inversely for catecholamine-induced lipolysis. On the contrary, scarce and elusive data focused on comparing lipogenesis in visceral and subcutaneous fat depots, with a strong cofounding effect of age and sex. Although, it seems clear that adipocytes from subcutaneous adipose tissue are larger than in visceral fat depots. In reference to adipogenesis, preadipocytes isolated from subcutaneous adipose were shown to be more proliferative than omental counterparts, although their differentiation capacity appeared to be the same (reviewed in Lafontan and Girard, 2008). It should be pointed out that these studies were conducting *in vitro* that may not entirely reflect the regulatory signalling pathways that operate *in vivo*.

2.2.2. Stroma-vascular fraction

The second fraction, termed the stroma vascular fraction (SVF), is composed of many heterogenous cell types that juxtapose the adipocytes in a network of extracellular matrix as illustrated in Figure 4. This SVF mainly includes preadipocytes, fibroblasts, endothelial cells from the vasculature, progenitor cells and as recently discovered, immune cells that all participate in a complex cross-talk with adipocytes. The nature of the SVF and extracellular matrix deposition can vary according to adipose tissue depots and physiological status as it

will be presented below. It is now well established that obesity can lead to changes in the cellular composition of the adipose tissue SVF and modulate individual cell phenotypes, likely to contribute to an altered inflammatory milieu. During my PhD, I focused on two predominant immune cell types of the human adipose tissue SVF, the macrophages and the lymphocytes.

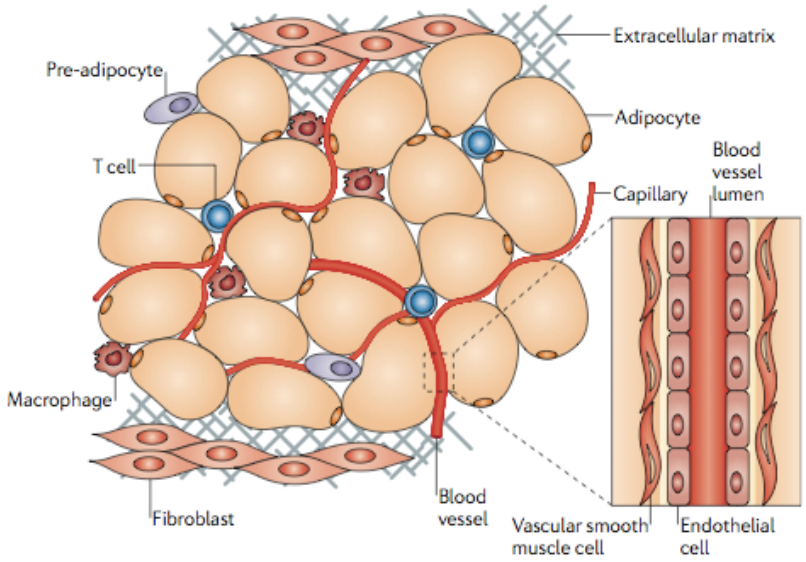

Figure 4 | Adipose tissue composition. In physiological conditions, adipose tisse is mainly composed of adipocytes but also preadipocytes, fibroblasts, blood vessel components and resident immune cells such as macrophages and lymphocytes. All these cells are embedded in an extracellular matrix allowing structural integrity of the tissue (from (Ouchi et al., 2011)).

2.3. Adipose tissue alterations in obesity

Adipose tissue is a dynamic tissue that has an exclusive capacity to undergo major morphologic changes in response to alterations of energy balance. In period of energy excess, adipose tissue undergoes massive remodeling. As defined by Lee and colleagues, the concept of adipose tissue remodeling refers to the turnover of cells and the renovation of the extracellular matrix in response to requirements for growth and expansion. This is illustrated in Figure 6.

2.3.1. Cellular alterations

As already mentioned, fat mass is the product of both adipocyte volume and adipocyte number in humans. Adipose tissue can be defined in two ways: hyperplasia, that is, an increase in adipocyte number and hypertrophy, that is, an increase in adipocyte volume. In

obesity, excess of calorie intake is stored in adipocytes leading to huge increase in fat cell size. Thus, hypertrophy has become a basic feature of adipose tissue in many, if not all, overweight and obese patients. Larger adipocytes are generally observed in subcutaneous adipose tissue compared to visceral fat depot. Recently, an increase in number of adipocytes has also been reported in obese subjects with childhood obesity. Currently, it is not known if the situation is similar in common obesity, developping later on with aging (Spalding et al., 2008). Diet-induced obesity (DIO) mice models describe that the increase in adipocyte volume precedes the increase in adipocyte number. These observations underlie the hypothesis of the « critical fat cell size » according to which once the maximum adipocyte volume is reached, it triggers a subsequent increase in cell number. Contrary to adipocyte size that can change throughout life, adipocyte number is subject to little variation during adulthood despite 10% annual turnover in humans. This implies that any alterations in fat mass during adulthood are credited to modulations of adipocyte hypertophy. Thus, in human, increased fat cell number would reflect a population of individuals predisposed (by their childhood fat cell number) to become obese. It rises the question to know whether or not having too many adipocytes predisposes to development of severe obesity (reviewed in (Arner and Spalding, 2010 ; Lonn et al., 2010).

In their study, Arner and colleagues suggests that the occurrence of subcutaneous adipose hypertrophy or hyperplasia was not influenced by sex or body weight, suggesting that both conditions are equally common among men and women and evenly distributed among lean, overweight, or obese subjects in the general population (Arner et al., 2010). In the population of morbid obese subjects that we follow at the laboratory, subcutaneous hypertrophy and hyperplasia were well distributed among women and men. Interestingly, hypertrophy appears to be associated with the diabetic status of obese subjects as illustrated in Figure 5. Thus, the occurence of subcutaneous hypertrophy or hyperplasia is likely to be related to alterations of glycemic parameters rather than obesity *per se*. Although, such analyses should also be performed in visceral adipose tissue.

Beside a modulation in cell number or cell size, adipocyte phenotype is altered as cell size influences adipocyte biology. Adipocyte hypertrophy is indeed associated with changes in gene expression and secretory functions as well as activation of many inflammatory pathways. Studies based on a technique allowing adipocyte separation according to their cell size showed that only very large adipocytes were dysregulated. Adipocyte hypertrophy was associated with increased secretions of acute-phase protein such as SAA, and pro-inflammatory cytokines and chemokines such as, IL-6, IL-8 and CCL-2. (Jernas et al., 2006); (Skurk et al., 2007).

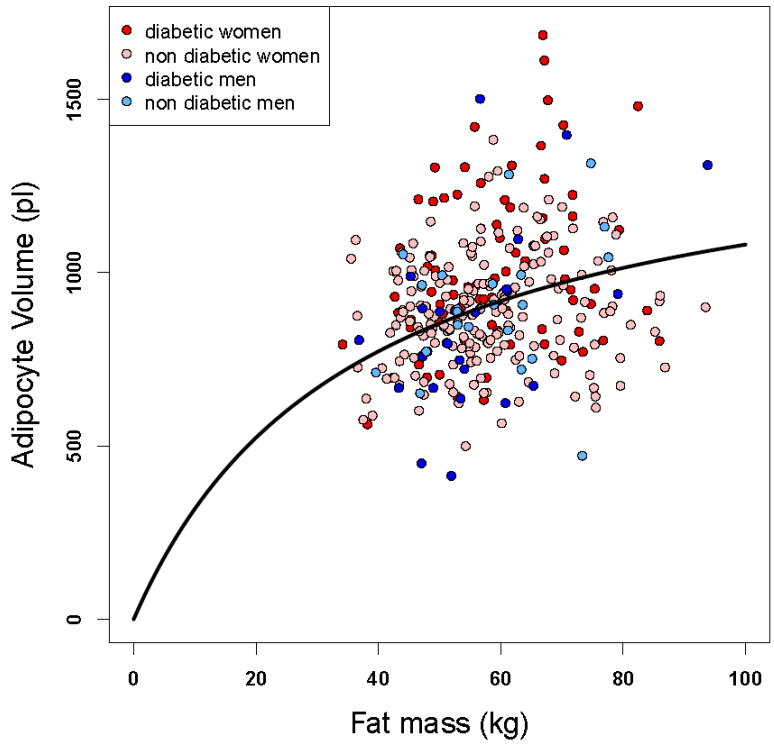

Figure 5 | Curve-linear relationship between fat cell volume and fat mass in our morbid obese population (Aurélie Cotillard, unpublished data).

These changes have been attributed to cellular stresses including hypoxia, endoplasmic reticulum stress and oxidative stress (Furukawa et al., 2004) (Hummasti and Hotamisligil, 2010). Overall, these pathways utimately induce cellular inflammatory cascades mainly through the NF-κB activation, likely to interfere with insulin signalling. In obesity, adipocytes become insensitive to insulin-induced inhibition of lipolysis leading to an increase in circulating FFAs. In obesity as well, visceral adipocytes were found to be more lipolytic and less capable of lipogenesis than subcutaneous counterparts (Lafontan et Girard, 2010 ; Ortega et al., 2010). Eventually, adipocyte hypertrophy can lead to adipocyte death. In a DIO mice model, the frequency of adipocyte death increases progressively during DIO and positively correlates with adipocyte cell size (Strissel et al., 2007).

One of the major discoveries in obesity pathophysiology is the accumulation of immune cells within the adipose tissue. Immune cells dialogue with stressed or moribund adipocytes contributing to the development of a pro-inflammatory microenvironment. Among immune cell types, macrophages and T lymphocytes were shown to massively infiltrate adipose tissue, while minor subsets such as mastocytes, NKT cells or B lymphocytes were also described. This part will be extensively discussed in introductory section B. In is interesting to

note that immune cell infiltration is generally increased in visceral adipose tissue compared to subcutaneous depots, underlying an increased pro-inflammatory status of the visceral depot. Other SVF components that are modulated during weight gain are the endothelial cells. It has been proposed that adipose tissue microenvironment can activate endothelial cells that, in turn, may promote leukocyte infiltration through up-regulation of adhesion molecules (Curat et al., 2004). Beside, endothelial cells isolated from visceral adipsoe tissue appeared in a premature senescent state characterized by increased expression of pro-inflammatory genes, compared to subcutaneous counterparts (Villaret et al., 2010).

2.3.2. Tissular alterations

Adipose tissue contains a dense network of blood capillaries, ensuring adequate supply in oxygen, nutrients as well as systemic lipids that will be stored in adipocytes. Thus, expansion and reduction of the fat mass partly relies on the adipose tissue circulation. Angiogenesis, that is, the growth of new vessels from pre-existing ones, appears to be crucial in the modulation of adipose tissue growth in the setting up of obesity. It is thought that angiogenesis is increased in obesity in both humans and animal models, and inhibitors of angiogenesis prevent obesity in murine models (Cao et al., 2003; Lemoine et al., 2012). Several immunohistochemistry-based studies tried to compare capillary densities between subcutaneous and visceral adipose tissue, although results are controversial (Ledoux et al., 2008; Galeakman et al., 2012). Discrepancies highlight the difficulties to 1/ find specific markers of endothelial cells, 2/ take into account the smallest adipocyte size in visceral compared to subcutaneous fat depots and 3/ comprehend the three dimentional aspect of the adipose tissue vasculature. In flow cytometry, endothelial cells were found to be more abundant in visceral adipose tisse than subcutaneous fat depot. Besides, positive correlations were found between adipose tissue vascular density and both BMI and adipocyte size, suggesting that adipose tissue vascularization supports its own expansion. One mechanism could be the development of regional areas of hypoxia that triggers angiogenic responses in adipose tissue. Therein, adipocyte hypertrophy might compromise effective oxygen supply from the vasculature and favor a hypoxic microenvironment. Several studies described the presence of hypoxia areas in adipose tissue through the induction of one key hypoxia regulator, hypoxia-inducible factor 1 (HIF1). Oxygen delivery to adipocyte becomes suboptimal as the blood flow per adipocyte declines with weight gain (reviewed in Lee et al., 2010). It could be hypothesized that “good” adipose tissue expansion occur when growing adipocyte size is concomitant with enhanced vascularization (Figure 6). The question of whether or not hypoxia occurs in adipose tissue has been well documented in diet-induced or genetically obese mice, using of pimonidazole as a hypoxia marker. Yet, the

real mechanism underlying hypoxia in human adipose tissue remains controversial and need clarification (Goossens et al., 2011). In human studies, one important issue could be that adult subjects are obese since many years and are usually weight stable (weight stabilization being required for gastric surgery). Hypoxia and hypoxia-induced angiogenesis might be involved in the dynamics of weight gain, which is missed in human obesity. Although, in visceral adipose tissue, endothelial cells showed increased hypoxia-related gene expression and exhibited more potent angiogenic and inflammatory properties (Villaret et al., 2010). Beside angiogenesis, hypoxia was also found to be associated with inflammation and extracellular matrix deposition (Halberg et al., 2009). This latter is of primordial importance in obesity-induced adipose tissue remodeling, as it can directly modelate adipocyte physiology.

Adipose tissue cells are maintained within a network of extracellular matrix (ECM) whose deposition is impaired during obesity. To accommodate to changes induced by increased adipocyte size in obesity, remodeling of the ECM occurs by degradation of the existing matrix and production of new matrix components. The consequences of ECM modification and pathological growth of adipose tissue have been mostly investigated in mice with knock-out models of ECM components that are, collagen VI and SPARC. Both studies show increased adipose mass and adipocyte size in the knockout compared to control mice, suggesting a primordial role for ECM to control adipocyte size in response to excess caloric intake. In humans, adipose tissue remodeling and fibrosis are poorly documented but it is thought that ECM accumulation occur during obesity. In morbid obese individuals, the presence of different patterns of fibrous depots, either pericellular fibrosis, *i.e.* around adipocytes and fibrosis bundles, was reported. Both total fibrosis content and pericellular fibrosis was significantly increased in obese subject compared to lean controls and fibrosis negatively correlated with adipocyte size (Divoux et al., 2010; Henegar et al., 2008). Besides, extracellular matrix deposition was found to be increased in subcutaneous adipose tissue compared to visceral counterpart in both lean and obese subjects (Divoux et al., 2010). Fibrosis is typically considered a fibroproliferative disorder with the uncontrolled production of ECM components, mainly by fibroblasts activated by an inflammatory microenvironment. In morbid obese patients, fibrosis depots were associated with the presence of immune cells such as mast cells but also macrophages and lymphocytes. Of note, it is still unknown whether immune cells directly participate in matrix deposition. In obesity, adipose tissue fibrosis is correlated with adipose tissue inflammation in both mice and human studies (Henegar et al., 2008; Pasarica et al., 2009a). On a physical point of view, fibrosis is likely to induce adipocyte dysfunction by exerting mechanical pressure on the cell to limit its size.

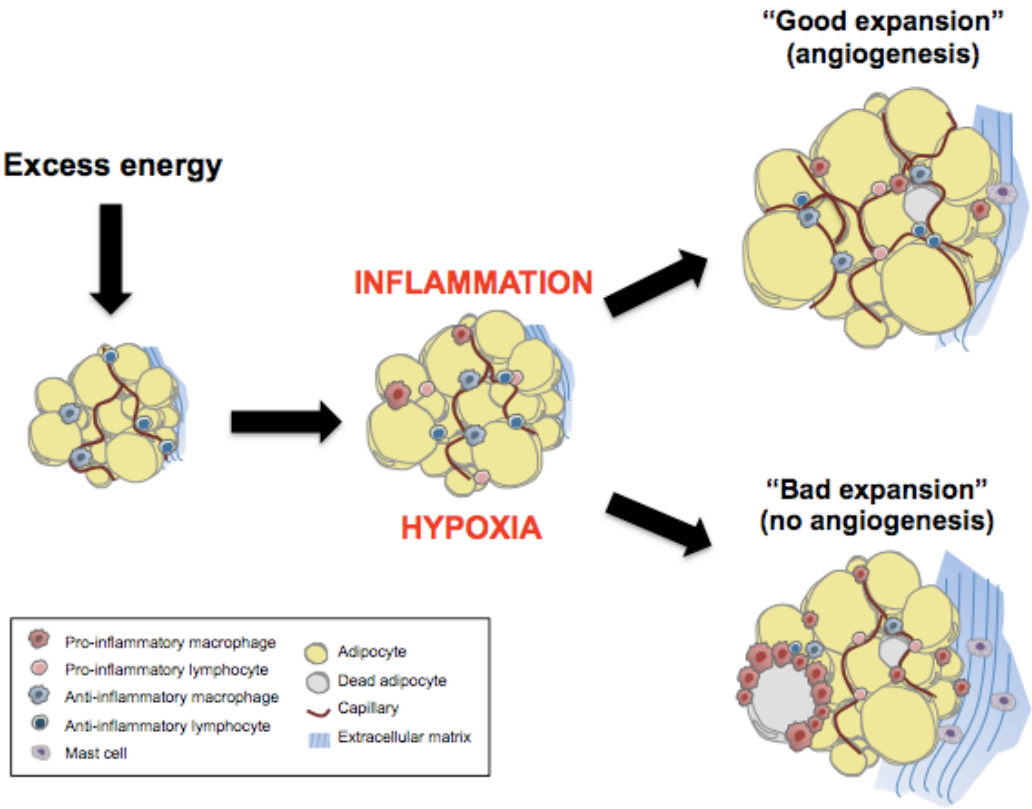

Figure 6 | Adipose tissue remodeling during obesity. Upon caloric excess, adipose tissue undergoes expansion, through adipocyte enlargement, leading to local hypoxia and a need for angiogenesis for healthy tissue function. « Good expansion » results in increased adipose tissue vasculature, with potentially higher adipocyte size and relative low immune cell infiltration. « Bad expansion » implies inflammatory cascades and insufficient adipose tissue vasculature, adipocyte dysfunction, inflammation through marked immune cell infiltration and massive fibrosis deposition (supposing smaller adipocytes). Of note, it is still debated whether hypoxia triggers inflammation, or inversely.

Thus, in obesity, adipose tissue is a site of intense modifications including cellular composition, cellular phenotype and tissular remodeling, in response to energy excess. Adipocyte dysfunction and overall inflammation appear to be the two key features of the obese adipose tissue. One can now wonder how local adipose tissue alterations can explain obesity-associated comorbidities. It should be kept in mind that adipose tissue depots show distinct metabolic and inflammatory profiles depending on their regional localization. Since insulin resistance and, utimately T2D, are the most prevalent obesity-associated complications, it is interesting to understand how obesity causes insulin resistance. Hence, in this introduction, I decided to focus on the different hypotheses that rationalize this phenomenon. Whereas they are treated separately, it is clear that they may act simultaneously and synergistically to bring about whole-body insulin resistant and eventually lead to T2D.

3. Theories of how obesity causes insulin resistance

3.1. The expandability hypothesis

The expandability hypothesis states that adipose tissue has a defined limit of expansion for any given individual. When the subcutaneous depot is not able to store the excess calories anymore, the storage shifts to other sites. These include the visceral adipose tissue and, ectopic sites such as the liver and muscle. Subcutaneous adipose tissue is thus considered a « metabolic sink » to buffer energy surplus, while excess visceral adipose tissue might reflect subcutaneous adipose tissue failure. For any given amount of total body fat, individuals with a selective excess of visceral adipose tissue are at higher risk of being insulin resistant or develop T2D (Pasarica et al., 2009b ; McLaughlin et al., 2011). Klöting and colleagues showed that insulin-resistant obese patients had significantly more visceral area but less subcutaneous depots than BMI- and age-matched insulin-sensitive obese subjects (Klotting et al., 2010). Considering its high lipolytic activity and its strategic localization draining the portal vein, accumulated visceral adipose tissue leads to excess FFA delivery in the circulation, whose blood concentration is also considered risks factor for T2D.

Subcutaneous adipose tissue failure could be explained by an insufficient total adipocyte number. This is supported by the fact that in the laboratory, T2D patients tend to show hypertrophy (as opposed to hyperplasia) in their subcutaneous adipose tissue (Figure 5). A 25-year prospective study further identified subcutaneous abdominal adipocyte size as a risk factor for T2D (Lonn et al., 2010). Both subcutaneous and visceral adipocytes were significantly larger in insulin-resistant obese patients compared to insulin-sensitive counterparts (Kloting et al., 2010). This insufficient adipocyte stock could originate from insufficient adipocyte number when set up during childhood or impaired adipogenesis in subcutaneous fat. The former hypothesis implies that individuals with rather low subcutaneous adipocyte number are predisposed to T2D (supposing potential genetic makeup). The latter hypothesis suggests that subcutaneous adipogenesis is dysfunctional due to local inflammation and/or metabolic alterations in obesity. Several studies suggest that abdominal obesity and obesity-induced T2D are associated with impaired adipogenesis in subcutaneous preadipocytes (Tchoukalova et al., 2006 ; Tienen et al., 2011 ; Park et al., 2011). Differentiation capacity of subcutaneous preadipocytes was found to be associated with surrogates of insulin resistance and visceral adipocyte size (Park et al., 2011). These observations suggest that impaired adipogenic capacity of subcutaneous depots might lead to visceral fat deposition and subsequent, visceral adipocyte hypertrophy.

Thus, while visceral fat is deleterious, subcutaneous adipose tissue may have many beneficial properties and is protective for insulin resistance (McLaughlin et al., 2011). This is supported by several arguments. For instance, lipodystrophies, which are often characterized by loss of subcutaneous fat depots, are associated with T2D. In a lipodystrophic mice model, diabetes was reversed by surgically implanting subcutaneous adipose tissue (Despres and Lemieux, 2006). In the same way, transplantation of subcutaneous fat of donor mice into the visceral cavity of recipient mice improved the insulin sensitivity of the recipient animals. Lesser effect was obtained when the transplantation was achieved in the subcutaneous area. These data implies that subcutaneous adipose tissue can have direct and beneficial effects on control of metabolism through combination of its own physiological properties and the visceral localization with direct access to the portal circulation (Tran et al., 2008). Another example is the thiazolidinedione treatment that induces a shift of fat repartition from visceral to subcutaneous adipose depots that could participate in improvements in peripheral tissue insulin sensitivity (Miyazaki et al., 2002). In humans, omentectomy that is, removal of visceral fat, resulted in decreased glycemia and insulinemia while subcutaneous fat removal, *i.e.* liposuccions, did not (Tran et al., 2008).

3.2. The adipokine hypothesis

The second hypothesis focused on the endocrine role of adipose tissue. Adipose tissue produce various factors that are collectively referred to as adipokines, although the exact definition is not clear. In this manuscript, I chose to define as adipokines all molecules that are mainly produced by adipocytes. Obesity is associated with an alteration in the secretion profile of these adipokines. In the obese condition, adipocytes secrete more adipokines that cause insulin resistance than adipokines that would promote insulin sensitity. Thus, among adipokines, the two most-studied, leptin and adiponectin, are thought to provide an important link between obesity and insulin resistance (reviewed in (Ouchi et al., 2011; Tilg and Moschen, 2006).

Leptin is an anorexigenous hormone that regulates energy dependent processes such as food intake, metabolic rate and fertility through the central nervous system. Mice with a mutation in the gene encoding leptin (*ob*/*ob* mice) or the gene encoding the leptin receptor (*db*/*db* mice) show hyperphagia, obesity and insulin resistance. Leptin infusion to *ob*/*ob* mice drastically reduced their food intake and improved their metabolic abnormalities. Circulating leptin levels increase in proportion to fat mass and leptin release is positively associated with increased adipocyte size (Skurk et al., 2007). Obese patients show increased levels of

circulating leptin without the anorexic reponse, suggesting the occurrence of a leptin resistance process. Leptin is considered a pro-inflammatory factor, structurally similar to other pro-inflammatory cytokines such as IL-6. It is at the frontier of metabolism and immunity and is now defined as a powerful immuno-regulator (reviewed in (La Cava and Matarese, 2004)). Thus, high leptin levels are likely to participate in obesity-associated inflammation, the third hypothesis.

Adiponectin is an anti-inflammatory adipokine that acts as a insulin sensitizer and overall protects against obesity-associated metabolic complications. Circulating levels of adiponectin are decreased in patients showing visceral obesity, and inversely correlate with insulin resistance. DIO mice deficient in adiponectin develop exacerbated insulin resistance. Interestingly, the overexpression of adiponectin in *ob*/*ob* mice markedly improves their glucose metabolism despite increased adiposity, providing strong support for the expandanility hypothesis (Kim et al., 2007). In human, low circulating adiponectin levels were shown to be predictive for the development of T2D (Skurk et al., 2007). The production of adiponectin by adipocytes is stimulated by PPAR-γ agonists and inhibited by pro-inflammatory factors such as TNF-α and IL-6, along with hypoxia and oxidative stress. Similiar to leptin, adiponectin may also play a role in modulating immune responses. Adiponectin was shown to greatly influence macrophage polarization. It inhibits the TLR-mediated NF-κB activation, and subsequent production of pro-inflammatory cytokines and chemokines, while up-regulating IL-10 and IL-1ra production. Beside, adiponectin is likely to act on adaptive immunity as its receptor was shown to be expressed on T lymphocytes and down-regulated during obesity (Alberti et al., 2007).

3.3. The inflammation hypothesis

Last but not least, the third hypothesis is based of the fact that obesity is considered an inflammatory condition characterized by low-grade chronic inflammation. The concept of inflammation was elegantly defined by Medzhitov as discussed in Box 1. Obesity associates with abnormal cytokines production, increased acute-phase reactants and other mediators that contribute to the activation of a network of inflammatory signalling pathways. As mentionned by Hotamisligil, chronic inflammation can lead to vicious circles as it intrinsically connects inflammation to the pathophysiological process it accompanies (Hostamisligil, 2006). At the systemic level, obesity associates with increased circulating levels of the acute-phase proteins CRP and SAA as well as many other pro-inflammatory cytokines and

chemokines such as IL-6, IL-8 and CCL-2 (Despres and Lemieux, 2006). Systemic inflammation is a risk factor for developping comorbidities. For instance, elevated levels of IL-6 and detectable levels of IL-1β were found to predict future risks of T2D (Spranger et al., 2003).

Part of this systemic inflammation originates from the adipose tissue that is known to be a site of inflammation itself. This was demonstrated for the first time by Hotamisligil and colleagues in 1993 (Hotamisligil et al., 1993). Their study showed an increased production of TNF-α by adipose tissue of DIO mice models and the improvement of insulin sensitivity in these animals when injected with a TNF-α neutralizing antibody. Of note, TNF-α is thought to play a key role in local inflammation while systemic levels were not increased in obese patients. From this moment, numerous studies focused on the production of pro-inflammatory mediators by adipose tissue, whether they would come from the adipocytes or the adipose SVF. It is now appreciated that not only TNF-α but also an array of inflammatory cytokines are increased in obese tissues, including IL-6, IL-1β, CCL2, and many others. Visceral adipose tissue is more pro-inflammatory than subcutaneous adipose tissue (Cancello et al., 2006). Additionally, ectopic depots also display pro-inflammatory features, as it was shown for perivascular adipose tissue in a DIO mice model or epicardial adipose tissue in human studies (Mazurek et al., 2003; Ouchi et al., 2011). Since a decade now, it is well appreciated that adipose tissue is infiltrated by different immune cell types during obesity. Predominantly, macrophages and T lymphocytes accumulate in close interactions with adipocytes and adjacent cells from the SVF. Thus, the “inflammation hypothesis” includes the different feed-forward cross-talks that together maintain an pro-inflammatory microenvironment in adipose tissue. Immune cell accumulation and their potential triggers in adipose tissue will be extensively review in introductory section B.

3.3.1. Hypertrophic adipocytes sustain inflammation

In obesity, adipocytes display increased secretion of pro-inflammatory cytokines, proportional to their cell volume (Skurk et al., 2007). Adipocyte hypertrophy may eventually lead to necrosis-like adipocyte death. In that case, cell content and alarming messages are released in the extracellular space where they trigger inflammatory response from neighboring cells, especially immune cells (Cinti et al., 2005). Whether immune cell infiltration is strictly secondary to concomitant adipocyte hypertrophy of death during fat expansion remains elusive. Besides, increased lipolytic activities of visceral hypertrophic adipocytes lead to an efflux of FFAs that can bridge metabolism and inflammation.

Box 1 | The concept of inflammation

Inflammation is an adaptive response that is triggered by noxious stimuli or conditions. It involves a wide range of cellular and molecular events that form complex regulatory networks and can be classified into different functional categories: inducers, sensors, mediators and effectors. Inducers, whether they are exogenous or endogenous, are the signals that trigger and initiate the inflammatory responses. They activate specialized sensors, which in turn elicit the production of a variety of mediators in specialised leukocytes or cells present in a local tissue. These mediators finally affect target tissues or cells, that are the effectors of the inflammatory response (Medzhitov, 2008). The most well studied inflammatory response is the acute inflammation during which occurs a physiological reaction against infection or tissue injury, leading to healing processes to restore tissue homeostasis. This acute inflammation represents an extreme end of a spectrum of conditions that can trigger inflammatory responses and are characterized by the highest magnitude of inflammatory mediators. More generally, an inflammation occurs whenever tissue malfunction is detected, a condition that is more common but engages lower magnitude response. The concept of inflammation assumes that a pathological inflammatory state eventually has a (beneficial) physiological counterpart, which is, providing the organism protection against infections. As Medhzitov states in his review, « whatever the inducers of the inflammatory responses, its purpose is to remove the source of the disturbance, to allow the host to adapt to the abnormal conditions and, ultimately, to restore functionality and homeostasis to the tissue ». If the abnormal conditions are transient, then an acute inflammatory response can be succesful to return to tissue homeostasis. If the abnormal conditions are sustained, an on-going inflammation occurs in an attempt to restore homeostasis without success, and a chronic inflammatory state ensues that can become maladaptive and detrimental. Sustained tissue malfuction can result from genetic mutations but also, from changes in environmental conditions and lifestyles, for instance the western diet. Medzhitov refers to this state as « para-inflammation », *i.e.* an adaptive response induced by tissue stress or malfunction that is intermediate between basal and inflammatory states. Obesity and subsequent T2D might be good illustrations. It remains to know if obesity-related para-inflammation eventually leads to beneficial counterparts or if it implies, because of its chronicity, that beneficial effects are not to be reached, unless external interventions are performed, such as weight loss.

One mechanism by which FFAs have been proposed to directly influence immune cell signaling is through their recognition by pathogen-sensing molecules as it will be presented in the second part of this introduction. It has become clear that accumulation of macrophages

in adipose tissue is concomitant with insulin resistance during the course of a high-fat diet in mice. In humans, this link was not that obvious until very recent studies showed associations between adipose tissue macrophages and obesity-induced insulin resistance or T2D (Barbarroja et al., 2010; Bremer et al., 2011; Wentworth et al., 2010). Phenotypic differences between insulin-sensitive obese subjects and insulin-resistant counterparts were statistically explained by the association of macrophage accumulation in omental adipose tissue and decreased circulating level of adiponectin (Kloting et al., 2010). Moreover, this study showed a close relationship betwwen systemic and adipocyte insulin resistance in obese patients (Kloting et al., 2010). As mentioned earlier, leptin whose serum concentration is highly increased proportional to adiposity, is likely to sustain immune cell activation. Leptin is able promote both macrophage and T lymphocyte activation and release of pro-inflammatory cytokines. More importantly, leptin is likely to prerentially target and inhibit regulatory T lymphocyte (La Cava and Matarese, 2004). These cells, with popent regulatory and anti-inflammatory properties, are likely to play a key role in adipose tissue physiology as discussed later on.

3.3.2. Inflammation alters adipocyte biology

Adipose tissue-infiltrated immune cells secrete a panel of pro-inflammatory cytokines that are able to alter adipocyte biology. Most of pro-inflammatory factors such as TNF-α, IFN-γ and IL-1β can trigger insulin resistance in adipocytes. These molecules are known to interfere with insulin signalling through activation of serine/threonine kinases (Duffaut et al., 2009a; Feingold et al., 1992; Gregor and Hotamisligil, 2011; Jager et al., 2007; Kloting et al., 2010; Lagathu et al., 2006; McGillicuddy et al., 2009; Rocha et al., 2008). These include the two prominent JNK and IKK-β kinases that inhibit insulin-induced IRS-1 tyrosine phosphorylation by promoting its counter-regulatory serine phosphorylation (Tanti et al., 2004). Beside, kinases also regulate downstream transcription programs through AP-1 and NF-κB activation, resulting in increased adipocyte expression of pro-inflammatory cytokines that further up-regulate JNK and NF-kB pathways through feed-forward mechanisms. NF-κB inflammatory cascades in omental fat were found to be discriminative between insulin-sensitive and insulin-resistant obese patients (Barbarroja et al., 2010). Adipocyte insulin resistance keeps insulin from promoting lipogenesis and suppressing lipolysis, leading to an increased flux of FFAs from adipocytes to other target tissues (Attie and Scherer, 2009). Finally, effects of pro-inflammatory cytokines are not limited to insulin signaling and may impair adipogenesis through inhibition of PPAR-γ activity (Gregor and Hotamisligil, 2011; Pasarica et al., 2009b). Of note, pro-inflammatory macrophage conditioned media were shown to inhibit preadipocyte

adipogenesis while promoting a profibrotic phenotype in preadipocytes (Keophiphath et al., 2009). Of note, extracellular matrix deposition that is increased in obese adipose tissue might also interfere with adipogenesis (Divoux et al., 2011).

One question that arises from all these observations is whether or not it is relevant to develop drugs that target inflammation in order to break the link between obesity and its comorbidities. Publications relating disruption of inflammatory cascades in obesity are scarce, sometimes contradictory but overall still promising. Treatment with anti-TNF-α antibodies has been demonstrated to improve insulin sensitivity in DIO mice, but is less effective in humans. Yet, a very recent study reported that treatment of obese T2D patients with etanercept, a TNF-α antagonist, resulted in reduced blood glucose and increased adiponectin levels (reviewed in Gregor and Hotamisligil, 2011). Other important study using anakinra, a human recombinant IL-1 receptor antagonist (IL-1ra), observed improved glycemia and β-cell function while decreased systemic inflammation in obese T2D patients (Larsen et al., 2009; Larsen et al., 2007). Human trials using, for instance, anti-IL-6 or targeting lymphocyte-derived cytokines such as IFN-γ are missing to date. Aiming at inflammation may also include kinase and pathogen-sensing pathways. For instance, many studies focused on the down-regulation of JNK kinase pathway that resulted in recovering of insulin sensitivity in DIO mice, whereas human outcomes have to be evaluated. Finally, salsalate, a prodrug form of salicylate, was shown to improve glycemic control in T2D obese patients through the down-regulation of the IKK-β/NF-κB activity (Goldfine et al., 2008). These pioneer studies in humans are of major importance as they strongly support a direct link between obesity-induced inflammation and its comorbidities. It might be possible that future therapeutic manipulations could target TLRs or other pathogen-sensing receptors, for the treatment of obesity-related comorbidities. Hopefully, it is currently under study for autoimmune diseases such as rhumatoid arthritis or inflammatory bowel syndrom (Hennessy et al., 2010).
Thus, during obesity, adipose tissue handles excess energy by preferentially filling the subcutaneous depot until it reaches it maximal storage capacity. At this point, visceral and other ectopic depots are accumulating in association with increased risk of developing T2D. Failure of subcitenaous adipose tissue is likely to result from impaired adipogenesis and subsequent adipocytes hypertrophy in response to a pro-inflammatory and pro-fibrotic microenvironment. At the cellular level, adipocytes undergo extraordinary adaptive changes, becoming hypertrophic at the cost of their own insulin sensitivity. Increased lipolytic activity in visceral adipose tissue ensues an up-regulation in FFA efflux in the circulation. In the same time, visceral adipose tissue is a site of intense inflammation where numerous immune cells infiltrate, perpuating adipocyte dysfunction.. Although, it is widely accepted that alterations of adipose tissue biology contribute to metabolic complications of obesity, the actual molecular

actors linking altered adipose tissue to peripheral organ pathologies are not fully defined. Although maybe over-simplified, Figure 7 tries to summarize the metabolic disturbances and inflammatory cascades going on in adipose tissue from lean *versus* insulin resistant obese subjects, that might eventually contribute to peripheral organ dysregulation as commented in Box 2.

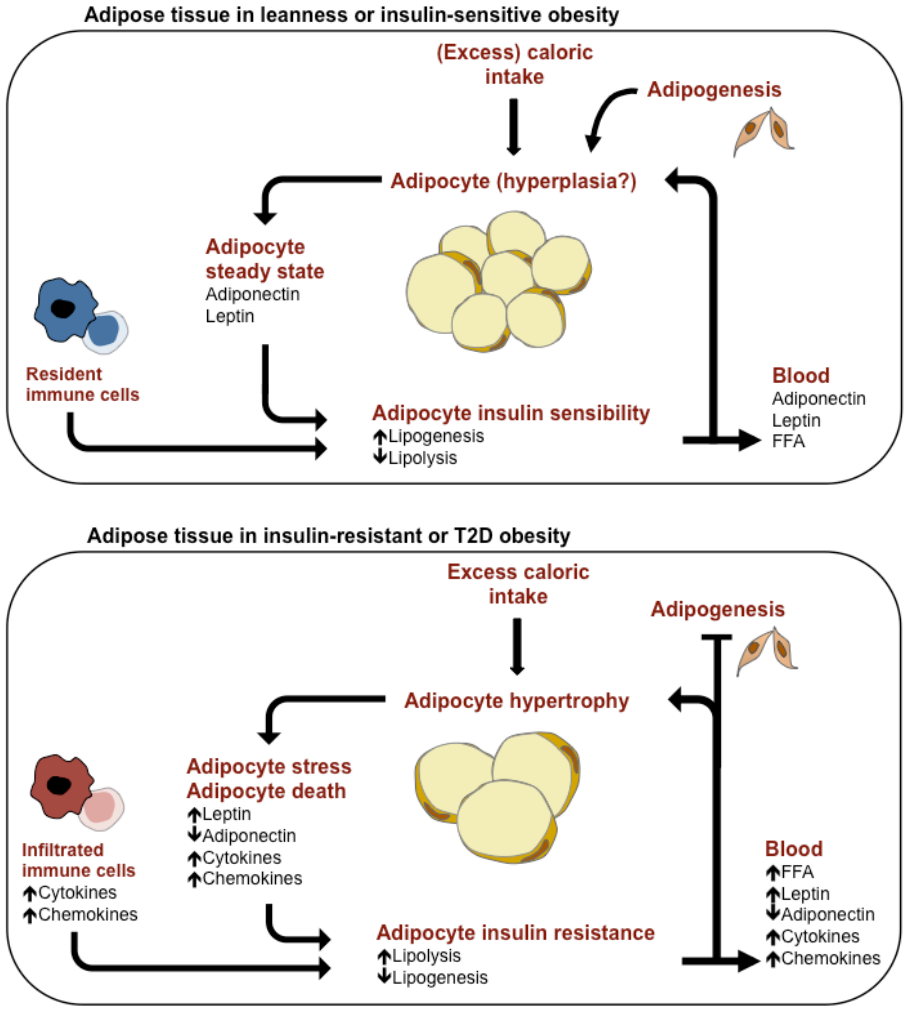

Figure 7 | Adipocyte inflammatory loop in obesity. In lean and insulin-sensitive obese individuals, adipose tissue is characterized by middle-sized adipocytes that are insulin sensitive and secrete insulin-sensitizing adipokines such as adiponectin. Resident immune cells ensure adipose tissue homeostasis while adipogenesis is maintained to provide new adipocytes in case of excess energy intake (leading to hyperplasia in the case of insulin-sentive obesity). Of note, it is my choice to extrapole that adipose tissue physiology is likely to be the same in lean and healthy obese individuals. In insulin-resistant obesity, adipocyte hypertrophy leads to cell stress and/or death and promotes the secretion of proinflammatory mediators that in turn, provok inflammatory immune cell infiltration and adipocyte insulin resistance. Beside, adipogenesis is blunted, perpetuating adipocyte hypertrophy. Increased FFAs, leptin and inflammatory mediators are released in the circulation.

4. Obesity and weight loss strategies

4.1. Weight loss outcomes in adipose tissue

Weight loss strategies are successful to reduce total fat mass in obese subjects, through the decrease of fat cell size but not cell number (reviewed in Arner and Spalding, 2010). Besides, it is widely accepted that weight loss, whether it is achieved by (very) low calorie diets or by digestive surgeries markedly improves inflammatory and glycemic status of obese subjects (Caiazzo et al., 2009). Weight loss is also associated with a decrease in local adipose tissue inflammation. *In situ*, cellular and molecular mechanisms relying these beneficial effects are not fully deciphered. Weight loss induced by a very low calorie diet decreases the expression of pro-inflammatory and macrophage markers in the SVF fraction of adipose tissue of obese subjects. In counterpart, this leads to the up-regulation of molecules with anti-inflammatory properties (Capel et al., 2009; Clement et al., 2004).

Then, the next question to answer is whether or not number of adipose tissue immune cells is also decreased during weight loss. Number of adipose tissue macrophage was unchanged during short-term caloric restriction but substantially decreased after a 6-month period of weight maintenance (Kovacikova et al., 2011). Following gastric surgery, weight loss was associated with a decreased content in macrophages in adipose tissue as soon as month 3 (Aron-Wisnewsky et al., 2009; Cancello et al., 2005). Interestingly, in a model of DIO mice submitted to caloric restriction, Kosteli *et al*. showed that macrophage recruitment in adipose tissue was initially increased following early caloric restriction, without concomitant increased in inflammatory mediators and, finally decreased. A series of experimental studies therefore indicated that these macrophages phagocytose excess lipids without causing inflammation, hereby contributing to restoration of local and systemic lipid homeostasis during the early phases of caloric restriction (Kosteli et al., 2010). In conclusion, overall weight loss leads to a marked improvement of systemic and adipose tissue inflammation in obese patients, with surgical interventions being the most effective to date. Limitation of weight loss studies lies in that only subcutaneous adipose tissue can be sampled during post-surgery follow-up, and yet, visceral adipose tissue appears to be the most deleterious fat depot in obesity.

Box 2 | Obesity-linked alterations in peripheral organs

Circulating FFAs, through a process of lipotoxicity, result in inappropriate triglycerides (TGs) accumulation in peripheral organs, causing adverse effects on cellular metabolism. While intra-cellular TGs could only represent a storage form of FFAs, fat intermediates and metabolites could directly be responsible for insulin resistance in peripheral organs. Fat metabolites include diacylglycerol, ceramides or acylcarnitine products of incomplete β-oxidation and are able to interfere with insulin signalling. In addition, chronically elevated glucose levels may also have detrimental effects, especially on pancreatic islets (Donath and Shoelson, 2011). Besides, circulating pro-inflammatory cytokines are up-regulated in obesity and it is obvious that these inflammatory agents may influence cell metabolism in a similar way to adipocyte insulin resistance.

Skeletal muscle is a key site of glucose uptake, glucose storage as glycogen and energy consumption in the body, contributing to glucose homeostasis. During obesity, TGs were observed to accumulate in myocytes inducing insulin resistance and local inflammation. This is likely to occur concomitant to macrophage accumulation, although publications do not agree concerning the presence or not of muscle macrophages (reviewed in (Kewalramani et al., 2010)).

Liver represents another major metabolic organ in its ability to control not only gluconeogenesis and glycogen storage, but also lipogenesis. In the liver, insulin resistance is selective and is defined as a failure to suppress gluconeogenesis while maintening FFA synthesis. Obesity and T2D are often associated with NAFLD, which is strongly correlated with insulin resistance, and interventions that enhance insulin resistance decreased fat liver content, suggesting that liver TGs cause insulin resistance (Gastaldelli et al., 2009). NAFLD is also associated with local inflammation that may cause hepatic insulin resistance. However, several mice models or human genetic diseases showed that NAFLD could occur in the absence of insulin resistance (Cohen et al., 2011; Verrijken et al., 2008). Thus, the mechanisms by which hepatic fat accumulation might lead to hepatic insulin resistance have not been resolved.

Pancreas is at the heart of glucose homeostasis through its endocrine function to produce hormones. Endocrine pancreas includes the islets of Langherans that are composed of many cells types, with β-cells being the insulin-secreting cells. Pancreatic islets compensate insulin resistance by enhancing their cell mass (islet number) and insulin secretary activity, leading to hyperinsulinemia. Yet, when β-cells fail to produce sufficient levels of insulin, T2D may develop, leading to hyperglycemia. The defect in insulin secretion in T2D is thought to be a combination of reduced functionnal β-cell mass and intrinsic β-cell dysfuntion (reviewed in (Donath and Shoelson, 2011)).

Recently, it has become evident that β-cell dysfunction implicates inflammatory processes, somehow similar to adipose tissue. During obesity, TGs might also accumulate in pancreas and functional adipocytes were even found near β-cells (Pinnick et al., 2008).

4.2. Our model: the gastric bypass

Unfortunately, caloric restrictions, with or without the help of medications, such as oral inhibitors of gastrointestinal dietary fat absorption, are relatively disappointing in term of long-term weight loss and metabolic improvements. A very recent study shows that conventional medical therapy (*i.e.* medications and lifestyle interventions) was far less effective than gastric surgery to control hyperglycemia in obese subjects (Mingrone et al., 2012). Thus, when classic weight loss strategies are insufficient, bariatric surgeries offer effective alternatives to treat obesity and T2D. Of note, « bariatric, » comes from the Greek and literally means « weight treatment ». Morbid obese subjects can undergo several types of bariatric surgeries; the most common include (adjustable) gastric banding and sleeve gastectomy that result in a diminution of the gastric pouch, or the gastric by-pass that leads to a diminution of the gastric pouch combined with a bypass of the upper intestine as illustrated in Figure 8. Surgery outcomes were extensively studied and comparing the three main bariatric interventions, bypass surgery appears to be the most effective way to treat T2D and other obesity-related comorbidities up-to-date.

Figure 8 | Three kinds of bariatric surgeries performed in clinical practice: the ajustable gastric banding, the sleeve gastrectomy and the Roux-en-Y gastric bypass.

Although all operated patients will eventually regain some weight, a 20-years weight loss follow-up study showed that gastric bypass induced the highest weight loss after the surgery and that it is roughly maintained throughout the years as illustrated in Figure 9 (Sjostrom et al., 2012). Of note, in reference to obesity-induced tissue alterations, an important factor that appears to modulate surgery-induced fat mass loss is the amount of subcutaneous adipose tissue fibrosis before surgery. Indeed, our group showed that the higher the amount of subcutaneous adipose tissue fibrosis, the lesser the fat mass loss.

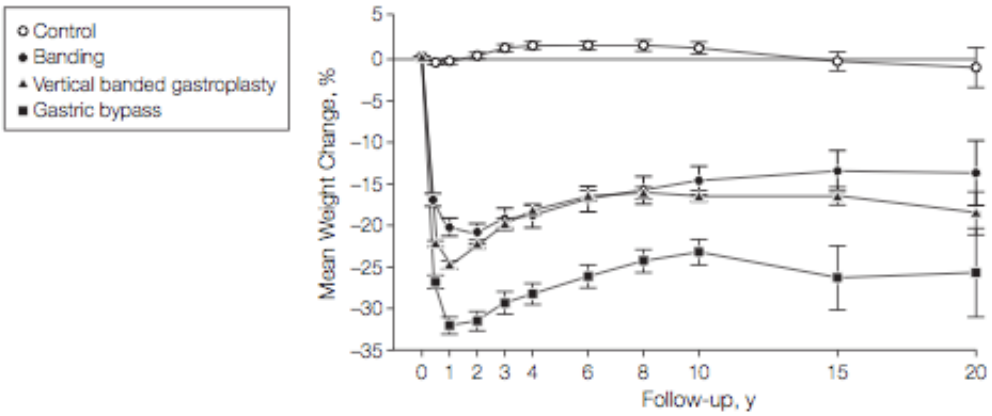

Figure 9 | Mean weight change percentages from baseline for controls and the three surgery groups over 20 years (Sjostrom et al., 2012).

Gastric bypass surgery is associated with substantial changes in total and regional adiposity. In our laboratory, total, trunk and appendicular fat masses were assessed using dual-energy X-ray absorptiometry (DXA) in a cohort of 92 morbid obese women and 12 morbid obese men. As illustrated in Figure 10, obese subjects preferentially lost abdominal adipose tissue. Note that DXA-derived trunk fat mass actually takes into account visceral adipose tissue (omentum plus mesenteric) as well as abdominal subcutaneous fat depots. In order to discriminate visceral from superficial fat depots, abdominal computated tomography scans would be required. Although, these sections are difficult to obtain, especially in morbid obese subjects, and could explain why scarce data are available in the literature.

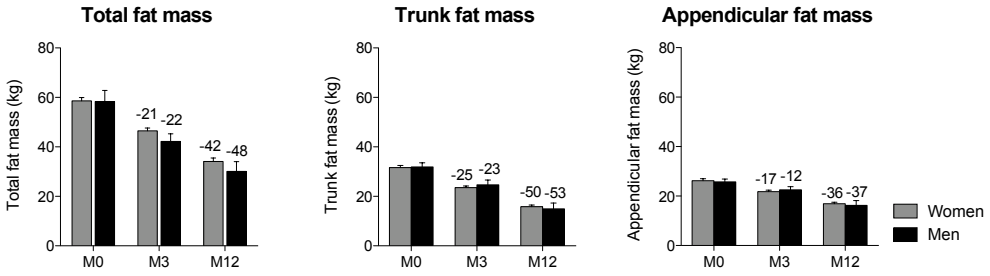

Figure 10 | Total and regional body composition before and during 3 and 12-month follow-up after gastric bypass in morbid obese women (n=92) and men (n=12). Negative value represent mean fat mass loss in percentages. (E. Dalmas, C. Poitou and J-M.Oppert, unpublished data).

Beside, a meta-analysis reviewing 136 weight loss studies compared comorbidity improvement following the 3 types of surgery. Gastric banding, gastric sleeve and gastric bypass resolved 48, 72 and 84% of T2D; 59, 74 and 97 of hyperlipidemia; 43, 69 and 68% of hypertension and 95, 78 and 80% of sleep apnea, respectively. The report strongly supports that gastric bypass is to date the most efficient intervention to resolve obesity-induced comorbidities, and especially T2D (Buchwald et al., 2004). Interestingly, T2D resolution sometimes occurs within days, if not hours, post-surgery, before any significant weight loss. Therefore, beside drastic weight loss, gastric bypass also acts through two potential mechanisms reviewed in (Caiazzo et al., 2009; Sandoval, 2011). The « foregut hypothesis » postulates that nutrient bypass of the upper gut hinders some yet undiscovered signals that promote insulin resistance, while the « hindgut hypothesis » suggests that increased delivery of nutrients to the lower gut stimulates the release of anorexic and incretin hormones with beneficial outcomes. Of course, surgery interventions always call for a risk-benefit assessment. Publications observed that gastric bypass operative mortality rates range from 0,25 to 0,5%, which is relatively low. Also, it was reported that 4,1% of patients experienced at least one major adverse complication. These include anastomotic leaks, wound infections, pulmonary events, hemorrhages and more often, nutritional complications such as vitamin and mineral deficiencies (Buchwald et al., 2004).

In our laboratory, we follow morbid obese subjects candidate for the Roux-en-Y gastric bypass surgery. Criteria for obesity surgery are defined as a BMI of more than 40 kg/m^2 or more than 35 kg/m^2 with a least one comorbidity (*i.e.* hypertension, T2D, dyslipidemia, or obstructive sleep apnea syndrome). Bariatric surgery is a suitable model of weight variation since weight gain studies, with significant magnitude, are not ethically feasible in humans. Gastric bypass, through laparoscopy, is a great opportunity for researchers to obtain several adipose tissue samples, both from the subcutaneous and visceral depots.

In conclusion, obesity is a complex pathophysiological state characterized by different cellular, tissular and systemic alterations that occur with different and yet undefined kinetics. At the heart of adipose tissue dysfunction lie adipocyte insulin resistance and inflammation. There is now considerable evidence supporting the hypothesis that chronic inflammation and immune cell infiltration are major risk factors for the development of obesity-related complications and notably, T2D. In 1998 already, and five years before the actual discovery of macrophages within adipose tissue, Pickup and Crook speculated and asked the question whether T2D was a disease of the innate immune system, mainly based on systemic low-grade inflammation observations. Now, with the on-going discovery of immune cell types co-infiltrating adipose tissue, the immune system, whether it is innate or adaptive, appears to play a key role in both adipose tissue homeostasis and pathopathology.

B. A new playground for the immune system: adipose tissue

1. Introducing the immune system

The immune system gathers different organs and tissues such as the spleen or lymph nodes that are composed of interdependent cell types. Collectively, they protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. Immune cells can engulf bacteria, kill parasites or tumor cells, or kill infected cells. It is interesting to note that the immune system can also face « sterile inflammation », as it was mentionned before. Immune cells activate one another and/or adjacent cells *via* inflammatory signals in the form of secretions formally known as cytokines, or more specifically interleukins. In vertebrates, two types of immunity are used to protect the host from infections: innate and adaptive. Cellular components of each system are shown in figure 11 below.

In this section, we will first focus on how the immune system and the adipose tissue have developed a complementary relationship and then, how the setting up of obesity specifically involves inflammatory immune cells. Among these latters, special interest will be given to the two main types of adipose tissue-infiltrated cells that are macrophages and T lymphocytes.

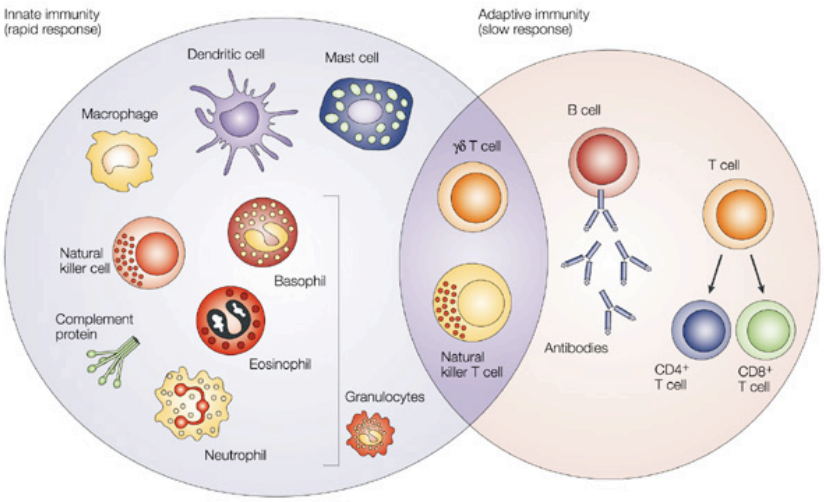

Figure 11 | The innate and adaptive immune cells (from (Dranoff, 2004)).

The innate immune system functions as the first line of defence and is genetically programmed to detect invariant features of invading microbes. It is composed of dendritic cells, macrophages and neutrophils among others. In contrast, the adaptive immune system, which is composed of T and B lymphocytes (and related soluble antibodies), employs antigen receptors that are not encoded in the germline but are generated *de novo* in each organism, making the adaptive immune responses slower but highly specific. In between, Natural killer T cells and gamma-delta T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.

2. Immune system and adipose tissue: a close relationship

Over the last decade, the immune system and inflammatory processes have been implicated in many diseases where their involvement had not previously been appreciated, such as type 1 diabetes and Alzheimer's disease. It is becoming evident that the immune system that was once considered to function independently of outside influence is in fact also regulated by many other systems, including the endocrine system. A very interesting example of system interconnection is the occurence of a transient insulin resistance during acute inflammation, allowing the redistribution of glucose to leukocytes that can have increased energy demand during infections (Medzhitov, 2008). First, many scientists assumed that adipose tissue through production of adipokines, *e.g.* leptin, could modulate the pathophysiology of immune reponse: metabolism disturbing the immune system (La Cava and Materese, 2004). Then, another idea arose that the immune system itself could play an important role in mediating adipose tissue alterations and metabolic complications: immune system sensing the

metabolism. Interestingly, Caspar-Bauguil and colleagues (2005) concluded that adipose tissue, containing both innate and adaptive immune cells, fits well the definition of organs such as the liver, whose « *primary function is not immunological but which often elaborate immunological mechanisms to mediate their functions* ».

Pioneer studies focused on adipose tissue surrounding lymph nodes that was thought to play a crucial role in immune responses. Lymph nodes are embedded in a thin layer of adipose tissue characterized by small adipocytes as compared to nodeless adipose tissue. These minor perinodal fat depots could serve to supply immune cells with the fuel and materials they need to ensure an effective response to foreign invasion. Prolonged, low-level immune stimulation induces the local formation of more adipocytes, especially adjacent to the inflamed lymph nodes (Pond, 2005). In the same way, the visceral adipose tissue has been shown to contain clusters of leukocytes embedded in the omental tissue, commonly termed milky spots, that monitor fluids, particulates and cells from the peritoneal cavity. Whether or not these milky spots may be considered as true secondary lymphoid organs is still debated. Recenly, clusters have been observed in both mouse and human mesenteric fat and could be colonized by unexpected novel Th2-type innate lymphocytes (Moro et al., 2010). The role of these milky spots is to be fully explored and curiously, no such structure was observed in visceral adipose tissue section in our laboratory yet.

Research on the link between metabolism and immunity started to intensify in the late 90's during which few but relevant hints were given to draw a connection between immune system and adipose tissue. Back in 1997, leukocytes appeared to play a role in regulating lipid metabolism and/or energy expenditure as mice deficient in ICAM-1, a major regulator of leukocyte migration, became spontaneously obese (Dong et al., 1997). In 2003, two major studies described for the first time the infiltration of adipose tissue by macrophages during obesity (Weisberg et al., 2003; Xu et al., 2003). A year later, another group found that adipose tissue also contains T lymphocytes (Robker et al., 2004). Together the presence of both innate and adaptive immune cells demonstrated the unique immune phenotype of adipose tissue. This also marked the beginning of a new exciting research field: the immunometabolism. From this moment, many studies focused on describing the accumulation and subtantial role of many different immune cell types in adipose tissue as illustrated in Figure 12.

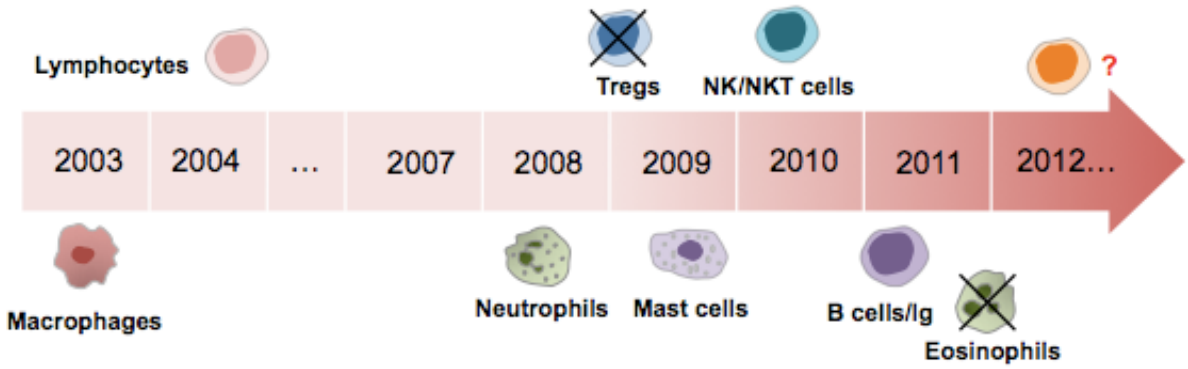

Figure 12 | Adipose tissue infiltrated immune cells. In the last decade, both innate and adaptive immune cells were found to infiltrate adipose tissue and play a major role in obesity in mice and/or humans. Black crossed-cells are subtypes whose number are decreased in obese adipose tissue. Ig, immunoglubulins.

Adipose tissue macrophages and lymphocytes were (and are still) extensively studied, as they appeared to be unexpected initiator and controller of obesity-induced inflammation. Other cells such as neutrophils, mast cells, NK cells and B cells were also described to participate in the setting up of obesity, while regulatory T cells and eosinophils showed anti-inflammatory and insulin sensibilizing properties (reviewed in (Cipolletta et al., 2011)). In the future, other immune cell subtypes will probably become new player in obesity. Most of immune cell infiltration and associated-inflammation in adipose tissue appear to coincide with the set up of insulin resistance suggesting that immune cell-dependent inflammation in adipose tissue can really affect systemic metabolism.

3. Adipose tissue & Innate immune system

During my PhD, I exclusively studied monocyte/macrophage lineage (and possibly dendritic cells as disccused later) physiology. For the sake of clarity, I chose not to present other innate immune cells, such as NK cells or granulocytes.

3.1. Antigen-presentation mechanisms

An antigen-presenting cell (APC) can be defined as any cell that expresses major histocompatibility complex (MHC) or related molecules (*e.g.,* CD1) that bind antigenic components, and can be recognized by the adaptive immune system. Additional co-stimulatory signal and soluble cytokines are then produced by the APCs, leading to activation of the accessory T cells. The two main types of APCs are dendritic cells (DCs) and macrophages that are also part of the mononuclear phagocyte family. In the case of

infections, mechanisms by which the inflammatory response is initiated have been well studied. Microorganisms are first sensed and recognized by specific receptors. Then, APCs mediate either cell-intrinsic (when APCs are infected) or cell-extrinsic innate immune recognition and present the antigen peptides at their surface for T cell interactions (Iwasaki and Medzhitov, 2010).

The best-characterized microbial sensors are the pattern recognition receptors (PRRs) of the innate immune system. PRRs detect relatively invariant molecular patterns found in most microorganisms of a given class that can be found in both pathogenic and non-pathogenic microorganisms. These are often called pathogen-associated molecular patterns (PAMPs). Depending on classification methodologies, there are four to six classes of PRRs that have been identified and categorized to date as shown in Table 1 (Chen and Nunez, 2010; Takeuchi and Akira, 2010). Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from stressed or damaged cells. These are called damage or danger–associated molecular patterns (DAMPs). In 1994, Polly Matzinger was the first to develop the « danger model » according to which DAMPs are normally kept inside the cells and yet, when cells are stressed or injured, these molecules are released into the extracellular environment and trigger inflammation under sterile conditions (Matzinger, 1998). DAMPs can also originate from degraded component of the extracellular matrix. Of course, the type of cell death determines the immunogenicity and immunostimulatory properties of the signal, with necrotic death, as opposed to apoptosis, inducing inflammatory cell infiltration and cytokine productions (Chen and Nunez, 2010; Matzinger, 1998).

In the context of obesity, it is often said that sterile inflammation occurs in adipose tissue, althouth the presence of specific obesity-related antigens is not excluded as debated later on in box 4. Several PRRs have been brought to light in obesity-associated inflammation. TLRs pathways were the first to be identified to play a role in adipose tissue inflammation (Gregor and Hotamisligil, 2011). Both TLR-2 and TLR-4 deficient mice show improvements in the metabolic syndrome associated with DIO (Himes and Smith, 2010; Shi et al., 2006). More specifically, $CD11c^{+}$ macrophage infiltrating adipose tissue in DIO mice were found to be stimulated with saturated FFAs *via* the two TLR-2 and TLR-4 pathways (Nguyen et al., 2007). TLR-mediated signal transduction leads to the activation of JNK and NF-κB signaling pathways, and subsequent production of pro-inflammatory cytokines. However, effect of TLRs in obesity might be more complex than simple macrophage activation. Indeed, it was shown that TLR-4 was also very much involved in the brain and notably, leptin pathway regulation and could act on food intake and body weight gain (reviewed in (Gregor and Hotamisligil, 2011)).

Name		Nature of ligands	Examples of ligands	Localization
Toll-like receptors	TLRs	PAMPS	Conserved microbial patterns such as LPS (TLR-1/2/4/5/6) and microbial nucleic acids (TLR-3/7/9)	Transmembrane (TLR1/2/4/5/6) or in endosomal organelles (TLR-3/7/9)
		DAMPS	Saturated lipids (TLR-2/4)	
NOD-like receptors	NLRs	PAMPS	Peptidoglycans, nucleic acids, toxins	Cytoplasm
		DAMPS	ATP, ROS, ceramides	
RIG-I-like receptors	RLRs	PAMPS	Viral nucleic acids	Cytoplasm
C-type lectin receptors	CLRs	PAMPS	Bacterial and viral proteins	Transmembrane
		DAMPS	OxLDL	
Absence in melanoma 2	AIM2	PAMPS	Bacterial proteins and viral nucleic acids	Cytoplasm
Collectins, ficolins (…)	-	PAMPS	Bacterial proteins and viral nucleic acids	Secreted

Table 1 | Categories of pattern recognition receptors, their ligands and cell localization.

More recently, NLRP3 inflammasome was found to be highly regulated in adipose tissue and other organs during obesity, controlling the secretion of IL-1β and IL-18 (Stienstra et al., 2011; Vandanmagsar et al., 2011). Contribution of NLPR3 inflammasome and related IL-1β in adipose tissue of morbid obese subjects will be the focus of Study 2. Beside, MINCLE, a C-type lectin receptor was also shown to be up-regulated in adipose tissue and contribute to NF-κB activation in obesity (Ichioka et al., 2011). So far, other receptors such as RLRs or AIM2, were not found relevant in obesity-induced inflammation yet. As mentioned earlier, possible DAMPs associated with obesity include endogenous and dietary lipid species (*e.g.* saturated fatty acids or ceramides), inflammatory cytokines (*e.g.,* TNF-α or IL-1β) or inflammatory products (*e.g.*, ROS). Beside, intracellular components (*e.g.,* ATP) that are released upon cell death, and probably adipocyte death, are likely to play a role in PRR activation. Endotoxemia, that is, level of circulating LPS, is known to be elevated in obese patients and to participate into adipose tissue inflammation. The origin of this LPS is debated, and one hypothesis consists in increased leakage of the intestinal epithelium and

translocation of gut commensal microorganisms or bacterial components (Burcelin et al., 2012). The question that arises is then to know whether it is still consider endogenous LPS and sterile inflammation. In conclusion, PRRs appear to be key players of obesity-induced inflammation and are likely to represent good potential therapeutical targets.

3.2. Antigen-presenting cells

3.2.1. Dendritic cells

Dendritic cells (DCs) are APCs specialized in the uptake, transport, processing and presentation of antigens to T cells. They represent a very heterogenous cell group; the two main subsets being the classical DCs and the plasmacyloid DCs (Geissmann et al., 2010; Liu and Nussenzweig, 2010).

Classical or conventional DCs (cDCs), also called myeloid DCs, are specialized antigen-presenting cells that exits in two functionnaly distinct states, immature and mature. Immature DCs are adept at endocytosis and express low levels of surface MHC class I/II and costimulatory molecules. Immature cDCs can take up antigens but do not present it efficiently to T cells. Most cDCs localized in peripheral tissues are in their immature form, for instance, the Langherans cells in the mucosa. Upon PAMPs or DAMPs detection, immature cDCs acquire the mature phenotype, with a reduced capacity for antigen uptake but now the exceptional capacity for cytokine production and T cell stimulation. In the meantime, proteins of the MHC class II are translocated from the endosomes to the cell surface and co-stimulatory or adhesion molecules are upregulated, including the integrin CD11c. Mature cDCs present numerous membrane extensions, similar to neuron' dendrites, that increase opportunities for T cell interactions. Mature cDCs are highly migratory cells that readily move from tissue to secondary lymphoid organs. They are generally short-lived and replaced by blood-borne precursors such as monocytes.

Plasmacytoid DCs (pDCs), also called lymphoid DCs, were recently added to the palette of DC heterogenous cell types. Contrary to cDCs, pDCs are long-lived and primarily reside in lymphoid tissues at the steady state. They develop in the bone marrow from a distinct progenitor cell line (or pro-DCs). pDCs are specialized in sensing viral infections (*eg*., foreign nucleic acids) and characterized by a massive production of type I interferons, such as IFN-α. pDCs display the round morphology of a lymphocyte, turn over relatively slowly and

express low level of MHC class II and costimulatory molecules. Of note, pDCS are low or negative for the integrin CD11c in mice and humans, respectively.

3.2.2. Macrophages

Compared to DCs, macrophages are more abundant and uniformely distributed throughout the body. In some strategic tissues, they represent 10 – 20% of all cells and become highly specialized cells such as Kupffer cells in the liver, osteoclasts in the bone or microglia in the brain. Macrophages are derived from circulating precursors, the monocytes. Monocyte/macrophage lineage is discussed in the review included. Briefly, circulating monocytes are released from the bone marrow as non-differentiated cells, circulate in the blood for a few days and are poised to rapidly extravate to inflamed tissues. Monocytes are known to display heterogenous phenotypes characterized by different markers. Based on flow cytometry analyses, subgroups of monocytes have been defined based on CD14 expression intensity and the surface marker CD16, also known as the FC receptor γIII. Upon local inflammation, circulating monocytes undergo extravasation, which involves rolling, adhesion and transmigration. Once in the inflamed tissue, monocytes readily differentiate into mature macrophages. As previously mentioned, macrophages are present all throughout the body and show a high degree of heterogeneity. Macrophages are versatile cells and can adopt some really specialized functions at particular tissue locations. They can adapt themselves and actively respond to environmental cues (Gordon and Taylor, 2005). Their amazing plasticity reflects the different phenotypes they can arbor. A M1/M2 macrophage activation classification was created where M1/M2 are a continuum of two extreme functional states. As Biswas and Mantovani discussed in their review, it is an “oversimplified but very useful macrophage classification”.

Stimulation of macrophages with cytokines such as IFN-γ alone or in concert with other cytokines (*e.g.* TNF-α) and bacterial stimuli (*e.g.* LPS) promotes maturation of "classicaly" activated macrophages termed M1. In general, M1 are characterized by high secretion of IL-12, IL-23, IL-6 or IL-1β, high production of toxic intermediates (*e.g.* reactive oxygen species, nitric oxydes) and high capacity to present antigens. In contrast, various signals (*e.g.* IL-4, Il-13, glucocorticoids, immunoglobulin complexes) induce distinct M2 functions able to tune inflammatory responses, scavenge debris, and promote angiogenesis and tissue remodeling. Beside, it was also published that M1 and M2 macrophages may be obtained using GM-CSF and M-CSF, respectively. It should be noted that it is not completely clear how these cells fit

within the M1/M2 paradigm (Lawrence and Natoli, 2011). From these data, many different classifications were elaborated; the one of Mosser and Edward appeared to be one of the most physiologically relevant as shown in Figure 13.

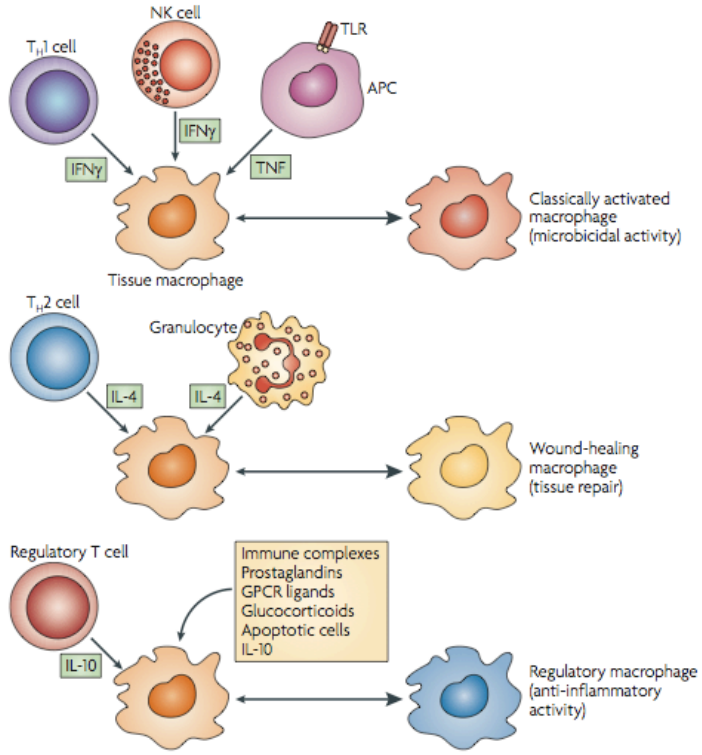

Figure 13 | Inflammatory microenvironment including cytokines produced by other immune cells can polarize macrophages with distinct physiologies. Classically activated macrophages are part of the M1 phenotype, while wound-healing and regulatory macrophages are two kinds of alternative M2-like polarization. GPCR, G-protein coupled receptor (from (Mosser and Edwards, 2008)).

The challenge of this M1/M2 nomenclature resides in finding good and specific M1/M2 markers in order to phenotype infiltrated macrophages in a given tissue. In the last decade, many studies focused on molecules that could play a key role in macrophage polarization, whether they are cytokines, transcription factors or enzymes. Role of transcription factors involved in macrophage polarization will be mentioned later. To assess their reproducibility, we tested human markers, either related to M1 or M2, during *in vitro* prototypical macrophage activation (Martinez et al., 2006). All of these markers have been discovered and validated in mice models. We differentiated monocytes from healthy donors into macrophages using M-CSF for 6 days and activated them with either LPS and IFN-γ (M1) or IL-4 and IL-13 (M2) for 18h. Table 2 shows overall results expressed in gene expression fold related to values obtained with RPMI control medium (M0).

Molecules		M1 (Fold relative to M0)	M2 (Fold relative to M0)
M1-like markers	**pro-IL-1β**	**23.5 ± 3.0**	0.4 ± 0.1
	IL-6	**239.8 ± 89.7**	1.8 ± 0.3
	TNF-α	6.5 ± 1.3	3.5 ± 1.1
	IL-12	**15,4 ± 3,1**	0,3 ± 0,2
	IL-23	**24,10 ± 8,8**	0.5 ± 0.1
	pro-IL-18	1.2 ± 0.2	0.5 ± 0.1
	CD40	**27.6 ± 4.4**	4.4 ± 0.9
	CD69	**80.2 ± 26.9**	1.8 ±0.3
	CD80	**86.3 ± 31.4**	1.8 ±0.3
	CD86	1,0 ± 0.2	1.4 ± 0.3
	CD11c	0.2 ± 0.1	0.9 ± 0.2
	MINCLE	**10.2 ± 2.3**	0.1 ± 0.1
	COX2	**119.2 ± 19.0**	1.0 ± 0.2
M2-like markers	**IL-1ra**	6.6 ± 1.5	2.9 ± 0.4
	IL-10	6.3 ± 2.5	0.6 ± 0.1
	CD206	0,1 ± 0,1	**2,0 ± 0,2**
	CD163	0,9 ± 0,3	0,5 ± 0,2

Table 2 | Gene expression folds obtained in human primary M1-like or M2-like macrophages as compared to resting M0-like macrophages. M1 induction was performed using LPS (100 ng/ml) plus IFNg (20 ng/ml) while M2 induction was done using IL-4 (10 ng/ml) and IL-13 (10 ng/ml) for 24h. M0 corresponds to unactivated macrophages.

As expected, pro-inflammatory cytokines such as pro-IL-1β, IL-6 and IL-23 gene expression were drastically up-regulated upon M1 activation while TNF-α was only slightly up-regulated in both M1 and M2 activation (Martinez et al., 2006). Interestingly, pro-IL-18, as compared to pro-IL-1β, did not seem to be modulated by these pro-inflammatory stimuli in agreement with published data (Netea et al., 2006). So-called anti-inflammatory cytokines such as IL-10 and IL-1ra were up-regulated along with pro-inflammatory cytokines and did not match with typical M2 phenotype as one could suppose, suggesting regulatory mechanisms (Bouhlel et al., 2007). M1 stimuli also sharply increased several membranous activation markers such as CD40, CD69, CD80 and MINCLE but not CD86 (Ichioka et al., 2011; Martinez et al., 2006). CD11c was down-regulated in both M1 and M2 activation, in contradiction with *in vivo* data, suggesting that CD11c regulation goes beyond typical M1 activation. Finally, the pro-inflammatory protein COX2 was very much increased upon M1 activation as expected (Martinez et al., 2006). In conclusion, there are many different markers that are used to define macrophage polarization and yet, some discrepancies exits depending on *in vitro* experimental conditions. It remains to wonder to which extend these markers are relevant *in vivo*, since all cells have to compose and process a mix of both M1-like and M2-like

inflammatory cues from their tissue microenvironment. It is interesting to note that all of these markers are not macrophage specific and many M1-like activation molecules are likely to be also found on other, if not all, APCs. This highlights the actual difficulties in distinguishing macrophages from DCs in a given tissue, as debating in box 3.

3.3. Antigen-presenting cells: guardians of adipose tissue

APCs were the first immune cells to be described in adipose tissue in obesity and to be associated with a myriad of metabolic and cardiovascular complications. We will refer to these cells as « macrophages » as it is done and tolerated since their discovery in 2003. Although, it is obvious that the presence of DCs cannot be ruled out (box 3).

Recent data on macrophage and their monocyte precursor phenotypes in adipose tissue are presented in the following review entitled: « Defining macrophage phenotype and function in adipose tissue». In this paper, we review mechanisms of monocyte recruitment to adipose tissue during the onset of obesity and how adipose tissue environment modulates the phenotype of macrophages. Other cells of the innate immune system such as neutrophils, NK cells or mast cells that play an additional role in adipose tissue are also mentionned. Finally, we raised questions about the true role of macrophages in adipose tissue, in both the lean and obese states. This review was also adapted for the French journal Médecine/Sciences and entitled: « Le tissu adipeux, un nouveau terrain de jeu pour les cellules immunitaires » that is enclosed in Annex 1.

More and more, research focusing on macrophage phenotypes goes beyond the oversimplied M1/M2 paradigm. In the context of obesity, two pioneer studies already stressed out that adipose tissue macrophages displayed M2-like markers while secreting amounts of pro-inflammatory cytokines (Zeyda et al., 2007 ; Bourlier et al., 2008). One question remains: is it really relevant to state that adipose tissue macrophages are of a M1/M2 mixed phenotype? Another simple example of model inconsistency is that experimental LPS-induced macrophage polarization can induce the production of both pro- (M1) and anti-inflammatory (M2) cytokines at the same time.

Box 3 | Macrophages and dendritic cells: fraternal twins?

The distinction between macrophages and DCs is a controversial topic. Confusion has arisen in part from the great plasticity of both cell types, their common myeloid precursors and the use of too many non-specific markers (Ferenbach and Hughes, 2008 ; Geissmann et al., 2010). For instance, the integrin CD11c was initially thought to be a marker of DCs, whereas most, if not all, macrophages also express low level of CD11c whose expression increases upon pro-inflammatory stimuli. In the same way, CD68 or F4/80, originally related to monocyte/macrophage lineage, does not distinguish between macrophages and DCs (Ferenbach and Hughes, 2008). The DC doctrine is that “DCs can uniquely present antigen to naïve T cells, so a cell that can do this is, by definition, a DC” (Geissmann et al., 2010). Macrophages were only given the property to activate memory T cells. However, several studies have now showed that macrophages can actually migrate to lymph nodes to present antigen to naïve T cells, just like a DC (Murray and Wynn, 2011). In the same way, DCs were known to be the cells expressing MHC class II the most and yet, macrophages were also described to do so, suggesting an additional key role for macrophages in antigen presentation. It is proposed that the term DCs could be exclusively used for the small APC subset that resides inside the T cell area of spleen or any lymphoid tissues, as these cells are known not to be phagocytic. Beside, Ferenbach and Hughes propose that macrophages and DCs should be part of a cellular spectrum of the mononuclear phagocyte system, “where cells may express variable sets of cell-surface markers and functions” at a given time (Geissmann et al., 2010). This nomenclature problem is not likely to be resolved at all and it is thus virtually impossible to truly distinct “macrophages” from “DCs”, notably in humans.

Thus, growing evidence suggests that what matters is the true role of macrophages in a given tissue. As already mentioned, macrophages contribute to adipose tissue microenvionment by secreting pro-inflammtory cytokines that can target adipocytes and SVF cells such as preadipocytes. Studies exploring the specific nature of macrophage secretions will probably give insights into macrophage role in adipose tissue. Interestingly, a recent paper highlighted that (white) adipose tissue macrophages were able to secrete catecholamines, potent inducers of adipocyte lipolysis, in response to cold environments (Nguyen et al., 2011). Although more work is needed to analyse this phenomenon in the context obesity, cathecolamines might represent a new pathway for exacerbated lipolysis in visceral adipose tissue in link to T2D. Besides, macrophages might play a substantial role in adipose tissue expansion through promotion of angiogenesis as it was already suggested in

experimental model of obesity (Pang et al., 2008). In the same way, macrophages could turn out to be unexpected regulator of adipose tissue fibrosis through direct interactions with fibroblasts for instance (Wynn and Barron, 2010). Finally, macrophages might orchestrate the dialogues between the different immune cell types that are present in the adipose tissue. Special interactions with T lymphocytes are to be expected. The last part of this introduction will focus on presenting the adaptive immune compartment in regards to adipose tissue.

Review

Defining macrophage phenotype and function in adipose tissue

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In obesity, chronic low-grade inflammation is thought to mediate the effects of increased adipose tissue mass on metabolic comorbidity. Of the different cell types that contribute to obesity-induced inflammation in adipose tissue, this review focuses on macrophages and their monocytes precursors. Mechanisms for monocyte recruitment to adipose tissue, and how both monocytes and macrophages are phenotypically modified in this environment in response to increasing fat mass, are considered. The versatile phenotype of adipose tissue macrophages might contribute not only to inflammatory and metabolic alterations, but could also help to maintain adipose tissue homeostasis in the setting of obesity.

Adipose tissue, a new playground for the immune system

Obesity is defined as an excess of white adipose tissue, and is considered a low-grade inflammatory state that is characterized by increased circulating concentrations of inflammatory cytokines and acute-phase proteins. It is now established that, in obesity, adipose tissue itself is a site of inflammation. For example, macrophages accumulate in adipose tissue of obese mice [1,2]: in lean mice, macrophage content is estimated at <10% of the total cell nuclei count in adipose tissue, but at >50% in extremely obese mice [2]. As a source of proinflammatory factors, macrophages might participate in inflammation and contribute to obesity-linked comorbidity. However, macrophages are versatile and can polarize to exert either pro- (M1) or anti-inflammatory (M2) effects, depending on the surrounding tissue microenvironment and the subset of circulating monocyte precursors from which they derive. Several immune cell types, including lymphocytes, mast cells and neutrophils have been identified in the adipose tissue of obese mice and humans. Therefore, both the innate and adaptive immune systems are now at the forefront of adipose tissue and obesity research. In this review, we consider recent data regarding the phenotype of monocyte precursors that give rise to adipose tissue macrophages, and how they are recruited to and polarize within adipose tissue. We examine the possible consequences of macrophage accumulation in adipose tissue during obesity and discuss how macrophages might contribute to adipose tissue homeostasis during changes in energy balance.

What triggers adipose tissue inflammation in obesity?

Although recent experimental and clinical data have established that adipose tissue is a site of inflammation in obesity (Box 1), the events that initiate inflammation are unclear. Adipocytes produce cytokines, chemokines and adipokines, which include leptin and adiponectin, and thus might initiate inflammation. A hallmark of obesity is adipocyte hypertrophy, which switches adipocyte secretion towards a proinflammatory profile. Eventually, hypertrophy can lead to adipocyte death and the release of cellular contents into the extracellular space. This is thought to trigger an inflammatory response from neighboring cells, macrophages in particular, which surround adipocytes that display features of dead cells to form crown-like structures (CLSs) [3]. There is, however, a relative paucity of CLSs in human adipose tissue compared to genetically or diet-induced obese mice, which suggests that increased adipocyte death in these animal models represents an extreme in the spectrum of adipose tissue inflammation.

Exogenous fatty acid- or lipopolysaccharide (LPS)-induced activation of Toll-like receptor (TLR)4, which is expressed on adipocytes and macrophages, can also provoke inflammation in adipose tissue [4,5]. Fatty acids released from hypertrophied adipocytes themselves serve as ligands for TLR4, and potentially form an inflammatory loop between the two cells types [6]. Activating transcription factor (ATF)3, a transcription factor of the ATF/cAMP response element binding protein family, and C1q/TNF-related protein-3 are negative regulators of TLR4 signaling and might represent a physiological, and potential pharmacological means to counterbalance macrophage activation in adipose tissue [6,7]. Local hypoxia, which can potently stimulate proinflammatory gene expression in adipocytes and macrophages, is an additional mechanism for chronic adipose tissue inflammation in obesity [8,9]. This list is not exhaustive, as shown by the recent identification of endoplasmic reticulum stress as a crucial factor underlying obesity-induced inflammatory responses [10].

Obesity might be viewed as a state of 'sterile' inflammation, that is, which occurs in the absence of any

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Box 1. Inflammation in obesity

Inflammation is a physiological response to defend the organism against injurious stimuli and to initiate healing processes to restore tissue homeostasis. A typical acute inflammatory response involves inducer molecules (e.g. LPS), which are recognized by cellular sensors (e.g. TLR4), which lead to the production of mediators [e.g. tumor necrosis factor (TNF)-α and IL-6] that trigger inflammatory response in target cells (e.g. endothelial cells, adipocytes, and hepatocytes). The inflammatory response ends with a highly regulated process of resolution that allows transition to the homeostatic state. When, for a given reason, the resolution phase cannot occur, a chronic inflammatory state ensues [66]. For a decade, obesity has been seen as an inflammatory disease that is characterized by a low-grade chronic inflammatory state. Obesity is associated with increased circulating concentrations of inflammatory cytokines and acute phase proteins. Tissue specific upregulation of genes that encode inflammatory factors has been described, associated with a marked accumulation of macrophages in enlarged adipose tissue [1,2,33,68].

microorganisms [11]. In this scenario, the relevant inflammatory cells might be stimulated by thus-far-unidentified non-microbial signals present in adipose tissue, including lipid species that are capable of directing an immune response [12]. Supporting this idea, natural killer (NK)T cells, which recognize glycolipid antigens, are increased in number in adipose tissue of diet-induced obese (DIO) mice, and adipose tissue macrophage infiltration is reduced in mice that lack NKT cells [13]. Similarly, genetic ablation of mast cells, which are mediators of the allergic response, reduces adipose tissue macrophage accumulation [14]. In the same DIO animal model, neutrophils transiently infiltrate adipose tissue shortly after initiation of high fat feeding, which suggests acute infiltration of immune cells in response to nutritional stress [15]. In addition to infiltration of innate immune cells into adipose tissue, T cell accumulation is also an early event in obesity [16,17]. One human study has reported an increased number of adipose tissue lymphocytes associated with an elevation of body mass index [18]. In DIO mice, changes in the relative frequency of lymphocyte subsets are observed in adipose tissue; mostly an increase in $CD8^+$ T effector cells and a reduction in forkhead box P3 regulatory T cells ($Foxp3^+$ Tregs) [17,19,20]. Immunological or genetic depletion of $CD8^+$ T lymphocytes [17], or induction of $Foxp3^+$Tregs [19–21], both lower macrophage accumulation in adipose tissue of DIO mice, which supports the idea that the adaptive immune system is relevant in adipose tissue inflammation. Of note, in global lymphocyte deficiency (Rag2 knockout mice), macrophage abundance is increased and not decreased in adipose tissue [22]. Mouse adipose tissue T cells express a restricted T-cell receptor repertoire, which suggests that they recognize yet unknown antigens within this tissue [19,20,23]. In this context, the presence of $Foxp3^+$Tregs that are enriched in adipose tissue of lean but not obese mice might help to keep under control an ongoing immune response, which remains to be defined [19,20]. Overall, although the initiating events remain elusive, these studies reveal a complex interplay between cells of both innate and adaptive immunity, which might sequentially intervene to trigger and maintain adipose tissue inflammation in obesity.

Monocyte precursors of adipose tissue macrophages

Bone marrow transplant studies have established that most macrophages found in the adipose tissue of DIO mice are derived from blood monocytes [2]; a concept confirmed by *in vivo* monocyte labeling [24]. Different subsets of circulating monocytes have been identified in humans and mice (Figure 1). Monocyte subsets differ in their migration and cell fate properties. The local microenvironment probably also contributes to the macrophage phenotype that is acquired by a monocyte upon arrival at target tissues [25]. Classical $Gr1^+Ly\text{-}6C^{high}$ monocytes are rapidly recruited to inflammatory lesions in several murine models of tissue injury. They are thought to be precursors of M1 macrophages, although their contribution to the pro- or anti-inflammatory macrophage compartment varies depending on the tissue and inflammatory status [26,27]. An increase in circulating $Gr1^+Ly\text{-}6C^{high}$ monocytes has been reported in DIO mice [28,29], but their cell fate in adipose tissue is not established. The lectin MGL1 (CD301) is crucial for the survival and extravasation of these cells [29] and the monocyte chemotactic protein (MCP)-1 receptor, chemokine CC receptor (CCR)2, is required for their mobilization from the bone marrow [28,30].

In humans, only one study has demonstrated an increase in $CD16^+$ monocytes; more specifically, the $CD14^{dim}$ $CD16^+$ monocyte subset, in overweight subjects [31]. $CD14^{dim}$ $CD16^+$ monocytes are implicated in local surveillance of damaged or virus-infected tissues [32]. Their contribution to adipose tissue macrophage accumulation in obesity remains an open question.

Monocyte recruitment into the adipose tissue occurs through diapedesis, and presumably involves the secretion of chemotactic molecules that are known to be overexpressed in mice and human adipose tissue depots (Box 2 and Figure 2). Several genetically modified mouse strains have helped to define the molecular components of monocyte diapedesis (Table 1), but this remains poorly explored in human adipose tissue. Human adipocytes release soluble factors that increase *in vitro* diapedesis of human blood monocytes across a layer of adipose-tissue-derived capillary endothelial cells [33]. Adipocyte-conditioned medium increases the amount of adhesion proteins, including platelet endothelial cell adhesion molecule (PECAM)-1 and intercellular adhesion molecule (ICAM)-1, expressed on human adipose tissue endothelial cells. This effect is reproduced using recombinant human leptin, albeit at supraphysiological doses. A subsequent study has reported that endothelial cells are in a more proinflammatory state in visceral compared with subcutaneous human adipose tissue [34], which could favor monocyte recruitment into visceral fat. To clarify the adhesion pathways that dictate monocyte infiltration into adipose tissue, adhesion molecule gene expression profiles have been examined in obese mice [35]. Among others, P-selectin and its monocyte ligand, P-selectin glycoprotein ligand-1, are increased in adipose tissue of genetically and DIO mice, and in the latter, *Psgl1* gene deletion markedly reduces macrophage accumulation. Blockade of α4 integrin signaling produces a

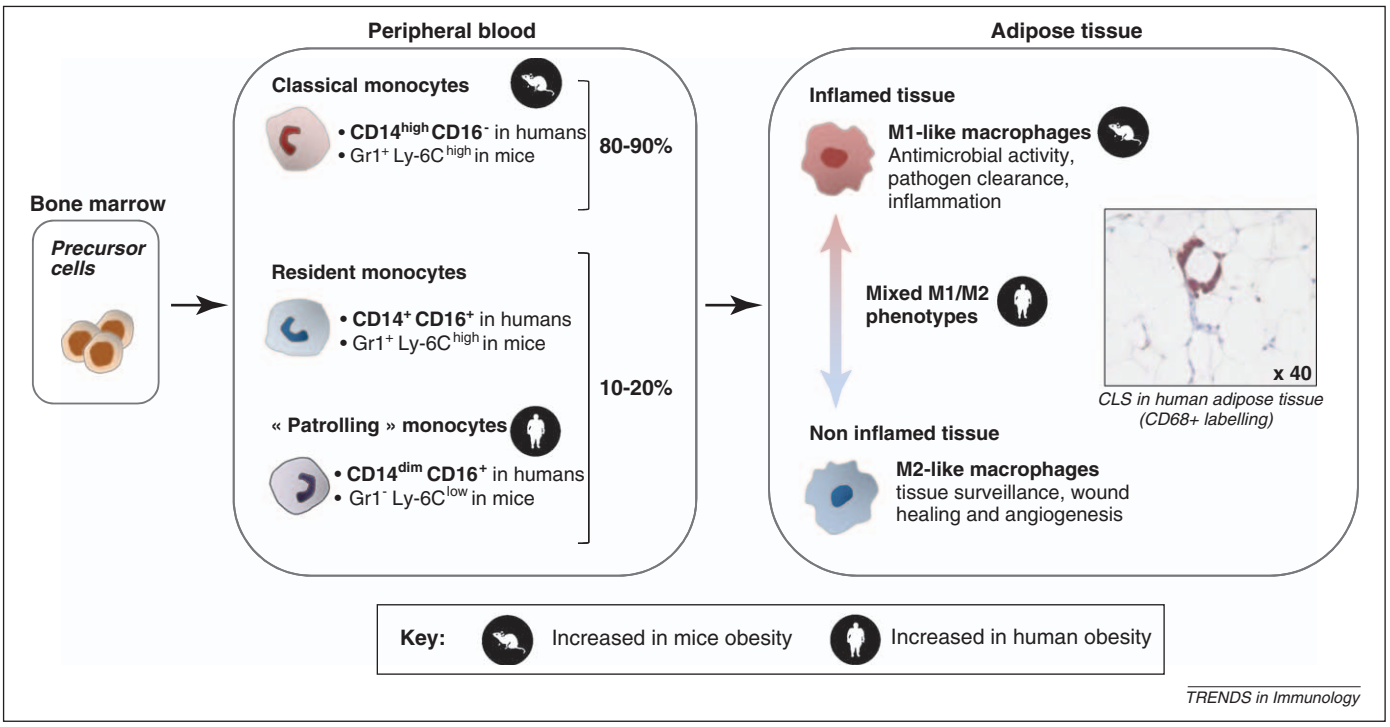

Figure 1. Trafficking of monocytes from bone-marrow to adipose tissue. In humans, subgroups of monocytes are defined based on the level of expression of a specific marker CD14. An additional separation relies on the surface marker CD16 (or FC receptor γIII), which is considered a marker of activation. The main monocyte population in humans is $CD14^{high}$ $CD16^{-}$ and constitutes classical monocytes recruited to inflamed areas. Less abundant $CD14^{+}$ $CD16^{+}$ monocytes are described to be more mature cells. Based on gene expression, $CD14^{+}$ cells resemble $Gr1^{+}$ Ly-$6C^{high}$ murine monocytes that also express high levels of CCR2. A third population of monocytes $CD14^{dim}$ $CD16^{+}$, so-called 'patrolling' monocytes, shares common features with the $Gr1^{-}$ Ly-$6C^{low}$ $CCR2^{low}$ mice monocytes. Once within tissues, these monocytes undergo differentiation into M1, M2, or macrophages with mixed M1/M2 phenotypes in response to local microenvironment. Obesity increases specifically distinct subsets of monocytes in humans and mice, and preferentially promotes accumulation of M1-like macrophages in mouse adipose tissue. In humans, the phenotypes of adipose tissue macrophage are not fully defined.

similar effect [36]. In mice deficient for caveolin-1, a membrane-associated protein implicated in endothelial barrier function, rescue of caveolin-1 in endothelial cells reduces macrophage content in adipose tissue. This highlights a crucial role for caveolins in the control of monocyte extravasion in this tissue; potentially through regulation of endothelium permeability [37]. Clearly, further studies are required to identify the whole spectrum of molecular components that participate in diapedesis and specifically contribute to endothelium activation in obesity.

Box 2. Chemotactic mediators in adipose tissue

Human studies and mouse models have both been used to identify the chemokines and associated receptors that are elevated in obese adipose tissue [69], and which contribute to monocyte recruitment. In mice, manipulation of the MCP-1α/CCR2 system affects macrophage accumulation in some [57–59] but not all models [70,71], which leaves debated the pathophysiological relevance of MCP-1 to promote adipose tissue inflammation. Using primary human cells, the contribution of chemokine CC ligand (CCL)5, also known as RANTES, in mediating monocyte/macrophage adhesion and transmigration though endothelial barrier has been described [72]. chemokine CXC ligand 14-deficient mice have impaired macrophage accumulation in adipose tissue in response to a high fat diet [73] and so do mice deleted for the chemokine-like matrix glycoprotein osteopontin [74]. By contrast, although macrophage inflammatory protein (MIP)-1α, also referred as CCL3, and its potential receptors CCR1 and CCR5 are overexpressed in obese mice adipose tissue, MIP-1α-deficient mice are not protected from macrophage accumulation [75]. Further studies should give insights into the hierarchy among an increasing number of chemotactic molecules promoting macrophage infiltration in the adipose tissue in obesity.

Unraveling complexity of adipose tissue macrophages

Increased macrophage accumulation in adipose tissue has now been extensively described both in mouse models and in human obesity. In this context, the phenotype acquired by macrophages in the setting of obesity has been examined using membrane and intracellular markers that are considered specific for M1 or M2 polarized macrophages (Box 3). Obesity induces a phenotypic switch from an anti-inflammatory M2 polarized state to a proinflammatory M1 state [38]. A population of $CD11c^{+}$ macrophages that expresses the proinflammatory cytokine interleukin (IL)-6 and inducible nitric oxide synthase has been identified in the adipose tissue of DIO mice, whereas $CD11c^{-}$ resident macrophages that express Arg1 and the anti-inflammatory cytokine IL-10 are found in lean mouse adipose tissue. To ask whether, during obesity, M1 macrophages are recruited to adipose tissue or if M2 resident cells repolarize, pulse PKH26 labeling experiments have been performed during the course of DIO in mice. Adipose tissue macrophages were PKH26 negative, and because monocytes do not take up this dye, it has been concluded that macrophages are newly recruited from monocyte precursors. These PKH26-negative macrophages display an M1-like phenotype and preferentially accumulate in CLSs [24]. CLS macrophages contain many oil-red O-staining vesicles [1], which indicates intracytoplasmic lipid accumulation that is consistent with phagocytic activity. By contrast, the presence of M2-like resident $MGL1^{+}$ macrophages localized in interstitial space is not altered by high fat feeding [24,39]. In another DIO mouse study, obesity induced an

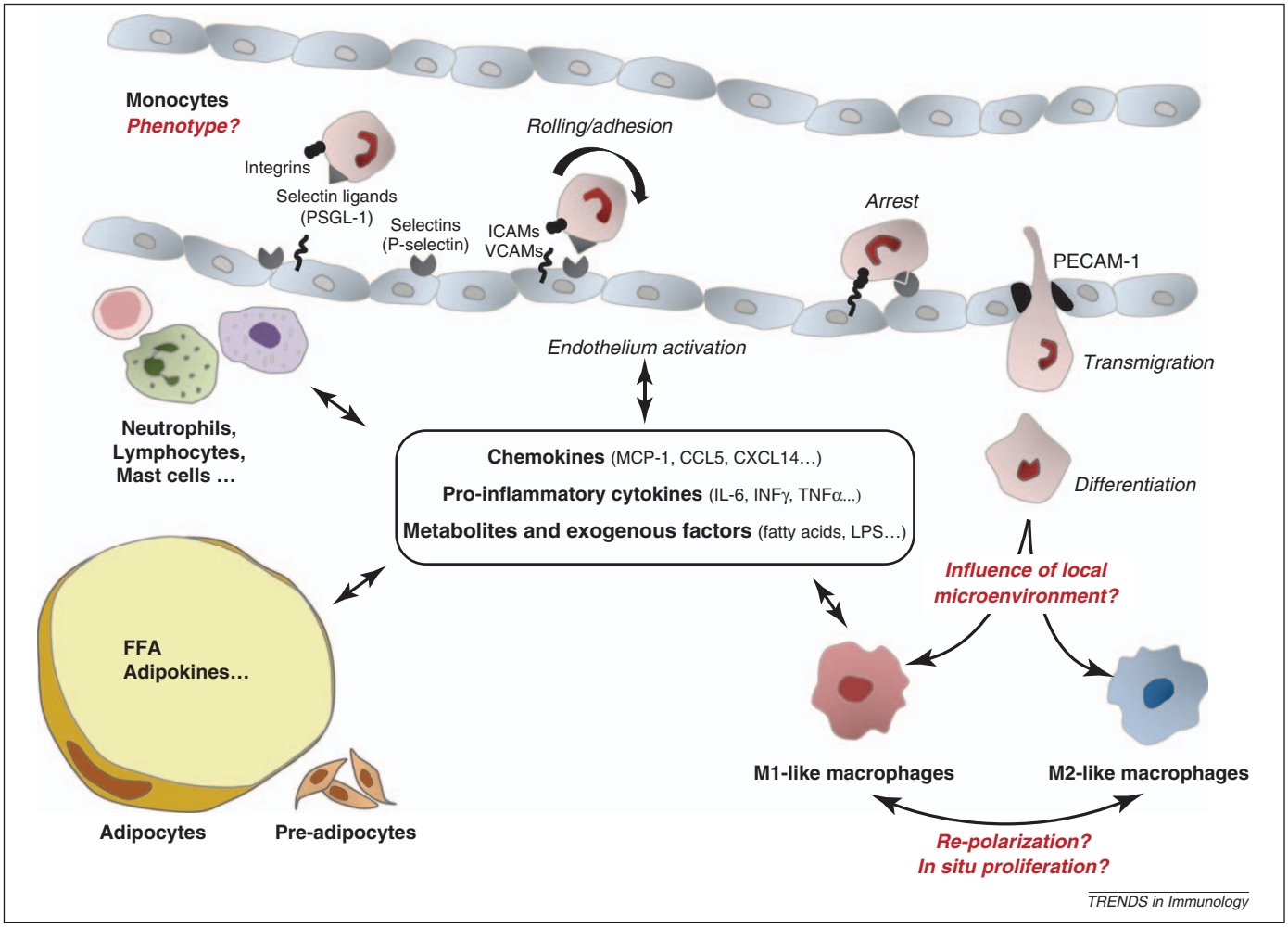

Figure 2. Monocyte recruitment into the adipose tissue. Upon obesity, adipose tissue macrophages together with hypertrophic adipocytes, preadipocytes and other immune cells produce a panel of chemokines, proinflammatory cytokines and metabolites that participate in endothelial activation. This causes endothelial cells to produce various cellular adhesion molecules, including selectins, ICAMs, vascular cell adhesion molecules and PECAM-1. Through a rolling/adhesion process, monocytes slow down and eventually bind to endothelial adhesion proteins via selectin ligands and integrins. Subsequently, monocytes undergo actin-dependent spreading, polarization, and integrin-dependent lateral migration on the luminal surface of the endothelium. Then, the cells breach and transmigrate across the endothelium, a process referred to as diapedesis, either through para-or transcellular routes [67]. Inside adipose tissue, monocytes undergo differentiation towards M1- or M2-like macrophages according to their initial circulating phenotype and/or in response to local microenvironmental stimuli. Whether M1 macrophages can repolarize into M2 macrophages and conversely, and whether macrophages proliferate within the adipose tissue remain open questions. Double arrows suppose that cells are both producers and targets of the associated chemokines and cytokines.

increase in M1 and M2 macrophages, although the M1/M2 ratio was switched towards the M1 phenotype [40]. Thus, obesity in mice is associated with accumulation of newly recruited proinflammatory M1 macrophages in the adipose tissue, which occurs in parallel to the maintenance or slight elevation of anti-inflammatory M2 macrophages (Figure 3).

Identification of the signaling pathways that control macrophage polarization is a challenging issue, because numerous cellular and exogenous factors might intervene.

Table 1. A selection of knock-out mouse models showing alteration in adipose tissue macrophages.

Factors related to	Targeted protein	Adipose tissue macrophages in DIO mice	Macrophage polarization	References
Diapedesis	Psgl-1	↓	↓ M1 ↑ M2	35
	α4 integrin[a]	↓	ND	36
	Caveolin-1[b]	↑	↔ M1 ↑ M2	37
Chemotactism	MCP-1	↔	ND	70, 71
	MCP-1	↓	ND	57
	CCR2	↓	↓ M1 ↔ M2	24, 59
	CXCL14	↓	ND	73
	Osteopontin	↓	ND	74
	MIP-1α	↔	ND	75
Transcription	PPAR-δ	↑	↑ M1 ↓ M2	42

[a]α4 integrin functional deletion was obtained through (Y991A) point mutation.

[b]macrophage changes were reported in chow fed mice. DIO: diet-induced obese. ↑ increased; ↓ decreased; ↔ no change; ND: not determined.

Box 3. Macrophage phenotypic nomenclature

Macrophages are well known to be versatile cells that can adopt specialized functions at particular tissue locations. They adapt themselves and actively respond to the local microenvironment [76]. Their plasticity is reflected by the different phenotypes that they can harbor. In line with the current understanding of monocyte heterogeneity, and in an effort to mimic the T cells Th1/Th2 nomenclature, an M1/M2 macrophage activation classification has been created in which the M1 and M2 phenotypes are the extreme of a continuum of functional states. Stimulation of macrophage with bacterial stimuli (e.g. LPS), Th1 cytokines, such as interferon γ, or other factors including TNF-α and granulocyte–macrophage colony-stimulating factor promotes maturation of classically activated M1 macrophages. These cells are characterized by high secretion of IL-12 and IL-23, high production of toxic intermediates (e.g. reactive oxygen species) and high capacity to present antigens. In contrast, various signals, including IL-4, IL-13, glucocorticoids and adiponectin [77] induce distinct M2 functions that can tune inflammatory responses and promote angiogenesis, tissue remodeling and wound healing. Other anti-inflammatory molecules such as IL-10 can be regarded as macrophages deactivating factors [78]. However, the M2 term is used in a confusing way and three definitions have been proposed in the M2 nomenclature: (i) M2a, induced by IL-4 or IL-13 and involved in killing or encapsulation of parasites; (ii) M2b, induced by exposure of immune complexes and involved in immunoregulation; and (iii) M2c, induced by IL-10 and glucocorticoids and preferentially implicated in matrix deposition and tissue remodeling [79]. In the meantime, a new foundation for macrophage classification has been recommended based on their functions: host defense (close to an M1 phenotype with microbicidal activity); wound healing (promoted by IL-4 produced byTh2 cells); and immune regulation (preferentially induced by IL-10 produced by Tregs) [80].

Studies in genetically modified mice have identified that the transcription factors peroxisome proliferator-activated receptor (PPAR)γ [41], PPARδ [42] and interferon regulatory factor (IRF)4 [43] are required for M2, and IRF5 [44] for M1 activation. Exogenous lipids might also promote M1 macrophage polarization through the fatty acid–TLR4–nuclear factor-κB pathway [45,46]. Progressive lipid accumulation within macrophages contributes to the M2 to M1 phenotypic switch that occurs with age in adipose tissue of genetically obese mice [47]. Surprisingly, macrophages in which triglyceride storage capacity is increased through overexpression of diacylglycerol acyltransferase 1 are resistant to M1 activation, probably because of protection against proinflammatory lipid species, such as free fatty acids and diacylglycerols [48].

The 'M1/M2 paradigm' and how it fits with the DIO mouse model might be more complex than initially proposed. A third macrophage population has been identified in obese mice that has an intermediate phenotype and a mixed M1- and M2-like gene expression pattern [49]. In a kinetic study, the frequency of adipocyte death was increased during the first 16 weeks of a high fat regimen, which was coincidental with maximum expression of CD11c and proinflammatory genes in adipose tissue [50]. At later time points, a state of adipocyte hyperplasia was accompanied by reduced adipocyte death and downregulation of CD11c. Thus, adipocyte death in adipose tissue is a progressive event that is temporally linked to M1-like macrophage recruitment. Eventually, M2-like polarization seems to occur, which potentially corresponds to an adaptive response that is geared to restore adipose tissue homeostasis.

Several groups have examined the phenotype of adipose tissue macrophages in humans. A population of $CD14^+$ macrophages has been identified that expresses CD206, CD163, αVβ5 and anti-inflammatory cytokines, which are characteristics of an M2-like phenotype, and produces proinflammatory molecules that are characteristic of M1-like polarization [51]. Similarly, human $CD14^+$ adipose tissue macrophages have also been reported to express M1 and M2 markers [52]. In a different human study, adipose tissue macrophages in the parenchyma were $CD11c^-$ $CD206^+$, whereas CLS cells were $CD11c^+$ $CD206^{low}$ and were characterized by high amounts of HLA-DR and CD86 expression and proinflammatory mediators [53]. Recently, the inflammatory nature of CLS macrophages that express the M1 markers CD86 and CD40, and the M2-oriented phenotype of interstitial macrophages that express CD206 and CD150 in human adipose tissue have been confirmed [54]. In a population of massively obese compared to normal weight subjects, increased numbers of $CD40^+$ cells were identified, whereas the numbers of $CD206^+$ and $CD163^+$ cells were unchanged with obesity [55]. In human obesity, it is virtually impossible to distinguish recently recruited cells from the resident pool of macrophages and to unequivocally identify M1 or M2 polarization. Thus, whether a mixed M1/M2 phenotype results from repolarization of resident M2 cells in M1-like macrophages remains an open question (Figure 2). Such a phenotypic switch has been proposed during development of atherosclerotic lesions [56]. Similarly, recruitment of M1 macrophages to adipose tissue, as described in PKH26 mouse experiments, might not be entirely relevant in human obesity. Additionally, it is currently unknown if resident macrophages proliferate in adipose tissue as reported in other tissues [26].

What is the role of adipose tissue macrophages?

It is well established that obesity is associated with a myriad of metabolic and cardiovascular complications. Currently, clinical studies and experimental evidence have suggested a link between macrophage adipose tissue infiltration and systemic insulin resistance, cardiovascular risk, and hepatic alterations. In animal models, a role for adipose tissue macrophages in inducing insulin resistance has been demonstrated through diet-induced, genetic or pharmacological manipulations of macrophage numbers in adipose tissue [1,2,57–59]. In these studies, increased numbers of macrophages in adipose tissue have been consistently associated with deteriorated glucose homeostasis and vice versa. However, in humans, the pathological consequences of macrophage infiltration into adipose tissue are more difficult to prove. In contrast to previous human studies, in which no association was found with adipose tissue macrophages, a positive association between systemic insulin resistance and a proinflammatory $CD11c^+$ $CD206^{low}$ minor population has recently been reported [53]. In another clinical study, macrophage accumulation specifically in visceral adipose tissue was positively associated with the severity of fibro-inflammatory lesions in the liver [60]. The factors that convey inflammatory signals from adipose tissue to the liver must be identified. Increased IL-6 concentrations measured in

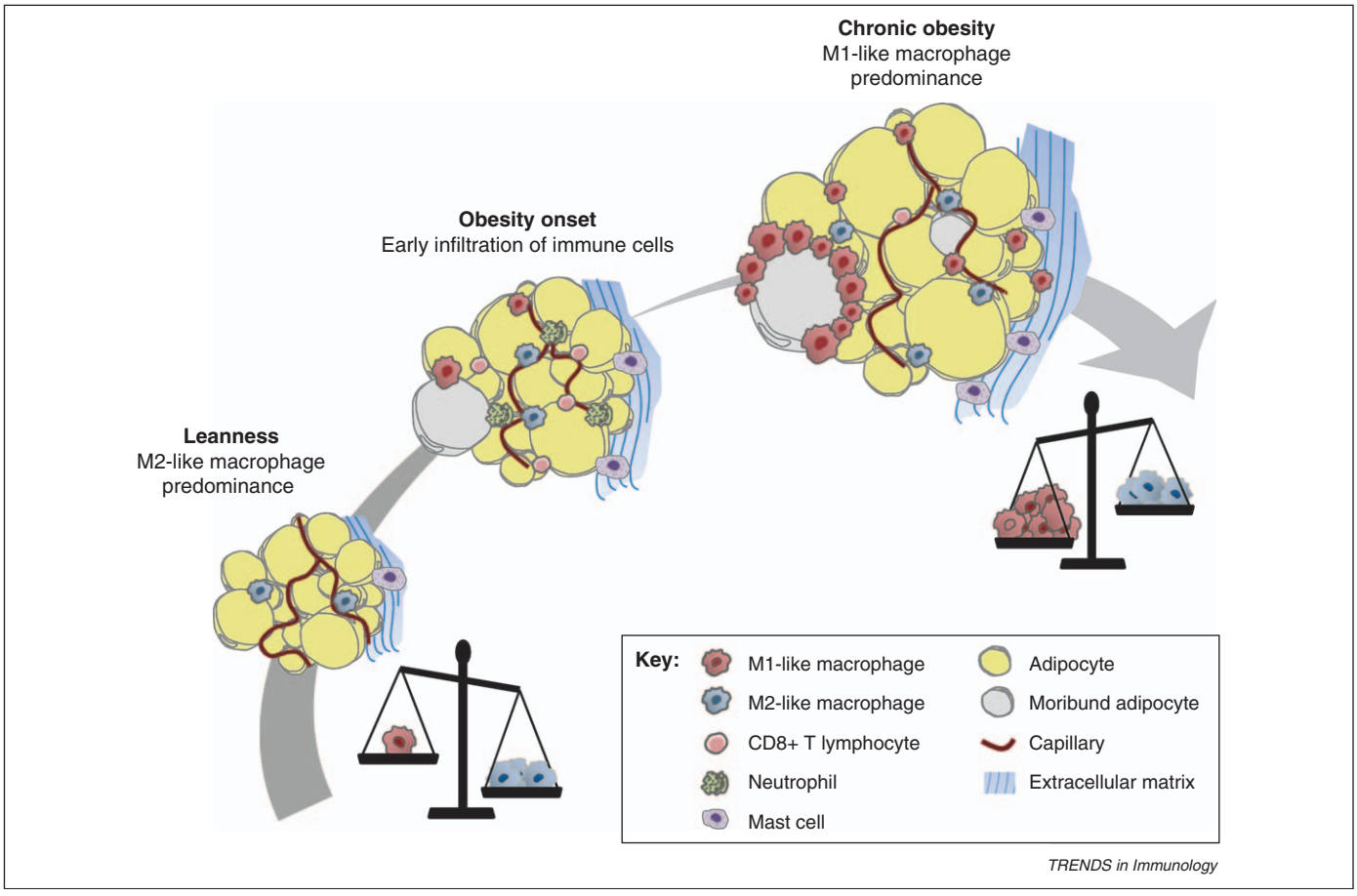

Figure 3. Cellular alterations in adipose tissue in obesity. Healthy lean adipose tissue contains resident M2-polarized macrophages that help to maintain tissue homeostasis. Based on kinetic studies in DIO mice, various immune cell types, including neutrophils, $CD8^+$ T lymphocytes and mast cells, are thought to infiltrate the adipose tissue before recruited macrophages of proinflammatory M1-like phenotype. In addition, hypertrophic adipocytes become inflammatory and/or necrotic, thereby attracting M1-oriented macrophages organized in crown-like structures. Obesity is associated with a switch in the M1/M2 macrophage balance, with M1 macrophages overwhelming the number of M2 macrophages.

the portal vein of obese subjects suggest that this proinflammatory cytokine might link enlarged visceral adipose tissue and liver damage [61].

Could macrophage accumulation be beneficial to adipose tissue homeostasis? One hypothesis is that M1-like macrophages help to limit adipocyte hypertrophy. This is illustrated in *Cccr2*$^{-/-}$ mice, which show decreased macrophage content, associated with increased adipocyte diameter [24,38,59]. Human preadipocytes exhibit impaired adipogenesis and increased extracellular matrix deposition when cultured with conditioned medium from obese adipose tissue macrophages [62]. Hence, inhibition of adipogenesis combined with a profibrotic phenotype of preadipocytes strengthens the role of proinflammatory macrophages to restrain adipocyte size. Fibrous depots in human adipose tissue have been detected, with macrophages and mast cells being the main immune cells found in fibrotic lesions [54,63]. As reviewed elsewhere [64], macrophages are considered master regulators of fibrosis in various tissues, where they are thought to exert pro- or antifibrotic activity depending on their phenotype. Whether adipose tissue macrophages promote or resolve fibrosis is unclear. Finally, macrophages might play a classical role to phagocytose debris from dead adipocytes and to remove fatty acids to prevent lipotoxicity and inflammation. In DIO mice given a restricted diet, macrophage recruitment is unexpectedly increased in adipose tissue during the early phase of weight loss [65]. An increase in macrophage number is concomitant with increased lipolysis in adipose tissue and is not associated with upregulation of inflammatory genes. In fact, these macrophages display phagocytic activity against lipids without causing inflammation, thereby contributing to restore local lipid homeostasis. In this way, macrophages could contribute to ensure adipose tissue homeostasis and remodeling in response to changes in energy balance and body weight alterations.

Concluding remarks

Adipose tissue inflammation and macrophage infiltration are well-established features of obesity. The whole spectrum of initiators and pathophysiological consequences of this inflammation is yet to be defined. The complex process of monocyte diapedesis and subsequent differentiation into M1 or M2 macrophages in the obese adipose tissue appears to be substantially different in mouse and human obesity, which emphasizes the need for investigation in humans. Understanding the regulatory mechanisms that lead to M1/M2 phenotypic switch could help to define new ways of controlling adipose tissue inflammation (Box 4). Obesity,

Box 4. Outstanding questions

- What is the temporal hierarchy of signals that provoke inflammation in adipose tissue in the setting of obesity?
- What is the phenotype of the circulating monocytes that contribute to macrophage accumulation in adipose tissue?
- What is the relative influence of the circulating monocyte phenotype versus the adipose tissue microenvironment to determine the polarization state of macrophages?
- Is obesity truly a 'sterile' inflammation, and what is the importance of adaptive immunity in adipose tissue inflammation?
- Could macrophage infiltration in adipose tissue be beneficial to restore adipose tissue homeostasis?

which is associated with different stresses such as nutrient excess or adipocyte hypertrophy, could be considered an illustration of 'para-inflammation', which refers to an adaptive response induced by tissue stress or malfunction intermediate between basal and acute inflammation [66]. In this context, the high numbers of macrophages recruited to adipose tissue might be part of the adaptive response that is aimed at restoring adipose tissue functionality. Thus, whether or not macrophage depletion represents a suitable tool to restore adipose tissue homeostasis in obesity remains an open question.

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4. Adipose tissue & Adaptive immune system

Adaptive immune response is composed on two distinct pathways: the cell-mediated immune reponse in which T cells are intimately involved and the humoral immune reponse based on the secretion of immunoglobulins by B cells. An effective adaptive immune response requires organs that enable the maturation of lymphocytes (primary lymphoid organs), specialized innate immune cells that present antigenic peptides to lymphocytes (APCs), organs that allows the optimized meeting between lymphocytes and APCs (secondary lymphoid organs) and finally, antigen-specific lymphocytes that recirculate throughout the body. In this manuscript, only the T cell' adaptive immune response will be fully developped. The humoral response and unclassical innate-like lymphocytes will be very briefly presented.

4.1. T lymphocytes or the cellular immunity

T lymphocytes, characterized by the general marker CD3, are first produced in the bone marrow before travelling to the thymus, from which they derive their name and where they mature. In secondary lymphoid tissues, specific antigen recognition occurs through their T cell receptor (TCR) composed of an α and a β chains. T cell activation occurs when APCs display the appropriate set of ligands to naive T cell, which include proper MHC-peptide complex and co-stimulatory molecules, predominantly CD80/CD86 interacting with CD28. Mature αβ T cells belong to two major types: $CD4^+$ T cells, which recognize antigenic peptides presented by MHC class II molecules, and $CD8^+$ T cells, which recognize MHC class I molecules. Whereas $CD4^+$ T cells have a predominantly regulatory role, $CD8^+$ T cells are essentially responsible for killing infected cells through cytotoxic functions.

TCR/MCH connexion leads to the formation of an immunological synapse as described in Figure 14. Membranous proteins at the T cell surface form organized structures, and corresponding counterstructures occur on the APC surface. This is followed by cytoskeletal rearrangements and the transcription of specific genes that are turned on. Eventually, T cells acquire the ability to sense chemotactic cues to reach site of inflammation and the qualification to produce cytokines that will act on T cell themselves, APCs and other succeptible target cells, or in the case of cytotoxic T cells, a licence to kill (Boes and Ploegh, 2004).

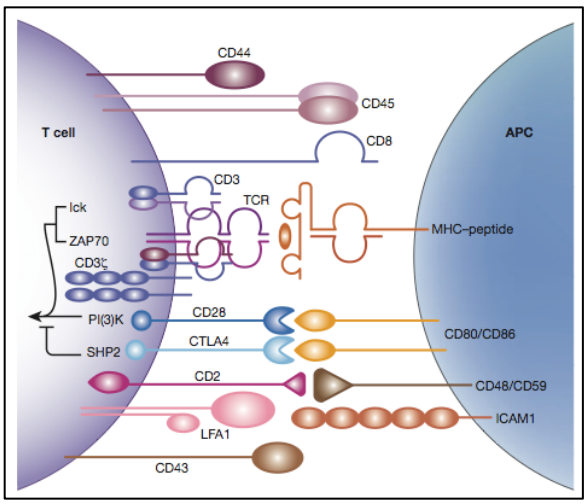

Figure 14 | The T cell-APC synapse (Boes and Ploegh, 2004).

Immunological synapse initiates proliferation and activation of naive T cells. Cell differentiation pathways can be influenced by additional signals from the environment to result in various phenotypic and functional outcomes. Absence or presence of inflammatory cues acts as a switch that determines whether TCR and co-stimulatory signals lead to tolerance or an effector response. The inflammatory response leads to a strong effector function, enhanced survival and memory population formation. Thus, in addition to TCR (signal 1) and co-stimulation (signal 2), the cytokine environment plays a central role in fate determination and effector function of T cells and it has been termed « signal 3 » (Curtsinger and Mescher, 2010).

In 2005, a first study showed that lymphocytes represents 10% of the SVF of adipose tissue from a lean mice (Caspar-Bauguil et al., 2005). Subsequently, extensive research focused on defining the role of lymphocyte subsets in the onset of obesity and related-metabolic complications. In many publications focusing on obesity, the role of lymphocytes was assessed using the Rag^{null} mice model that lacks adaptive immunity. In this introduction, special attention will be given to $CD4^+$ and $CD8^+$ T lymphocytes in both blood and adipose tissue of obese mice and individuals. The ongoing question of a possible « fat antigen» is debating in Box 4. Mice under high fat feeding were shown to significantly increase their number of $CD3^+$ T cells in adipose tissue in obesity as opposed with their lean counterparts (Rocha et al., 2008; Wu et al., 2007). $CD3^+$ cell infiltration was concominent with early insulin resistance suggesting a role for lymphocytes, in addition to macrophages in obesity-induced metabolic complications (Kintscher et al., 2008). In human, number of $CD3^+$ T cells was also found to be increased in both subcutaneous and visceral adipose tissue of obese patients (Duffaut et al., 2009b; Yang et al., 2010). Immunohistochemistry analyses showed that

lymphocytes were localized in proximity of, or crowning around adipocytes, predicting specific interactions between the cells, and potentially macrophages (Duffaut et al., 2009a; Yang et al., 2010). Several studies described that lymphocytes, both T and B cells, accumulated earlier than macrophages in adipose tissue during the development of obesity, suggesting a central role for T cells in initiating adipose tissue inflammation. Once should keep in mind that a classical immune response may be composed of a primary response (innate immunity) and a secondary response (adaptive immunity) coming later on. Thus, it seems quite odd that lymphocytes might be the cells initiating adipose tissue inflammation.

Box 4 | Are lymphocytes still looking for the fat antigen(s)?

Obesity might be viewed as a state of 'sterile' inflammation, which occurs in the absence of any microorganisms. Cues that trigger adipose tissue inflammation such as cytokines, intracellular content of moribund adipocytes or exogenous lipid species, are all part of DAMPs and are likely to induce an inflammatory response in immune cells, as described earlier. On the other side, the presence of lymphocytes in the adipose tissue raises an important issue: do lymphocytes recognize adipose tissue-associated antigen(s) ? It is true that the presence of T lymphocytes and maintenance of their activation suggests the continual persistance of antigenic stimuli in adipose tissue. In the steady state, that is, in lean adipose tissue, the astonished high number of $Foxp3^+$Treg cells that are subsequently depleted in obesity, might help to keep under control an ongoing immune stimulation (Feuerer et al., 2009). Beside, several groups focused on the study of TCR rearrangements in adipose tissue T cells. They agreed to state that obesity is associated with a strinking loss of T cell TCR diversity. This restricted distribution is not monoclonal but they discribed repeated selection of T cells with similar antigen receptors occurring in both $CD8^+$ and $CD4^+$ subsets, including Tregs. These data suggest that T cell expansion could be antigen-driven in either lean or obese adipose tissue, and that this TCR biais increases during obesity (Feuerer et al., 2009; Winer et al., 2009a; Yang et al., 2010). Of note, a TCR biais is a common feature in organ-selective autoimmune disorders such as type 1 diabetes or rheumatoid arthritis (Turner et al., 2006). Comparing insulin resistant with insulin sensitive patients, several circulating IgG autoantibodies were identified and specifically associated with the metabolic status of subjects, although, the corresponding antigens were not adipose tissue specific (Winer et al., 2011). Thus, it remains important to go on with studies focusing on the identification of yet unknown adipose tissue-associated antigens. Antigens could be from many different sources such as immunogenic lipid species, viral particles or bacterial peptides from gut microbiota translocation as it was recently proposed for obesity (Burcelin et al., 2012; Henao-Mejia et al., 2012).

One possibility is that resident adipose tissue macrophages that are found in fat depot, might be the cells to give the very first signal for T cells to accumulate, participating in an inflammatory loop and attracting new macrophages in a later phase (Lumeng et al., 2007; Murray and Wynn, 2011).

Considering $CD4^+/CD8^+$ subset distribution, one study reported that there were threefolds more $CD4^+$ cells than $CD8^+$ in VAT of lean mice (Feuerer et al., 2009). During obesity, accumulation of both $CD4^+$ and $CD8^+$ T cells have been reported, but contradictions remain over the extent to which the two subsets contribute to obesity and obesity-induced inflammation and insulin resistance. Most of mice studies reported an increase in $CD8^+$ cells in adipose tissue in response to high fat feeding, trending towards a very low CD4/CD8 ratio in obesity (Deiuliis et al., 2011; Nishimura et al., 2009; Rausch et al., 2008; Winer et al., 2009a; Zuniga et al., 2010). In contrast, other groups described significant $CD4^+$ T cells accumulation as compared to $CD8^+$ subsets (Rocha et al., 2008; Yang et al., 2010) while one publication did not find any difference between the two subsets (Strissel et al., 2010). These results can be explained by multiple differences in the fat diet protocols including the age of the mice, the length of the regimen and especially, the fat composition of the diet that was found to range from 35 to 60% calories from fat. It is interesting to note that these results were found in VAT since SAT did not seem to specifically response to high fat diet in term of $CD4^+/CD8^+$ accumulation. Conflicting results also exist in humans. While $CD8^+$ cells were found to be increased in VAT of obese subjects in two studies, another group found a predominence of $CD4^+$ T cells (Duffaut et al., 2009b; O'Rourke et al., 2009; Yang et al., 2010). Thus, it remains to be found which T lymphocyte subsets, or their possible interactions, are the key players in obesity and subsequent comorbidities.

4.1.1. $CD4^+$ Helper T cells

$CD4^+$ or helper T cells play a central role in the function of the immune system; they help B cells make antibody, enhance and maintain $CD8^+$ T cell action, regulate APCs, orchestrate immune responses against a wide variety of pathogens. They also regulate and suppress immune responses both to control autoimmunity and to adjust the magnitude and persistence of responses. Thus, $CD4^+$ cells play critical roles in orchestrating the adaptive immune responses, mainly through the release of cytokines and chemokines that activate and recruit target cells. Based on the set of cytokines they secrete, T helper cells were classified in different subsets as described in Figure 15. The different Th lineages require

specific « signal 3 » that were mainly assessed by *in vitro* studies upon polyclonal stimuli such as anti-CD28 and anti-CD3 (Zhu and Paul, 2008).

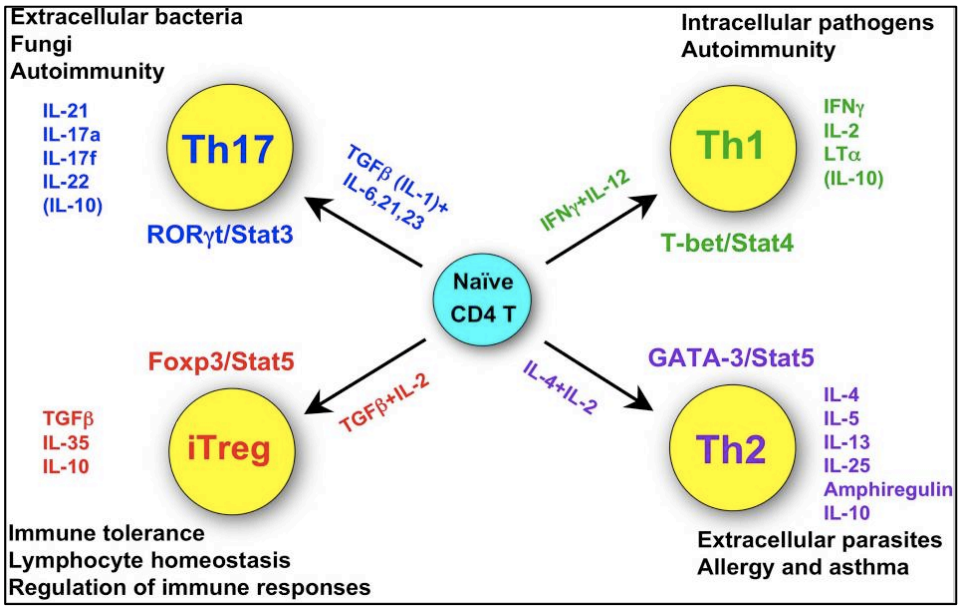

Figure 15 | Summary of the main CD4 T helper cell fates: their functions, secretions, characteristic transcription factors and immunogenic conditions that critically determine their differentiation fates (Zhu and Paul, 2008).

(a) Th1 cells

Th1 cells produce IFN-γ as their signature cytokine and also uniquely produce lymphotoxin. They appear to be good producers of IL-2 and some of them also release TNF-α. *In vitro* experiments showed that naive $CD4^+$ T cells could differentiate into Th1 upon IL-12 addition. The master transcription factor inducing Th1 lineage is Tbet that induces production of IFN-γ and expression of IL-12 receptor (Zhu and Paul, 2008). Based on the known biological function of the cytokines they produce, Th1 cells act to activate macrophages, enhancing their microbicidal activities including phagocytosis but also on $CD8^+$ favoring their activation and proliferation. Strong and persistent Th1 responses appear to be involved in chronic inflammatory disorders such as Crohn's disease and rheumatoid arthritis but this concept was challenged by the recent discovery of Th17 as presented below (Zhu and Paul, 2008).

In obesity, Th1 cell number and IFN-γ production are increased in VAT of DIO mice fed a high fat diet (HFD) as compared to standard diet (Kintscher et al., 2008; Rocha et al., 2008; Strissel et al., 2010; Winer et al., 2009a; Yang et al., 2010; Zuniga et al., 2010). Th1 cells are activated quite early in obesity with increased production of IFN-γ as soon as 6 weeks of diet

(Strissel et al., 2010). Human obesity is also associated with enrichment in Th1 cells (Duffaut et al., 2009b; O'Rourke et al., 2009; Zeyda et al., 2011). Interestingly, studies focusing on circulating levels of IFN-γ did not found a difference between lean and obese patients but a specific increase associated with T2D (Jagannathan-Bogdan et al., 2011; Sumarac-Dumanovic et al., 2009) (van der Weerd et al., 2012). IFN-γ appears to play a key role in obesity-associated inflammation and insulin resistance as DIO mice deficient in IFN-γ display improved glucose tolerance and insulin sensitivy compared to wildtype animals (O'Rourke et al., 2009; Rocha et al., 2008). This phenotype was confirmed in DIO IL12p35-null mice (Winer et al., 2009a). There are two complementary ways that IFN-γ may initiate and promote insulin resistance in obesity. First, IFN-γ can directly act on adipocytes to alter their metabolism. Rocha *et al*, showed that IFN-γ selectively up-regulated inflammatory gene expression, especially chemokines, in adipocytes (Rocha et al., 2008). Others demonstrated that T cell-derived IFN-γ blocked differentiation of preadipocytes into their mature phenotype, impaired adipocyte insulin-stimulated uptake of glucose and reduced adipocyte insulin-mediated upregulation of lipogenic enzymes (Duffaut et al., 2009b; McGillicuddy et al., 2009). Then, IFN-γ can polarize and maintain a M1-phenotype in adipose tissue macrophages, participating in an obesity-associated inflammatory loop (Mosser and Edwards, 2008; O'Rourke et al., 2009). Consistently, IFN-γ knock-out mice show decreased adipose tissue inflammation with reduced M1-macrophage number and crown-like structures in VAT in response to high fat feeding (O'Rourke et al., 2009; Rocha et al., 2008). Indeed, these results indicate a crucial role for Th1 cells in regulation of the inflammatory response within adipose tissue and insulin resistance in the context of obesity. IFN-γ appears to be a major mediator of adipose tissue T cells directly acting on adipocyte metabolism and potentially, on macrophage polarization.

(b) Th2 cells

Contrary to Th1 subset, Th2 cells fail to produce IFN-γ but predominantly release IL-4, IL-5 and IL-13. It was described that Th2 cells were obtained in the presence of IL-2 and their own secreted product : IL-4. Transcription factor GATA3 and STAT6 promotes Th2 differentiation through initiating and selectively stimulating the growth of Th2 cells, while suppressing Th1 commitment. Of interest, Th2 mainly produce cytokines with known anti-inflammatory properties (typically IL-4 and IL-13) that can antagonize the macrophage-activating action of IFN-γ. Thus, Th2 lineage aims at limiting acute and chronic inflammation which is consistent with the fact that Th1 response occurs early while Th2 cells accumulate at later stages, when the immune response progresses (Zhu and Paul, 2008).

Few studies focused on Th2 lineage and their cytokines IL-4 and IL-13. In a DIO model, obesity was associated with a decrease in IL-4 gene expression and in the proportion of Th2 among T cells in VAT (Deiuliis et al., 2011; Strissel et al., 2010; Winer et al., 2009a). In human, studies are only based on gene expression and show contradictory results: GATA3 was found either decreased or increased in VAT of obese patients compared with lean controls (Deiuliis et al., 2011; Zeyda et al., 2011). The number of circulating Th2 was found to be increased in obese patients, although serum levels of IL4 was not different when compared to lean controls (van der Weerd et al., 2012). In their study, Winer *et al*. used the $Rag1^{null}$ mouse model that lacks lymphocytes (Winer et al., 2009a). DIO $Rag1^{null}$ mice showed increased weight gain, adipocyte hypertrophy and impaired glucose and insulin tolerance as compared to wildtype animals, suggesting an overall protective role for lymphocytes in obesity. While transfer of $CD4^+$ T cells in the $Rag1^{null}$ recipient mice improved their insulin sensitivity, $CD4^+STAT6^{null}$ T cells did not, suggesting metabolic effects of Th2 cells. This CD4+ T cell transfer model was accompanied by a sharp increased in both IL-4 and IL-13 (Winer et al., 2009a). DIO mice treated with IL-4 showed improved glucose tolerance and reduced inflammation in VAT (Ricardo-Gonzalez et al., 2010). Interestingly, it was recently shown that the major source of IL-4 in VAT may be eosinophil-derived, with only a small pourcentage derived from Th2 cells as show in IL-4 GFP reporter mice (Wu et al., 2007). So far, few and conflicting results exist on the role of Th2 cells in obesity. Whether Th2 cells and related IL4 directly influence adipose tissue homeostasis through its anti-inflammatory properties and could contribute to the regulation of insulin sensitivity remain to be determined.

(c) Th17 cells

In 2005, a third major effector population, Th17 cells, was identified and characterized by the production of IL17A, IL-17F, IL-21 and IL-22 as signature cytokines. Of note, these cells specifically express the chemokine receptors CCR6 and CCR7. Unlike Th1 and Th2 cells, differentiation of Th17 is still debated, greatly differs between mice and men and shows some flexibility in differentiation depending on the priming environment. Pioneering studies indicated that IL-6 and TGF-β were the two key cytokines for the *in vitro* differentiation of mouse Th17 cells (Mangan et al., 2006). Additional work has revealed that IL-1β, in possible synergy with IL-6 and IL-23 (and perhaps TGF-β), leads to Th17 differentiation, in humans. Beyond polarization, IL-1β and IL-23 were found to also influence Th17 cell, possibly through cell proliferation, survival and stimulation of IL-17 production to maintain their Th17

differentiated state (Acosta-Rodriguez et al., 2007; Lalor et al., 2011; Wilson et al., 2007; Zhu and Paul, 2008). Mice defective in either IL-23 or IL-1β signaling have reduced IL-17 production and are resistant to disease progression in Th17 cell-dependent experimental autoimmunity. At the transcription level, Th17 express RORγt in mice/RORc in humans (Wu et al., 2007). Recently, Th17 cells biology has been linked to many autoimmune conditions including psoriasis, multiple sclerosis and rheumatoid arthritis as well as allergy and transplantation rejection in mice and humans (Peters et al., 2011).

It has been a couple of years since Th17 lineage and IL-17 particularly drew research's attention in the context of obesity, although, compared to IFN-γ, less data are still available. While in two publications, IL-17 was either not detectable or not increased in adipose tisse, a very recent recent study showed an increased in $CD4^+$IL-17 producing cells in fat of DIO or db/db mice (Bertola et al., 2012; Winer et al., 2009b; Yang et al., 2010). Besides, DIO mice showed 3-folds more IL-17^+ cells in their spleen as compared to controls without affecting other T cell lineages. Winer *et al*. used a model of combined obesity and experimental multiple sclerosis through DIO and MOG immunization (Winer et al., 2009b). They showed that, in lymph nodes, obesity generated a sharp increase in the number of Th17 and the production of IL-17 as compared to lean mice. Thus, obesity induced a more pronounced Th17 biais in autoimmune diseases (Winer et al., 2009b). Interestingly, the major source of IL-17 in both SAT and VAT appears to come from γδ T cells rather than $CD4^+$ αβ T cells in mice. Young IL17-deficient mice showed greater adiposity and improved glucose tolerance as compared to controls; although, these beneficial effects were lost with later stages of obesity (Zuniga et al., 2010). Of note, DIO TCRγδ-deficient mice showed no difference in metabolic profiles compared with wild-type mice, suggesting compensary source of IL17 (Zuniga et al., 2010). In humans, only one study showed an up-regulation of Il-17 producing cell number in subcutaneous adipose tissue of overweight and obese subjects compared to lean group (Bertola et al., 2012). Interestingly, adipocytes were shown to secrete the chemokine CCL-20, while lymphocytes appeared to express the cognate CCR6 receptor, already suggesting the presence of Th17 cells in human adipose tissue in obesity (Duffaut et al., 2009). In non-diabetic obese patients, a study futher showed no difference in circulating Th17 number nor serum IL-17 levels when comparing lean counterparts, and yet another one described a significant increase in both IL-17 and IL-23 serum levels (Sumarac-Dumanovic et al., 2009; van der Weerd et al., 2012). Interestingly, Jagannathan-Bogdan and colleagues demonstrated that blood Th17 subset was specifically associated with T2D in moderatly obese patients. Stimulated peripheral blood mononuclear cells (PBMC) from diabetic patients secreted more IL-17 than lean controls and this production was correlated with severity of

glucose tolerance (Jagannathan-Bogdan et al., 2011). Adipocytes were found to express IL17 receptor (IL-17R) suggesting that IL-17 could affect adipocyte metabolism. Indeed, treatment of preadipocytes with IL-17 inhibited cell differentiation and insulin-induced glucose uptake (Zuniga et al., 2010). These results suggest a pathogenic role for Th17 in obesity and especially T2D, although further studies are needed to fully define the contribution of IL-17 in adipose tissue inflammation. Human studies are especially required as it was shown that TCR$\gamma\delta$ cells represent only a negligeable percentage of adipose tissue T cells, suggesting massive discrepancies between mice and humans (Duffaut et al., 2009b).

(d) Treg cells

Regulatory T cells (Tregs) are a subpopulation of T cells that is known to be involved in lymphoid homeostasis, maintenance of tolerance to self and control of autoimmune deviation. Tregs can be of two origins. Natural Tregs (nTregs) are generated in the thymus during T cell development. On the other hand, induced Tregs (iTregs), also known as adaptive or converted Tregs, are developped from naive T cell precursors in peripheral lymphoid organs (or *in vitro*) after antigen exposure and processing of environmental immunogenic triggers. Both nTregs and iTregs are characterized by the expresion of the Foxp3 transcription factor whose continuous expression is required to maintain the suppresive activity of such cells (Feuerer et al., 2009). Tregs are also characterized by the secretion of the immunoregulatory cytokine: IL-10 (Zhu and Paul, 2008). Differentiation of both nTRegs and iTregs is dependent of TGF-β that is required, in combination with IL-2, for the survival and function of Treg cells even after they have differentiated. As mentionned above, TGF-β is involved in both Th17 and Treg differentiation in mice, suggesting a close relationship between those two lineages. Both an excess of Th17 function or a defect in Treg function may trigger inflammatory disorders and contribute to automimmunity. Thus, a balance between Th17 and Treg is particularly crucial for immune homeostasis (Long and Buckner, 2011).

In adipose tissue, regulatory Foxp3 T cells have been extensively studies in the last few years. The pioneer study by Fueurer *et al*. showed that both SAT and VAT of lean mice were enriched with Tregs that account for 10 and 50% respectively, of the total CD4+ T cell compartment (Feuerer et al., 2009). This represents a much higher fraction than in lymphoid and non-lymphoid tissues, already suggesting a strong and specific role for Tregs in adipose tissue. While fat Tregs produce a massive amount of IL-10, the rest of adipose tissue lymphocytes belongs to the Th1 cells as they produce IFN-γ and TNF-α and express Tbet

(Feuerer et al., 2009). In obesity, Tregs fraction drastically drops in VAT but not in SAT nor spleen. Tregs are localized between adipocytes or in crown-like structures around single adipocyte, predicting possible interactions with fat macrophages in DIO mice (Feuerer et al., 2009; Winer et al., 2009a). During the course of HFD, the Th1/Tregs ratio goes from 1,5 in lean mice to 6 in obese mice (Feuerer et al., 2009; Nishimura et al., 2009; Winer et al., 2009a). Proportion of Tregs among total $CD4^+$ cell compartment is fourfolds decreased during high fat compared low fat diets. This decrease appears to be inversely correlated with the presence of $CD11c^+$ macrophages (Deiuliis et al., 2011). Interestingly, in all studies, Treg number was comparable between groups when normalized to adipose tissue weight, suggesting that freshly recruited Th1 and $CD8^+$ cells progressively "dilute" the number of Tregs in adipose tissue (Winer et al., 2009a). Transient depletion of Tregs induces an increase in inflammatory genes in adipose tissue and raises insulinemia. On the other hand, adoptive transfer of Tregs induced more IL10 transcripts in VAT and tended to lower blood glucose (Feuerer et al., 2009). In the same way, VAT Treg number could be restored in DIO mice by treatment with an anti-CD3 specific antibody and significantly improved fasting glucose and insulin levels. This was concomitant with an induction of IL-10-producing M2-like macrophages while decreasing M1-like macrophages in VAT (Ilan et al., 2010). In human, data are rare and sometimes contradictory. VAT gene expression ratio of Th1/Foxp3 ranged from 6 in lean controls to 12 in obese patients (Winer et al., 2009a). Foxp3 appeared more expressed in SAT than in VAT and the drop in Tregs in SAT versus VAT correlated with BMI (Feuerer et al., 2009). However, another study shows that Foxp3 expression was increased in both SAT and VAT of obese compared to lean subjects (Zeyda et al., 2011). Number of circulating Tregs was found to be increased in non-diabetic obese individuals compared to lean subjetcs but drastically decreased in T2D obese patients and inversely correlated with BMI (Jagannathan-Bogdan et al., 2011; van der Weerd et al., 2012). Hence, loss-of-function and gain-of-function experiments indicated that Tregs cells guard against excessive inflammation in the adipose tissue and its downstream systemic consequences.

4.1.2. $CD8^+$ Cytotoxic T cells

Cytotoxic T cells play an important role in controlling viral, bacterial and parasitic intracellular infections by directly killing infected cells and producing pro-inflammatory cytokines. They have a unique ability to survey the host for intracellular perturbations and restore homeostasis. In order to acquire effector functions naive $CD8^+$ T cells undergo a series of events which also include recognition of antigen *via* MHC class I complexes (Signal 1), co-stimulatory signals (signal 2) in presence of inflammatory cytokines (signal 3) leading to activation, proliferation and differentiation. Like their $CD4^+$ counterparts, the local

microenvironment directs the multiple possible fates that $CD8^+$ T cells can adopt. Of note, as almost all cells express MHC class I molecules, it is clear that there is a great potential for $CD8^+$-derived tissue damage. The importance of $CD8^+$ T cells in autoimmune disease such as type 1 diabetes or multiple sclerosis is becoming increasingly evident (Zhu and Paul, 2008).

Upon antigen recognition, naive $CD8^+$ T cell undergo changes in gene expression. At the transcription level, $CD8^+$ T cell are also prone to be regulated by different master regulators. Mainly, T-bet expression in $CD8^+$ cells is required for IFN-γ production and cytolytic gene expression. It is interesting to note that it has recently been shown that $CD8^+$ effector cells can give rise to different subsets, mimicing $CD4^+$ classification. Thus, IL-17-producing $CD8^+$ (Tc17) or regulatory $CD8^+$ T cells arise as interesting populations, although mechanisms of their exact regulation and function are unclear (Cox et al., 2011). When effector $CD8^+$ T cells encounter infected cells, they use both cytotoxic and non-cytotoxic functions to affect their targets: cytotoxic molecules (*e.g.* granzyme and perforin) mediate direct contact-dependent cytotoxicity, expression of Fas ligand (CD95L) induces apoptosis in a Fas–Fas ligand-dependent manner and secretion of pro-inflammatory cytokines (*e.g.* IFN-γ and TNF-α) sustains local inflammation. $CD8^+$ cells appear to be highly regulated by their $CD4^+$ counterparts. It was shown that $CD4^+Foxp3^+$ Tregs could inhibit the differentiation of islet-reactive cytotixic $CD8^+$ T cells in the pancreas, thereby preventing type 1 diabetes progression (Cox et al., 2011).

Although, results are conflicting, it appears evident that accumulation of $CD8^+$ T cells in VAT plays a crucial role in obesity-induced inflammation and insulin resistance. Nishimura *et al.* showed that as soon as 2 weeks of DIO, the proportion of $CD8^+$ cells specifically increased in VAT, before macrophage accumulation. $CD8^+$ cells co-localized with macrophages in crown-like structures (Nishimura et al., 2009). Depletion of $CD8^+$ cells obtained by injected anti-CD8 antibody during the course of DIO and in pre-established DIO, significantly alleviated adipose tissue macrophage infiltration and overall inflammation, while improving insulin sensitivity. Besides, CD8a-deficient DIO mice did not show adipose tissue inflammation, including macrophage infiltration that was restituted upon adoptive transfer of functional $CD8^+$ T cells. $CD8^+$ T cells might contribute to macrophage accumulation though chemokine secretion as indicated in co-culture experiments (Nishimura et al., 2009). As mentioned earlier, Winer *et al*. used the $Rag1^{null}$ mouse model that lacks lymphocytes and are characterized by increased weight gain and insulin resistance compared to controls. Using adoptive cell transfer, they showed that while $CD4^+$ T cell reconstitution lowered weight gain and normalized glucose tolerance, transfer of cytotoxic $CD8^+$ T cells did not. Once again, the pathogenic role of $CD8^+$

T cells in obesity is highlighted (Winer et al., 2009a). Thus, overall data show that $CD8^+$ T cells are definitely required for the initiation, propagation as well as maintenance of adipose tissue inflammation.

4.2. B lymphocytes or the humoral immunity

B lymphocytes develop in the bone marrow (hence the B) and are central to the adaptive humoral immune system. They are responsible for mediating the production of antigen-specific antibodies or immunoglobulins (Igs) directed against invasive microbial agents. One B cell produces immunoglobulins of a single specificity. Membrane-bound Ig, or IgM, on the B-cell surface serves as the cell's receptor for antigen, known as the B-cell receptor (BCR). Eventually, Igs of the same antigen specificity are secreted in great amounts by terminally differentiated B cells, termed the plasma cells, usually in conjunction with T-cell help. The secretion of antibodies, which bind pathogens or their toxic products in the extracellular spaces of the body, is the main effector function of B cells in adaptive immunity (Vaughan et al., 2011). B cell–dependent immune responses have now been shown to be involved in a variety of (auto)immune-mediated inflammatory diseases (Vaughan et al., 2011).

Beside a key role for T lymphocytes in obesity and insulin resistance, recent attention was given to B lymphocytes. DIO mice models showed that B cells also infiltrate adipose tissue, and this occurs quite early in the set up of obesity, before any other immune cell accumulation (Duffaut et al., 2009b; Winer et al., 2011). Adipose tissue B cells were associated with a rise in serum IgG concentration in obesity (Winer et al., 2011). Immunohistochemistry analysis revealed that B cells and their secreted Ig were also localized in crown-like structures, next to other T cells or macrophages located around dying adipocytes (Winer et al., 2011). B-cell-deficient (B^{null}) mice on high fat feeding showed significant improvement in glucose tolerance, insulin sensitivity and adipose tissue inflammation compared with wildtype controls. Furthermore, transfer of functional B cells or serum IgG purified from DIO mic, in B^{null} recipient mice brought the insulin resistance and inflammatory phenotype back. On the contrary, glucose metabolism was not impaired when transfering functional B cells in total lymphocyte-deficient ($RAG1^{null}$) recipient mice under HFD. These pioneer data demonstrated that B cells play a pathogenic role in obesity, but B cells require adjacent T cells to fully promote altration of metabolic parameters. This occurs in a MHC-dependent way, probably through B cell antigen-presentation to T cells. Finally, DIO mice were treated with a B cell depleting CD20 antibody. CD20-treated mice showed improved glucose metabolism and relieved adipose tissue inflammation compared to vehicle-

treated mice. Thus, it is likely that IgG from B cells can have an effector function in promoting insulin resistance in DIO mice and that manipulating B cells could be an interesting therapeutical target. Although, it seems that B^{null} mice suffer from T cell abnormalities that could bias B cell experimental outcomes (Cipolletta et al., 2011). Besides, very few studies exit on B cells in humans and if so, their role is not so clear. Relatively low number of $CD19^+$ B cells was identified in both subcutaneous and visceral adipose tissues (Duffaut et al., 2009b). B cells also localized in crown-like structures in human adipose tissue (McDonnell et al., 2012). At the circulating level, B cells showed increased production of IL-8 and surface expression of TLR-4 in diabetic patients compared to non-diabetic controls. Thus, diabetes *millitus* may be associated with activated circulating B cells (Jagannathan-Bogdan et al., 2011). Much more studies should be performed to unravel the role of B cells in adipose tissue in human obesity.

4.3. Innate-like lymphocytes

Two lymphocyte subsets represent a bridge between innate and adaptive immunity: the γδ-T cells and Natural killer T (NKT) cells. They express the $CD3^+$ T cells marker but show distinct innate-like characteristics. NKT cells recognize various glycosphingolipid antigens (including the prototypical NKT cell antigen: α-galactosylceramide or α-GalCer) through the MHC class I–like molecule CD1d receptor of APCs. This unusual specificity is intimately linked with their expression of a very limited TCR repertoire. Unlike their T cell cousins, NKT cells produce very large amounts of cytokines, such as IFN-γ and IL-4 upon stimulation, with the ability to drive immune responses in both pro- and anti-inflammatory directions (Godfrey and Rossjohn, 2011). Besides, γδ-T cells gain their name from their TCR receptor composed of γ and δ chains (instead of common αβ TCR) and some rectriction in their repertoir similar to NKT. Molecular cues determine the commitment of γδ-T cells to distinct transcriptional programs: γδ-T1 express Tbet and secrete IFN-γ, and γδ-T17 express RORγt and secrete IL-17, similar to $CD4^+$ T cells. γδ-T cells are usually found in epithelial-rich tissues such as the skin and intestines and have recently been assumed to also play a role in autoimmunity (Korn and Petermann, 2012).

As mentionned earlier, γδ lymphocytes are the predominent source of IL-17 secretion in adipose tissue in DIO mice; and yet, they represent a negligeable cell percentage in human adipose tissue (Duffaut et al., 2009b; Zuniga et al., 2010). On the other hand, the possible role of NKT cells in obesity appear much more complex. Among different types of NKT cells, type 1 or invariant NKT (iNKT) cells are the most abundant and express a highly restricted

TCR. Many studies have now showed that iNKT cells are selectively decreased in liver and adipose tissue upon high-fat feeding (Kotas et al., 2011). Two recent studies using the $CD1d^{null}$ mice model, demonstrated the lack of metabolic effect in NKT-deficient mice following high fat feeding. Thus, deletion of NKT cells alone was not sufficient to protect mice against obesity and obesity-associated alterations (Kotas et al., 2011; Mantell et al., 2011). Specific role of iNKT was further studied using a DIO mice model deficient in iNKT but not CD1d ($J\alpha^{null}$) that did not recapitulate the phenotype (Kotas et al., 2011). These data suggest that iNKTs play a minimal role in metabolism whereas CD1d-restricted non-iNKT cells could have a protective role in obesity. αGalCer-mediated activation of NKT cells enhanced M2-like macrophage polarization in adipose tissue and improves glucose homeostasis in DIO mice, through the IL-4/STAT6 signalling axis (Ji et al., 2012). In human, visceral adipose tissue was shown to contain the highest amount of $CD1d^+$ cells and iNKT cells in the body with dual Th1/Th2 cytokine secretion profiles. Omental iNKT-cell numbers and CD1d expression decreased in obese patients compared to controls and negatively correlated with insulin resistance and fasting glucose (Ji et al., 2012; Lynch et al., 2009). Thus, publications overall suggest a protective role for CD1d-restricted NKT cells in obesity; although cellular mechanisms, let alone (glyco)lipid recognition, are still unclear. Additional studies should be lead to elucidate a potential role for NKT in metabolism.

In conclusion, adipose tissue appears to be a site of intense immune cell regulations. Impact of obesity on the adaptive immune cell types in mice and humans is summarized in table 3. There are many contradictions among the aforementioned studies on the contributions of $CD4^+$ and $CD8^+$ cells to obesity-associated inflammation and metabolic complications. It should be pointed out that methods used to identify/describe/quantify T cells differed in the various studies. First, technical discrepancies can appear while isolating the cells (enzymatic digestion, percoll, serum addition…). Then, normalization of cell counts is of major importance. In flow cytometry, both percentages and absolute number per gram of fat are required. In reference to cytokine secretions, T cells required activation upon isolation that can differ in the studies. Gene expression performed in whole adipose tissue does not necessarily reflect regulation of the cells of interest. For mouse studies, it should be kept in mind that there are multiple divergences in the high-fat diet protocols used, including age of the mice at the onset of the diet, fat content and length of time on the regimens. Perhaps, modulations of the gut microbiota will soon have to be taken into account. In humans, much more work is needed to decipher the true role of adaptive immune cells in adipose tissue and their contribution to alteration of glycemic parameters. Considering discrepancies between mice and humans and the overall importance of IL-17 in autoimmune-like inflammation, the infiltration of IL-17 producing cells in adipose tissue is an important question that needs to

fully adressed. Besides, studies should unravel the functions of B and NKT cells in obesity, likely to play unexpected key role in obesity. Finally, the Tregs question is now to be addressed in humans: considering their abundance in lean mice, their function might be crucial.

	Mice		Humans	
	Changes	**References**	**Changes**	**References**
$CD4^+$ Th1	↑	(Kintscher et al., 2008; Rocha et al., 2008; Strissel et al., 2010; Winer et al., 2009a; Yang et al., 2010; Zuniga et al., 2010)	↑	(Duffaut et al., 2009b; O'Rourke et al., 2009; Zeyda et al., 2011)
$CD4^+$ Th2	↓	(Deiuliis et al., 2011; Strissel et al., 2010; Winer et al., 2009a)	?	(Deiuliis et al., 2011; Zeyda et al., 2011)
$CD4^+$ Th17	?	(Winer et al., 2009a; Winer et al., 2009b; Zuniga et al., 2010)	↑	(Bertola et al., 2012)
$CD4^+$ Tregs	↓	(Deiuliis et al., 2011; Feuerer et al., 2009; Nishimura et al., 2009; Winer et al., 2009a)	?	(Feuerer et al., 2009; Winer et al., 2009a; Zeyda et al., 2011)
$CD8^+$	↑	(Deiuliis et al., 2011; Nishimura et al., 2009; Rausch et al., 2008; Winer et al., 2009a; Zuniga et al., 2010)	↑	(Duffaut et al., 2009b; O'Rourke et al., 2009; Yang et al., 2010)
B cells	↑	(Winer et al., 2011)	?	(Duffaut et al., 2009b; McDonnell et al., 2012)
γδ T cells	↑	(Zuniga et al., 2010)	⊘ ?	(Duffaut et al., 2009b)
NKT cells	↓	(Ji et al., 2012; Kotas et al., 2011; Lynch et al., 2009; Mantell et al., 2011)	?	(Ji et al., 2012; Lynch et al., 2009)

Table 3 | Impact of obesity on proportions of adipose tissue adaptive immune cells.
↑, up-regulation; ↓, down-regulation ; ⊘, absence ;**?**, unknown or controversial.

Obesity is thus associated with alterations in the balance between different myeloid cells, predominantly from the monocyte/macrophage lineage and lymphocyte subsets in strong connections with metabolic alterations as illustrated in Figure 16. Beside, studies looking at defining the precise interactions and regulations between all these different immune cells mixed within the adipose tissue are crucial. Part of my PhD work lies in understanding how macrophages can influence T cells in adipose tissue in regards to T2D.

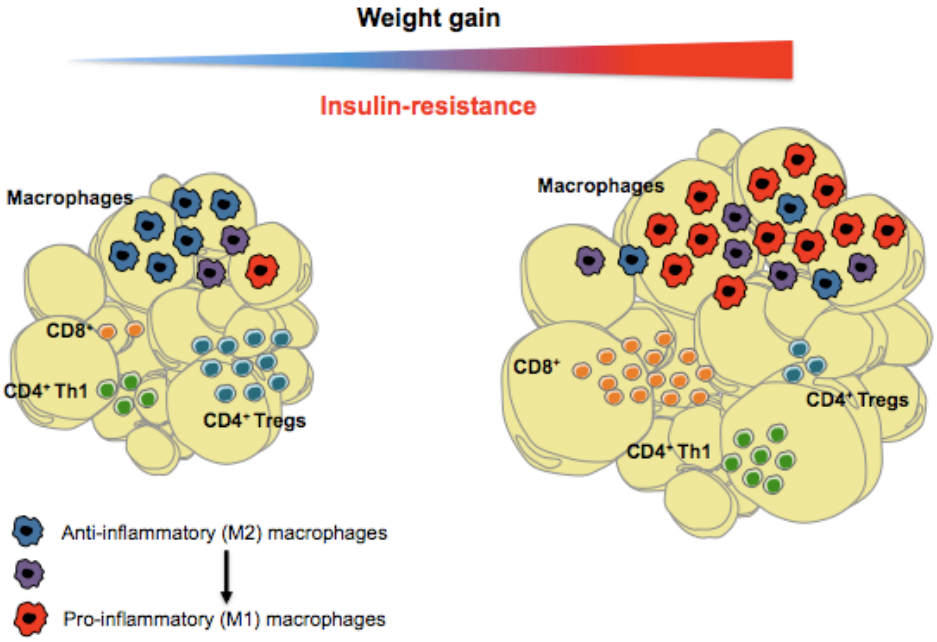

Figure 16 | Cellular alterations in visceral adipose tissue during obesity. Lean state, that may be extrapolated to insulin-sensitive obesity, is characterized by resident M2-like macrophages and Tregs predominance that are thought to maintain adipose tissue homeostasis and secrete molecules with insulin-sensitizing properties. On the other hand, weight gain ensues a shift in macrophage phenotype that acquires pro-inflammatory traits while Treg cells are « diluted » through a massive accumulation of $CD8^+$ and $CD4^+$ Th1 lymphocytes that all contribute to adipose tissue altered microenvironment associated with insulin-resistant obesity (adapted from (Cipolletta et al., 2011; Duffaut et al., 2009b; Yang et al., 2010),al).

THESIS WORK

C. Study 1: Circulating inflammatory mediators and effectors in obesity and during gastric surgery-induced weight loss

It is now well accepted that obesity is associated with increased circulating levels of inflammatory mediators that may contribute to the development of obesity-induced metabolic complications. Serum levels of inflammatory factors partly originate from circulating leukocytes or adipose tissue depots. Here, we decided to focus on the regulation of these circulating mediators and effector cells that are the monocytes, in morbid obese patients during bypass surgery-induced weight variations.

1. Article #1: Variations of circulating inflammatory factors are related to changes in calorie and carbohydrate intakes early in the course of surgery-induced weight reduction

In this first article, we described the effect of bypass surgery-induced weight loss on the regulation of several circulating markers that are, adipokines, CRP, cytokines and chemokines in 51 morbid nondiabetic and diabetic obese patients. We hypothesised that circulating markers would have different regulation patterns depending on their pro- or anti-inflammatory properties. These changes were analysed in light of fat mass loss, improvement in glycemic parameters and caloric intake. Expectedly, serum levels of CRP and leptin decreased while adiponectin was up-regulated during weight loss. We observed that most of circulating cytokines and chemokines showed a biphasic pattern of variation characterized by drastic decrease shortly after surgery, followed by a rebound and eventual stabilization at later time points. Similar kinetic patterns were described for energy intake and especially carbohydrates, which were significantly associated with changes in circulating factors.

Variations in circulating inflammatory factors are related to changes in calorie and carbohydrate intakes early in the course of surgery-induced weight reduction[1–3]

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ABSTRACT

Background: Obesity is considered a low-grade inflammatory state that improves with weight loss. In addition to acute-phase proteins, other cytokines might contribute to systemic inflammation.

Objective: Our objective was to compare serum concentrations of a large panel of inflammation-related factors in obese and normal-weight subjects and to determine kinetic changes induced by caloric restriction.

Design: The cohort comprised 14 normal-weight women and 51 obese women who were followed over 2 y after Roux-en-Y gastric bypass. Multiplexed proteomics were used to simultaneously assay 27 cytokines and growth factors in serum.

Results: Concentrations of interleukin (IL)-9, IL-1-receptor antagonist, IL-10, interferon-γ–inducible protein 10, macrophage inflammatory protein 1β, monocyte chemoattractant protein 1, IL-8, RANTES (regulated upon activation, normal T cell expressed and secreted), monokine induced by interferon-γ, and vascular endothelial growth factor were found to be elevated in obesity. IL-10 was further elevated in diabetic obese patients, whereas eotaxin was found to be higher only in diabetic subjects. After surgery, many factors showed a biphasic pattern of variation, decreasing sharply at month 3 before rising back to presurgical values at month 6; these changes closely tracked similar kinetic changes in calorie and carbohydrate intake. After 1 y, an overall reduction in cytokines accompanied the reduction in body mass index and an amelioration in metabolic status.

Conclusions: Obesity is associated with elevated circulating concentrations of a large panel of cytokines. Coordinated kinetic changes during weight loss suggest an early influence of calorie and carbohydrate intakes, whereas a longer-term reduction in corpulence might prevail in regulating circulating cytokine concentrations. This trial is registered at clincaltrials.gov as NCT00476658. *Am J Clin Nutr* 2011;94:450–8.

INTRODUCTION

Increased adipose tissue mass in obese individuals contributes to the development of many comorbidities that can result in serious health consequences. Obesity is now recognized as a state of chronic low-grade inflammation, characterized by an increase in systemic acute phase proteins [C-reactive protein (CRP) and serum amyloid A (SAA)] and obesity-related inflammatory markers such as interleukin (IL)-6 and IL-1 (1). Part of the systemic inflammation originates from adipose tissue, in which inflammatory cells, mainly macrophages, accumulate and create local inflammation (2–4). Adipose-derived inflammatory factors produced by enlarged adipocytes and/or by adipose tissue macrophages are elevated in the serum of obese subjects and are thought to contribute to metabolic and vascular complications, including insulin resistance, atherosclerosis, and liver diseases (5). Other mechanisms are also implicated in obesity-related systemic inflammation, including the activation of circulating immune cells (6–8) and inflamed vascular endothelium (9), which are potentially influenced by nutrient excess, hyperglycemia, dyslipidemia, or oxidative stress.

Weight reduction resulting from nutritional intervention or gastric surgery significantly improves the systemic and adipose tissue inflammatory states associated with obesity (4, 10–12). This amelioration relies on a regular and continuous reduction in CRP, SAA, or IL-6 concentrations, correlating with a parallel decrease in body mass index (BMI) (13). Other recently described molecules, such as chemerin, a chemoattractant protein associated with inflammatory processes, also decrease markedly in the serum after Roux-en-Y Gastric Bypass (RYGB)-induced weight loss (14). Despite these results however, the large battery of obesity-related cytokines and their variations in relation to weight reduction is far from established. In the current study we tested the possibility that cytokines would show distinct patterns according to their known

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pro- or antiinflammatory function. Moreover, we sought to test whether changes in circulating cytokine concentrations rely on adipose mass reduction and/or on amelioration of metabolic factors, such as insulin resistance.

RYGB intervention is the most effective method for reducing body weight and improving metabolism in severely obese individuals. The bioclinical outcomes of RYGB include marked changes in quantitative and qualitative food intake, reduced body fat mass, and amelioration of blood glucose, insulin resistance, and lipid variables. Here, we took advantage of the drastic and rapid RYGB-associated weight loss, coupled with caloric restriction and significant variations in macronutrient intake, to explore the kinetics of circulating inflammation-related factors. We used multiplexed proteomics to simultaneously assess a large panel of cytokines, chemokines, and growth factors in the serum at 4 consecutive short- and long-term time points after RYGB. Correlation analyses with bioclinical measures were performed to address the potential regulatory factors contributing to variations in systemic inflammation in human obesity and weight loss.

SUBJECTS AND METHODS

Subjects

For this study, 51 obese women involved in a gastric surgery program were prospectively recruited between 2007 and 2009 in the Department of Nutrition, Center of Reference for Medical and Surgical Care of Obesity (CREMO; Pitié-Salpêtrière Hospital, Paris, France). Patients meeting the criteria for obesity surgery included those having a BMI (kg/m^2) $\geq$40 or $\geq$35 with at least one comorbidity (hypertension, type 2 diabetes, dyslipidemia, or obstructive sleep apnea syndrome). The preoperative evaluation of patients included a detailed medical history and physical, nutritional, metabolic, cardiopulmonary, and psychological assessments. The weight of the subjects included had been stable (variation of less than $\pm$ 2 kg) for $\geq$3 mo before surgery. Subjects did not show evidence of acute or chronic inflammatory disease, infectious diseases, cancer, and/or known alcohol consumption (>20 g/d) at any time point. Patients displaying surgical complications during the first year after surgery were subsequently excluded. Clinical and biological variables were assessed before RYGB (baseline) and 3, 6, 12, and 24 mo after surgery. Eighteen subjects were classified as having type 2 diabetes, defined as a fasting glucose concentration >7 mmol/L or the use of an antidiabetic drug. These 18 subjects (Ob/D group) were treated with metformin and hypolipemic drugs (either fibrates or statins). Two subjects were additionally treated with insulin. An oral-glucose-tolerance test was performed before RYGB and confirmed that all patients in the nondiabetic obese group had glucose concentrations <11 mmol/L (200 mg/dL) in the 2 h after a 75-g oral glucose challenge. One year after surgery, 11 of the 18 Ob/D individuals were reclassified as having normoglycemia (glycated hemoglobin <6.5%), which permitted them to cease antidiabetic treatment. Body fat mass was determined by dual-energy X-ray absorptiometry (DXA; GE Lunar Prodigy Corporation, Madison, WI) in obese and Ob/D subjects. Three obese individuals (n = 2 obese; n = 1 Ob/D), who had preoperative weight exceeding the limit of the analyzer (160 kg) or did not fit entirely within the DXA field-of-view were found in our population. An additional 14 normal-weight, nondiabetic, and healthy female volunteers living in the same area as the obese subjects were recruited as the control group (C group). The study was conducted in accordance with the Helsinki Declaration and was registered in a public trials registry. The Ethics Committee (CPP Ile-de-France 1) approved the clinical investigations for both obese and nonobese individuals. All subjects provided written informed consent.

Dietary and nutritional assessment

The estimated calorie intake (kcal/d) and the amounts of macronutrients (g/d) were recorded by a registered dietitian during the first year after RYGB. Multivitamins and iron supplements were provided to avoid deficiencies, which is a well-known secondary effect of bariatric surgery (15). Serum iron, ferritin, the coefficient of saturation of iron in transferrin, vitamins (A, D, E, thiamine, B-12, and B-9), micronutrients (selenium and zinc), and calcium were measured by using routine bioclinical tests. Serum measurements of these aforementioned variables showed that they conformed to the normal range at all time points in all subjects (data not shown).

Metabolic and inflammatory variables

Venous blood samples were collected in the fasting state at each time point for routine measurement of the biochemical variables outlined elsewhere (13). QUICKI (Quantitative Insulin Sensitivity Check Index) was determined by a mathematical transformation of fasting blood glucose and insulin measurements (16). Serum samples were stored at −80°C for later assessment of other biological components, including leptin, adiponectin, acute phase response markers measured in routine evaluations in our clinical department [high-sensitivity (hs)-CRP, IL-6], and inflammatory-related factors. Serum leptin and adiponectin were determined by using a radioimmunoassay kit from Linco Research (Saint Louis, MO) following the manufacturer's recommendations. The sensitivity was 0.5 ng/mL for leptin and 0.8 μg/mL for adiponectin. Intraassay and interassay CVs were <4 and <9% for leptin and adiponectin, respectively. Serum concentrations of IL-6 were measured by a high-sensitivity enzyme-linked immunosorbent assay system (Quantikine HS; R&D System Europe Ltd, United Kingdom). The sensitivity of this assay was <0.04 pg/mL, with intraassay and interassay CVs <8% for IL-6. hs-CRP was quantified by using an IMMAGE automatic immunoassay system (Beckman-Coulter, Fullerton, CA). The sensitivity was 0.02 mg/dL. The intraassay and interassay CVs were <5% and <7.5%, respectively.

Multiplexed assays of inflammation-related factors

A cytometric bead array (Human Chemokine Kit; BD Biosciences, Franklin Lakes, NJ) was used to assess serum concentrations of monocyte chemoattractant protein 1 (MCP-1), interferon-γ-inducible-protein 10 (IP-10), monokine induced by interferon-γ (MIG) and RANTES (regulated on activation, normal T cell expressed and secreted) in 10 obese and 14 control subjects. We also used the Human Cytokine 27-Plex Panel kit from Bio-Rad (Hercules, CA) to measure serum concentrations of IL-1, IL-1-receptor antagonist (IL-1-Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, fibroblast growth factor (FGF), eotaxin, granulocyte colony-stimulating

factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ, IP-10, platelet-derived growth factor BB, MCP-1, MIP-1α, MIP-1β, tumor necrosis factor-α (TNF-α), RANTES, and vascular endothelial growth factor (VEGF). Multiplex assays were performed according to the manufacturer instructions. Multianalyte profiling was performed on the Luminex-200 system and the Xmap Platform (Luminex Corporation, Austin, TX). Fluorescence data were analyzed with Xponent software by using standard curves obtained from serial dilutions of standard cytokines mixtures. Dilutions of 1:4 were used for serum sample analysis. The chemokine RANTES was readily measurable with the cytometric bead array, but was found to be outside of the standard curve for the 27-Plex assay. In contrast, the same ranges of concentrations were measured for both IP-10 and MCP-1 in both assays (data not shown). IL-8, IL-9, and IL-10 were readily detectable with the 27-Plex kit before surgery, but fell below the detection threshold for at least one point after surgery. This led us to exclude these data in the subsequent statistical analysis, which thereby reduces the sample size for this list of factors, as indicated in the tables.

Statistical analysis

The normal distribution of data was tested by using the Shapiro-Wilk test. Data were log transformed when required. All analyses were adjusted for age. Comparisons between groups were performed by analysis of variance (ANOVA). When the ANOVA procedure showed significant differences, Bonferroni correction was used for post hoc comparisons. Linear modeling allowed for the adjustment of comparisons for confounding variables. Relations between continuous variables were assessed by nonparametric Spearman rank correlation, with adjustment for age and diabetic status. To assess significant differences in postsurgery kinetics analyses, we used repeated-measures multivariate ANOVA for the obese group. Comparisons between preoperative baseline and postsurgery time points were obtained by using paired Student's t test, and P values were Bonferroni corrected. Relations between kinetic changes in inflammatory markers and clinical variables, including food intake (total calories and macronutrients), were tested by building linear mixed-effects models (LMEs) with subjects' identification as a random variable. The inflammation-related factors IL-8, IL-9, IL-10, and RANTES—having reduced sample sizes because of postoperative values below the detection threshold, were excluded for lack of power. All LME models were fit by maximizing the restricted log-likelihood of their estimated coefficients and were adjusted by age and diabetic status. LME modeling was performed by relying on functions available in the nlme package (17). Statistical analyses were performed by using R software (version 2.10.1, http://www.r-project.org) or JMP Start Statistics (version 7.0.1; SAS, Cary, NC). A difference was considered significant at $P \leq 0.05$.

RESULTS

Effect of obesity and type 2 diabetes on circulating inflammation-related factors

The obese and Ob/D women enrolled in the current study had anthropometric and metabolic abnormalities usually associated with severe obesity and type 2 diabetes (**Table 1**). Because the diabetic subjects were older than the obese and lean subjects, all statistical analyses were adjusted for age. We used multiplexed proteomics to investigate systemic inflammatory profiles in these subjects. In a first set of measures, the serum concentrations of 4 chemokines (IP-10, MCP-1, RANTES and MIG) were determined with a cytometric bead assay. These analyses were subsequently augmented with the Luminex 27-Plex assay, which measured 3 cytokines (IL-9, IL-1-Ra, and IL-10), 5 chemokines (IP-10, MIP-1β, MCP-1, IL-8, and eotaxin), and VEGF. Of these, IL-9, IL-1-Ra, IL-10, and IL-8 were below the detection threshold in the control women. After adjustment for age, the concentrations of all factors, except eotaxin, were found to be significantly higher in the obese than in the control women (Table 1). Concentrations of individual cytokines and chemokines were found to be highly variable, with MIP-1β having a range of $<$1.5-fold and IL-10 a range of $>$60-fold. IL-10 was found to be higher in the Ob/D group than in the obese and control groups, ie, a 2-fold increase over that in the obese subjects. Conversely, eotaxin was found to be elevated specifically in the Ob/D group compared with the obese group. Differences between the obese and Ob/D groups remained significant when adjusted for BMI (data not shown). These observations indicate that serum levels of a large panel of inflammation-related factors are increased in human obesity, with substantial variability and with additional effects of type 2 diabetes noted for some.

Kinetic evolution of bioclinical characteristics and inflammation-related factors after RYGB in obese subjects

As expected, RYGB resulted in a progressive and steady amelioration of BMI and fat mass and a reduction in fat-free mass, as expected (**Figure 1** and **Table 2**). Postoperative triglyceride and leptin concentrations gradually decreased, whereas adiponectin increased steadily. Circulating glucose and insulin decreased progressively with time, which indicated an amelioration of insulin sensitivity, as further evidenced by increased QUICKI values. Amelioration of glucose homeostasis was confirmed through a steady decrease of glycated hemoglobin. The reduction in total cholesterol was accompanied by an increased HDL-cholesterol concentration at 12 mo. These kinetic profiles were similar between the obese and Ob/D subjects (*see* Supplementary Figure 1 under "Supplemental data" in the online issue).

An analysis of total energy and macronutrients intakes showed a drastic caloric deduction 3 mo after surgery, with a major effect observed in carbohydrate intake (**Figure 2**, A and B). At 6 mo, energy and carbohydrate consumption rebounded to $\approx$70% of initial values and stabilized at 1 y (Table 2).

A similar biphasic profile of variation was observed for 8 cytokines in the serum. IL-9, IL-10, MIP-1β, MCP-1, IL-8, RANTES, eotaxin, and VEGF all sharply decreased by 3 mo, reaching $-$20% to $-$70% of the initial values before returning to near preoperative values at 6 mo (Figure 2C). In contrast, IP-10 and IL-1-Ra remained essentially stable over this period, and MIG concentrations steadily increased (Table 2 and Figure 2D). At 1 y, most of the measured cytokines remained stable or slightly below the baseline values (Table 2). Strikingly, these profiles did not parallel the decrease in the acute phase response protein hs-CRP and the inflammatory cytokine IL-6 (Figure 1D). The variation profiles were similar between the obese and Ob/D subjects for all measured factors (*see* Supplementary Figure 2 under "Supplemental data" in the online issue).

TABLE 1
Bioclinical characteristics and serum factor concentrations in nonobese, obese, and obese/diabetic (Ob/D) women[1]

	Nonobese (n = 14)	Obese (n = 33)	Ob/D (n = 18)	Overall P value[2]
Age (y)	38.6 ± 2.3[3]	39.7 ± 1.9	47.9 ± 2.2*,§	0.010[4]
BMI (kg/m^2)	21.5 ± 0.3	48.2 ± 1.4*	52.8 ± 2.0*	<0.001
Fat mass (% of body weight)[5]	28.8 ± 0.9	49.7 ± 0.7*	46.1 ± 1.6*	<0.001
Energy intake (kcal/24 h)	ND	2123 ± 20.4	1952 ± 31.7	—
Glucose (mmol/L)	4.2 ± 0.1	5.5 ± 0.1*	9.1 ± 0.9*,§	<0.001
Insulin (μU/mL)	3.3 ± 0.2	15.2 ± 1.6*	23.1 ± 3.0*,§	<0.001
QUICKI	0.420 ± 0.005	0.323 ± 0.004*	0.290 ± 0.007*,§	<0.001
Glycated hemoglobin (%)	ND	5.9 ± 0.1	7.9 ± 0.1½	—
Total cholesterol (mmol/L)	4.3 ± 0.20	5.2 ± 0.12*	5.1 ± 0.21*	0.005
HDL cholesterol (mmol/L)	1.5 ± 0.09	1.4 ± 0.06*	1.2 ± 0.08*	0.030
Triglycerides (mmol/L)	0.75 ± 0.09	1.4 ± 0.11*	2.3 ± 0.33*,§	<0.001
Leptin (ng/mL)	8.1 ± 0.9	70.6 ± 4.4*	59.8 ± 5.3*	<0.001
Adiponectin (μg/mL)	14.8 ± 2.6	6.6 ± 0.8*	6.7 ± 1.1*	<0.001
hs-CRP (mg/dL)	0.34 ± 0.08	0.85 ± 0.11*	1.0 ± 0.16*	<0.001
IL-6 (pg/mL)	3.0 ± 0.3	3.9 ± 0.5	4.9 ± 0.7*,§	0.050
Cytokines (pg/mL)				
IL-9[6]	< 2.5[7]	22.1 ± 11.2	17.5 ± 3.1	—
IL-1-Ra	< 5.5[7]	140.0 ± 19.4	179.2 ± 43.1	—
IL-10[6]	< 0.3[7]	20.5 ± 4.4	44.1 ± 6.4§	—
Chemokines (pg/mL)				
IP-10	328 ± 33	1077 ± 104*	1278 ± 213*	<0.001
MIP-1β	81.4 ± 11.6	108.6 ± 8.5*	138.3 ± 18.1*	0.010
MCP-1	43.4 ± 2.9	77.1 ± 10.3*	110.0 ± 19.9*	0.050
IL-8[6]	<1.0[7]	6.7 ± 1.0	10.1 ± 2.2	—
Eotaxin	64.3 ± 8.4	63.4 ± 8.5	139.3 ± 34.4*,§	0.030
RANTES[8]	2103 ± 105	16,795 ± 257*	ND	—
MIG[8]	325 ± 10.7	745 ± 39.4*	ND	—
Other factor (pg/mL)				
VEGF	69.8 ± 13.9	193.6 ± 28.6*	359.2 ± 64.5*	<0.001

[1] IL, interleukin; IL-1-Ra, IL-1-receptor antagonist; hs-CRP, high-sensitivity C-reactive protein; IP-10, interferon-γ–inducible protein-10; MIP-1β, macrophage inflammatory protein 1β; MCP-1, monocyte chemoattractant protein 1; RANTES, regulated upon activation, normal T cell expressed and secreted; MIG, monokine induced by interferon-γ; VEGF, vascular endothelial growth factor; QUICKI, Quantitative Insulin Sensitivity Check Index; ND, not determined. *Significantly different from nonobese, $P < 0.050$. §Significantly different from obese, $P < 0.050$.

[2] Obtained by one-factor ANOVA on log-transformed data adjusted for age. When the ANOVA showed a significant difference, Bonferroni correction was used for post hoc comparisons.

[3] Mean ± SEM (all such values).

[4] Nonadjusted.

[5] Data missing for 2 obese subjects and 1 Ob/D subject.

[6] Data available for 14 nonobese, 10 obese, and 10 Ob/D subjects.

[7] Below detection threshold indicated.

[8] Data available for 14 nonobese and 10 obese subjects.

Because we observed that changes in energy intake had a biphasic profile similar and concomitant to that of many serum factors measured in this study, we sought to test this relation using an LME model adjusted for age and diabetic status, with caloric intake and BMI at 0, 3, and 6 mo set as fixed effects. Significant associations between variations in energy intake and serum concentrations were detected for MIP-1β ($P = 0.002$), MCP-1 ($P = 0.002$), and VEGF ($P = 0.027$), but not for eotaxin ($P = 0.113$), IL-1-Ra ($P = 0.444$), or IP-10 ($P = 0.250$). Similarly, variations in carbohydrate intake were significantly associated with serum concentrations of MIP-1β ($P = 0.014$), MCP-1 ($P < 0.001$), VEGF ($P = 0.024$), and eotaxin ($P = 0.045$), but not with IL-1-Ra ($P = 0.526$) or IP-10 ($P = 0.128$). In contrast, variations in lipid intake were only associated with changes in MIP-1β concentrations ($P = 0.021$). No significant associations with protein consumption were found. This suggests that a decrease in carbohydrate consumption prominently influences the serum concentrations of a subset of inflammatory factors during the first 3 mo after RYGB.

A long-term assessment of metabolic and serum factors was performed in 19 obese subjects included in the initial population and followed up to 2 y after RYGB. As expected, this group of subjects had an amelioration of corpulence, metabolic status, and systemic inflammation, as evidenced by BMI, QUICKI, and hs-CRP values (**Figure 3**). This was paralleled by a reduction in IL-1-Ra, MCP-1 MIP-1β, VEGF, and eotaxin 2 y after surgery, whereas IP-10 remained unchanged.

DISCUSSION

Little previous information is available about the spectrum of circulating inflammatory factors modified by obesity and the

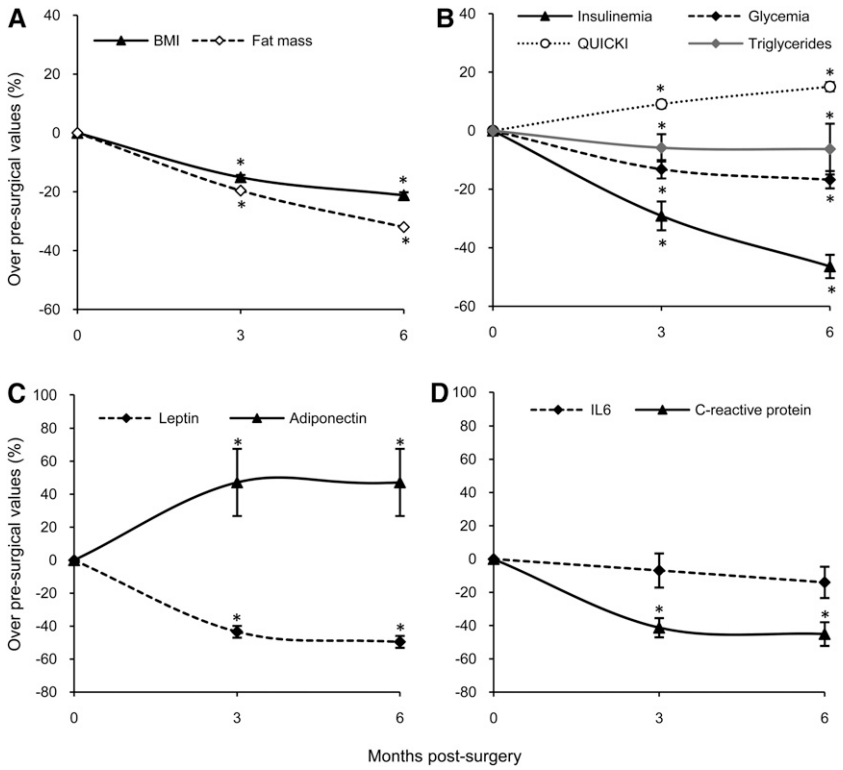

FIGURE 1. Early kinetic variations in anthropometric (A) and various glycemic (B) and inflammation-related (C and D) bioclinical variables in 51 obese women 3 and 6 mo after Roux-en-Y gastric bypass. Data are expressed as mean (±SEM) percentages over presurgical values. *Significantly different from preoperative values, $P < 0.05$ (Bonferroni corrected). QUICKI, Quantitative Insulin Sensitivity Check Index; IL6, interleukin-6.

potential improvement associated with weight reduction. In this study we sought to measure circulating concentrations of a large panel of inflammatory factors in a population of obese women during RYGB-induced weight loss. Contrary to our expectations, only 9 of 27 factors were readily measurable with the 27-Plex kit used. Of these, 3 cytokines (IL-9, IL-10, and IL-1-RA) and 1 chemokine (IL-8) were undetectable in the serum of normal-weight women. Except for eotaxin, all of the measured factors were found to be increased in obese individuals, although the amount of increase was strikingly different depending on the factor being measured. These results suggest that distinct sensitivity to regulatory factors or distinct regulatory mechanisms, which remain to be defined, contribute to determining the circulating concentrations of these inflammation-related proteins in obesity. Cytokines and chemokines are low-molecular-weight secreted proteins that can be categorized according to their pro- or antiinflammatory functions (18). Here, we found up-regulation of these factors in serum, regardless of their known proinflammatory (IL-8, IP-10, MIG, MCP-1, and RANTES) or antiinflammatory (IL-1-Ra and IL-10) role in human physiology. This suggests that obesity is associated with a general inflammatory response and is not restricted to proinflammatory factors.

As previously observed in populations of patients undergoing gastric surgery (13), 35% of the severely obese participants were categorized as having type 2 diabetes. In our study, only IL-10 was higher in the Ob/D women than in the obese women, and eotaxin was the sole factor to be exclusively increased in Ob/D. These data indicate that type 2 diabetes influences a limited number of inflammatory factors, in contrast with the more widespread effect of obesity. Previously, Herder et al (5, 19) reported a specific association of type 2 diabetes with serum concentration of RANTES and IL-8, but not with eotaxin, in a large population of normal and overweight men and women. Currently, we have no clear explanation for the discrepancy observed with eotaxin. It is possible, however, that specific factors related to sex and/or to the severity of the obese phenotypes in the 2 cohorts could have contributed to this difference in observations.

Several cytokines and chemokines that were elevated in our cohort of obese subjects have been implicated in the pathogenesis of inflammatory diseases, such as asthma, inflammatory processes in the vascular wall, and an increased risk of coronary artery disease and/or tumor development (20–25). Thus, it is tempting to speculate that elevated concentrations of these factors contribute to the high incidence of these diseases in obesity.

RYGB is a well-established procedure used to reduce body fat mass and ameliorate the metabolic status of severely obese subjects. The fat mass and fat-free mass losses observed in our study are consistent with those in previous studies (26, 27). The effects of surgery- or diet-induced weight-loss on circulating inflammation-related factors were previously reported in separate studies. Gastric surgery is known to reduce serum VEGF (28) and MCP-1 concentrations (29, 30). Serum eotaxin decreased after diet-induced weight reduction (31), whereas other factors, such as IL-10 (32) or IL-8 (33), were found to be less affected. In one study, changes in the serum concentrations of MCP-1, IL-1-Ra, and IP-10 were related to the amount of weight loss, whereas others, including IL-10 and IL-8, showed no significant correlation (34). For the first time, our study showed an unexpected pattern of acute variation for a large panel of cytokines and other inflammatory factors at early time points after RYGB. This

TABLE 2
Change in bioclinical characteristics and serum factor concentrations in 51 obese women after Roux-en-Y gastric bypass[1]

	Preoperative (baseline)	3 mo	6 mo	12 mo	Overall *P* value[2]
BMI (kg/m^2)	49.8 ± 1.1	42.4 ± 1.1*	39.3 ± 1.1*	36.4 ± 1.7*	<0.001
Fat mass (% of body weight)[3]	48.3 ± 0.8	46.1 ± 0.7*	42.9 ± 0.8*	39.3 ± 0.9*	<0.001
Fat-free mass (%)[3]	49.5 ± 0.8	52.0 ± 0.7	55.1 ± 0.7	58.3 ± 0.9	<0.001
Energy intake (kcal/24 h)	2057 ± 84	1042 ± 42*	1336 ± 53*	1446 ± 53*	<0.001
Protein (g/24 h)	87.2 ± 3.5	47.4 ± 2.4*	59.4 ± 2.7*	63.5 ± 2.7*	<0.001
Lipids (g/24 h)	87.0 ± 5.7	40.5 ± 2.3*	50.5 ± 2.8*	54.7 ± 3.0*	<0.001
Carbohydrates (g/24 h)	231.9 ± 10.6	126.9 ± 6.7*	163.2 ± 7.7*	168.4 ± 7.5*	<0.001
Glucose (mmol/L)	6.7 ± 0.4	5.4 ± 0.2*	5.1 ± 0.2*	4.8 ± 0.1*	<0.001
Insulin (μU/mL)	18.1 ± 1.6	10.9 ± 0.8*	8.2 ± 0.6*	6.6 ± 0.5*	<0.001
QUICKI	0.312 ± 0.004	0.340 ± 0.004*	0.358 ± 0.005*	0.372 ± 0.005*	<0.001
Glycated hemoglobin (%)	7.0 ± 0.3	6.1 ± 0.1*	5.9 ± 0.1*	5.7 ± 0.1*	<0.001
Cholesterol (mmol/L)	5.1 ± 0.1	4.4 ± 0.1*	4.4 ± 0.1*	4.5 ± 0.1*	<0.001
HDL cholesterol (mmol/L)	1.4 ± 0.05	1.3 ± 0.05	1.4 ± 0.05	1.6 ± 0.05*	<0.001
Triglycerides (mmol/L)	1.7 ± 0.15	1.3 ± 0.07*	1.3 ± 0.08*	1.0 ± 0.06*	<0.001
Leptin (ng/mL)	66.9 ± 3.5	36.5 ± 2.3*	31.6 ± 2.1*	26.2 ± 2.0*	<0.001
Adiponectin (μg/mL)	6.7 ± 0.6	8.0 ± 0.7*	8.9 ± 0.8*	9.8 ± 1.0*	<0.001
hs-CRP (mg/dL)	0.90 ± 0.10	0.51 ± 0.07*	0.44 ± 0.06*	0.38 ± 0.12*	<0.001
IL-6 (pg/mL)	4.3 ± 0.4	3.9 ± 0.3	3.8 ± 0.4	2.4 ± 0.2*	0.010
Cytokines (pg/mL)					
IL-9[4]	19.9 ± 6.0	11.1 ± 3.1*	13.8 ± 2.1	13.5 ± 3.4	0.01
IL-1-Ra	141.2 ± 18.1	161.2 ± 25.8	153.5 ± 42.2	48.2 ± 7.2*	<0.001
IL-10[4]	31.4 ± 5.0	11.2 ± 3.5*	32.4 ± 6.4	22.1 ± 5.3	0.015
Chemokines (pg/mL)					
IP-10	1107 ± 89	1030 ± 86	1127 ± 208	942 ± 72	NS
MIP-1β	119.4 ± 9.3	72.5 ± 5.0*	115.1 ± 9.1	87.0 ± 7.8*	<0.001
MCP-1	90.9 ± 10.5	34.7 ± 3.3*	70.9 ± 7.0	53.0 ± 6.3*	<0.001
IL-8[4]	8.5 ± 1.4	5.5 ± 0.8*	8.6 ± 0.9	6.9 ± 1.1	0.026
Eotaxin	90.0 ± 15.4	42.1 ± 3.6*	94.8 ± 11.0	89.7 ± 10.2	<0.001
RANTES[5]	16,795 ± 257	6624 ± 257*	15,825 ± 191	ND	<0.001
MIG[5]	745 ± 39.4	856 ± 39.8*	1087 ± 56.0*	ND	0.05
Other factors (pg/mL)					
VEGF	294.8 ± 44.8	78.6 ± 23.6*	276.4 ± 46.0	192.3 ± 40.3*	<0.001

[1] All values are means ± SEMs. IL, interleukin; IL-1-Ra, IL-1-receptor antagonist; IP-10, interferon-γ–inducible protein 10; hs-CRP, high-sensitivity C-reactive protein; MIP-1β, macrophage inflammatory protein 1β; MCP-1, monocyte chemoattractant protein 1; RANTES, regulated upon activation, normal T cell expressed and secreted; MIG, monokine induced by interferon-γ; VEGF, vascular endothelial growth factor; QUICKI, Quantitative Insulin Sensitivity Check Index; ND, not determined. *Significantly different from baseline, $P < 0.050$.

[2] Obtained by using repeated-measures multivariate ANOVA. Comparisons between preoperative baseline and each time point after gastric surgery were obtained by paired Student's *t* test. *P* values were Bonferroni corrected.

[3] Data available for 20 subjects.

[4] Data available for 10 obese subjects.

[5] Data missing for 3 subjects.

pattern was characterized by drastic decreases in measured molecules shortly after surgery, followed by a rebound and eventual stabilization at later time points. Three factors—IL-1-Ra, MIG, and IP-10—did not follow this generalized pattern over the course of the study. Thus, not all of the cytokines measured in the same serum sample had similar kinetics, which ruled out an influence of sample treatment. We also ruled out an effect of anesthesia or surgery. Indeed, patients who had complications related to anesthesia and/or surgery (abscess, fistula, or infections) were excluded from the study. Furthermore hs-CRP, an established marker of acute inflammation, was verified and was lower in all patients 3 mo after surgery and at all of the postoperative follow-ups, which ruled out a potential bias related to seasonal- or surgery-related infection.

In the search for bioclinical characteristics associated with early changes in serum cytokines, we observed similar and concomitant drastic variations in energy intake. These parallel profiles were further supported by a multivariate analysis that showed significant associations between kinetic changes in energy intake and selected circulating cytokines. These results indicate that a reduced energy intake is a regulatory factor influencing the systemic inflammatory profile of obese subjects during the first 3 mo after surgery. Furthermore, we highlighted here the influence of macronutrients, with associations between variations in carbohydrate intake and changes in the concentration of a distinct subset of measured cytokines. In clinical practice, it is well known that food intake is substantially modified after RYGB. In the first year, food habits are characterized by a lower consumption of sugars that are sometimes responsible for Dumping Syndrome. Compared with carbohydrate intake, lipid and protein intakes do not appear to have a major influence on serum cytokines concentrations. However,

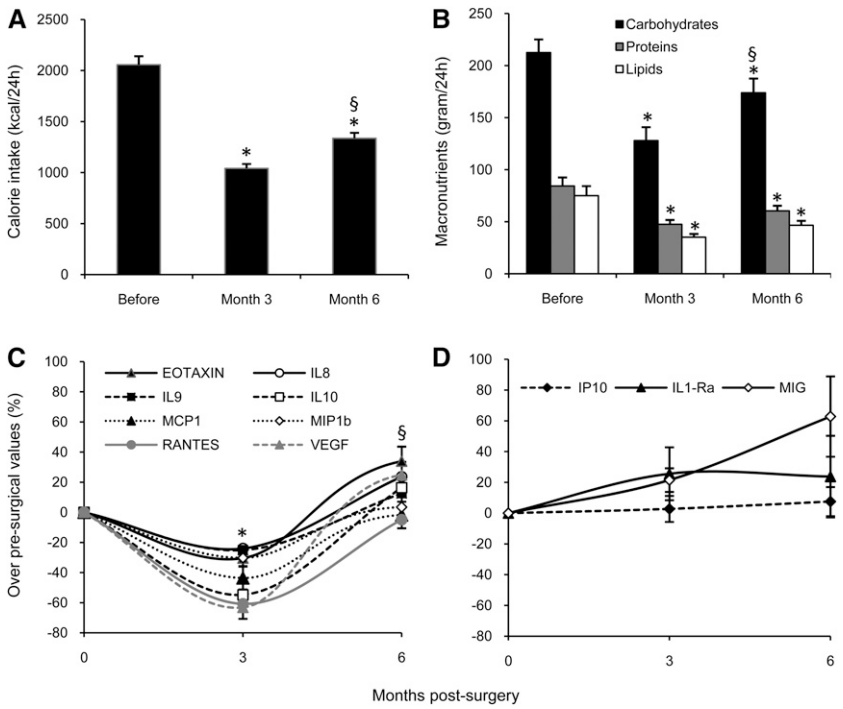

FIGURE 2. Early kinetic variations in calorie (A) and macronutrient (B) intakes and in circulating inflammation-related factors (C and D) in 51 obese women 3 and 6 mo after Roux-en-Y gastric bypass. Data are expressed as means ± SEMs. *Significantly different from preoperative values, $P < 0.05$ (Bonferroni corrected). §Significantly different from 3 mo, $P < 0.05$ (Bonferroni corrected). IL, interleukin; IL1-Ra, IL-1-receptor antagonist; IP10, interferon-γ–inducible protein 10; MIP1b, macrophage inflammatory protein 1β; MCP1, monocyte chemoattractant protein 1; RANTES, regulated upon activation, normal T cell expressed and secreted; MIG, macrophage-induced gene; VEGF, vascular endothelial growth factor.

our study on dietary intake was incomplete and requires further research to evaluate the role of different categories of amino acids and/or fatty acids. Indeed, it is suspected that amino acid intake is liable to impair immune function (35). Finally, modifications of polyunsaturated and unsaturated fats or ratios of ω-3 (n−3) to ω-6 (n−6) essential fatty acids were proved to directly participate in systemic and adipose tissue inflammation (36–38).

The long-term assessment of inflammation-related factors showed steady decreases in virtually all of the cytokines, except IP-10, which remained stable during the entire period of weight loss. BMI reduction and amelioration of metabolic status accompanied the overall reduction in cytokines, which suggests that beneficial effects of reduced corpulence might predominate in regulating long-term circulating cytokine concentrations.

An important outstanding question concerns the cell origin of circulating inflammation-related factors in obesity. It has been established that adipose tissue in obese subjects is infiltrated by immune cells (4) able to produce cytokines. Increased secretion of several cytokines and chemokines, such as MCP-1 (39), IL-10, RANTES, or IL-8 (40), by human adipose tissue has been reported in obesity. We and others have shown that decreased proinflammatory gene expression in adipose tissue is significantly associated with weight loss (12, 41, 42). Whether adipose tissue secretion contributes to the acute variations observed in this study with obesity and during weight loss needs to be further clarified. Circulating cytokines are also produced by blood cells. It has been shown that, in obese subjects, peripheral mononuclear cells are characterized by an inflammatory activation (6, 8) that also improves with weight loss (7, 43). In parallel, the induction of reactive oxygen species and the activation of endoplasmic reticulum stress occur and decrease after surgery-induced weight reduction (44). It is therefore possible that, in addition to adipocytes, circulating leukocytes and/or endothelial cells participate in the regulation of circulating inflammatory factors with obesity and weight reduction.

In conclusion, this study provides new insights into the overall inflammatory profile in obese subjects and its evolution during weight loss. We highlight coordinated changes in many serum factors, including several cytokines and chemokines, which are elevated in obese subjects and decrease with energy restriction. Our data emphasize that changes in a patient's nutritional status is an important contributor to systemic inflammation during the early phase of rapid and drastic weight loss, whereas reduced corpulence might prevail in regulating circulating cytokine concentrations at later time points. Additional studies are required to identify the molecular and cellular actors involved in the acute down-regulation of systemic cytokines that occurs shortly after RYGB-induced weight loss. Indeed, this study strongly suggests that distinct mechanisms might be involved in the amelioration of systemic inflammatory response at early and late time points during the course of weight reduction in obese subjects.

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The authors' responsibilities were as follows—CP, KC, and J-LN: designed the research; ED, C Rouault, C Rovere, J-LB, and CP: conducted the research;

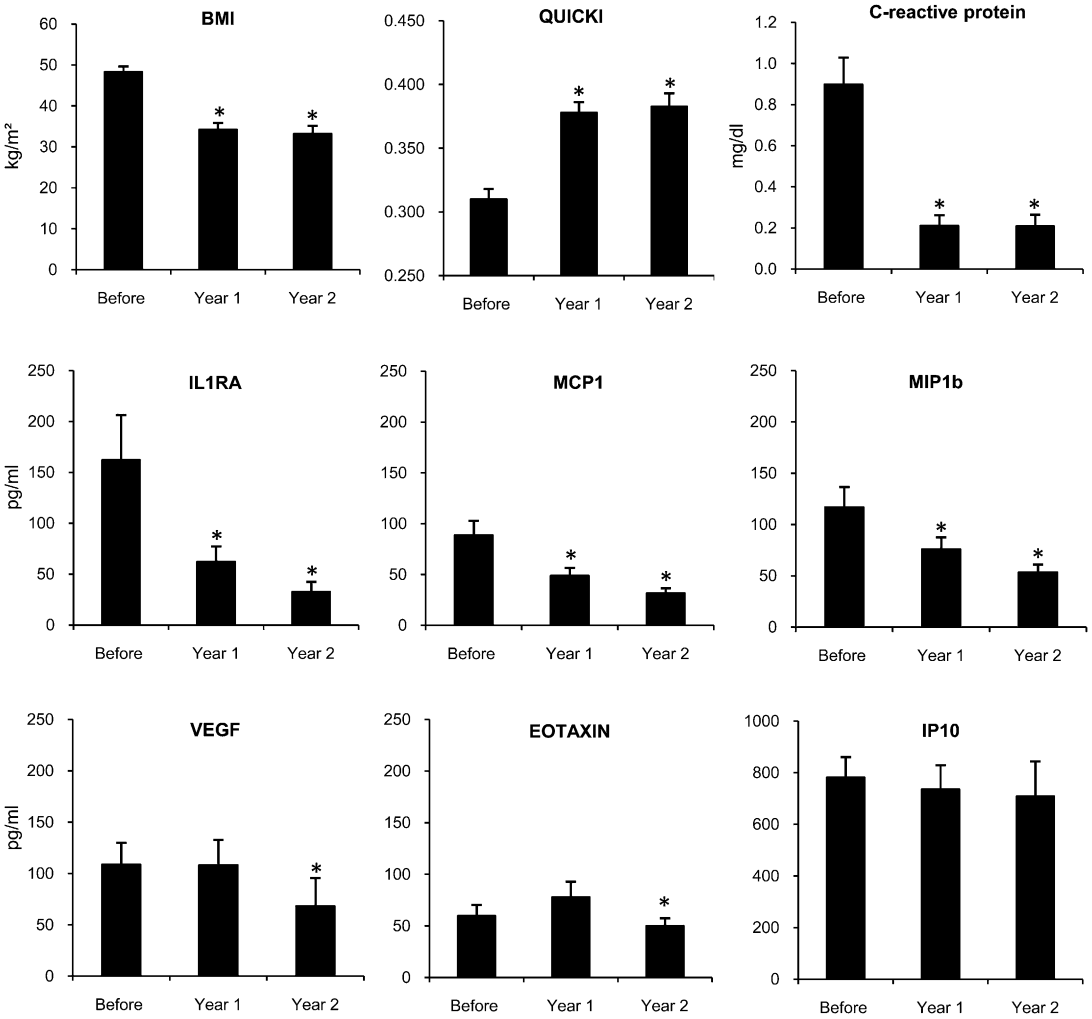

FIGURE 3. Late kinetic variations in bioclinical variables and circulating inflammation-related factors in 18 obese women. Data are expressed as means ± SEMs. *Significantly different from preoperative values, $P < 0.05$. IL1RA, interleukin-1-receptor antagonist; IP10, interferon-γ–inducible protein 10; MIP1b, macrophage inflammatory protein 1β; MCP1, monocyte chemoattractant protein 1; VEGF, vascular endothelial growth factor; QUICKI, Quantitative Insulin Sensitivity Check Index.

SR: provided the control cohort; CP, MA, ED, and AB-H: analyzed the data; CP, ED, MG-M, and KC: wrote the manuscript; and CP: had primary responsibility for the final content. All authors read and approved the final manuscript. None of the authors declared a conflict of interest.

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The American Journal of Clinical Nutrition

2. Article #2: $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes in obesity and during weight loss : relationships with fat mass and subclinal atherosclerosis

Monocytes display heterogenous phenotypes based on their expression of two membranous markers, CD14 and CD16. The following table summarizes the different monocyte subgroups existing in mice and human and some protypical features of each subset.

Subset	Markers	Chemokine receptors	Functions
Mouse			
$LY6C^{hi}$	$CD11b^{+}CD115^{+}LY6C^{hi}$	$CCR2^{hi}CX_3CR1^{low}$	Pro-inflammatory[7] and antimicrobial[3] roles
$LY6C^{low}$	$CD11b^{+}CD115^{+}LY6C^{low}$	$CX_3CR1^{hi}CCR2^{low}$	Patrolling[11]; early responses[11]; tissue repair[128]
Human			
Classical	$CD14^{++}CD16^{-}$	$CCR2^{hi}CX_3CR1^{low}$	Resemble $LY6C^{hi}$ monocytes based on gene-expression arrays[7,17,140]
Intermediate	$CD14^{++}CD16^{+}$	$CX_3CR1^{hi}CCR2^{low}$	Pro-inflammatory roles[12,15]
Non-classical	$CD14^{+}CD16^{++}$	$CX_3CR1^{hi}CCR2^{low}$	Patrolling[14]; antiviral roles[14]

CCR2, CC-chemokine receptor 2; CX_3CR1, CX_3C-chemokine receptor 1.

Table 4 | Mouse and human monocyte subsets (Shi and Pamer, 2011). In human, $CD14^{+}CD16^{++}$ cells are usually termed $CD14^{dim}CD16^{+}$ cells.

The two $CD16^{+}$ subpopulations were shown to be associated with different metabolic disorders, including obesity and cardiovascular events. In this study, we hypothesised that $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocyte subsets may be modulated in obesity and during weight loss. Also, we analysed whether monocyte populations coud be associated with obesity-induced complications including T2D and subclinical atherosclerosis as assessed by carotid intima media thickness (IMT). IMT is a measurement of the thickness of the artery walls, performed by ultrasonography, to detect the presence and to follow up progression of atherosclerotic plaques as illustrated in figure below.

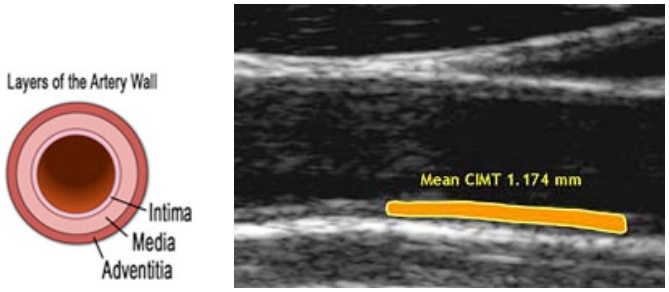

Figure 17 | Measure of carotid intima-media thickness
(http://www.medscape.org/viewarticle/561026)

The carotid arteries provide a “window” to the coronary arteries and carotid IMT is regarded as a marker of preclinical atherosclerosis and its following risks. Carotid IMT is known to be increased with obesity and T2D (Bots et al., 2007).

In this article, we showed that both $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocyte subsets were increased in obesity compared to lean subjects, with a significant enrichment of $CD14^{dim}CD16^{+}$ in T2D obese patients. Surgery-induced weight loss ensued a significant decrease in $CD16^{+}$ monocyte proportions that was greater for the $CD14^{dim}CD16^{+}$ subset. While decrease in $CD14^{dim}CD16^{+}$ number was associated with Hb1ac improvement, $CD14^{+}CD16^{+}$ decrease was significantly correlated to carotid IMT variations.

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$CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ Monocytes in Obesity and During Weight Loss : Relationships With Fat Mass and Subclinical Atherosclerosis

Christine Poitou, Elise Dalmas, Mariana Renovato, Vanessa Benhamo, Froogh Hajduch, Meriem Abdennour, Jean-François Kahn, Nicolas Veyrie, Salwa Rizkalla, Wolf-Hervé Fridman, Catherine Sautès-Fridman, Karine Clément and Isabelle Cremer

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$CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ Monocytes in Obesity and During Weight Loss

Relationships With Fat Mass and Subclinical Atherosclerosis

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Objective—Studies suggest the implication of $CD16^{+}$ subpopulations ($CD14^{+}CD16^{+}$, $CD14^{dim}CD16^{+}$) in inflammatory diseases. We aimed to determine the frequency of these subpopulations during weight loss in obesity and diabetes, conditions associated with changes in systemic inflammation, and we tested the link with subclinical atherosclerosis.

Methods and Results—$CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ frequencies were measured by flow cytometry in lean subjects, obese subjects before and after a hypocaloric diet or gastric surgery, and obese diabetic subjects before and after gastric surgery. Both monocyte subsets were increased in obese subjects, with a significant enrichment of the $CD14^{dim}CD16^{+}$ subpopulation in obese diabetic patients. Multivariate analysis demonstrated a link between the percentages of $CD14^{dim}CD16^{+}$ monocytes and glycemia, independent of fat mass. Drastic weight loss led to a sharp decrease of this subset, the variations of which were strongly related to fat mass changes. A reduction of at least 5% of fat mass was sufficient to observe a significant decrease of $CD14^{dim}CD16^{+}$ monocytes. A diminution of the $CD14^{+}CD16^{+}$ subset was also observed during weight loss and was associated with a decrease in intima-media thickness.

Conclusion—This work demonstrates a major impact of fat mass variations on $CD14^{dim}CD16^{+}$ monocyte subsets and that the decrease in the $CD14^{+}CD16^{+}$ subpopulation is linked to a reduction of subclinical atherosclerosis.

Clinical Trial Registration—URL: http://clinicaltrials.gov. Unique identifier: NCT00476658.

(*Arterioscler Thromb Vasc Biol*. 2011;31:2322-2330.)

Key Words: atherosclerosis ■ blood cells ■ diabetes mellitus ■ nutrition ■ obesity

Human obesity is associated with the development of cardiometabolic diseases, such as insulin resistance, dyslipidemia, diabetes, and cardiovascular injury. Obesity is characterized by the modulation of innate immunity. Obese subjects have increased systemic levels of inflammatory markers, such as acute-phase proteins (C-reactive protein [CRP] and serum amyloid A), cytokines, and interleukins.[1] Monocytes have a pivotal role in innate immunity, including phagocytosis, the secretion of inflammatory cytokines, and the production of reactive oxygen species, nitric oxide, and myeloperoxidase. Monocytes are involved in atherogenesis development[2] and metabolic regulation.[3] The monocyte count is positively associated with body mass index (BMI) and triglycerides (TG) and is negatively related to high-density lipoprotein cholesterol.[4] Furthermore, clinical studies report that the monocyte count is also associated with subclinical peripheral atherosclerosis[5–7] and the development of cardiovascular disease.[4] Overall, it is proposed that the mononuclear phagocyte system contributes to the pathophysiology of cardiometabolic diseases (review in[8]).

Monocytes display heterogeneous phenotypes characterized by different levels of expression of FcγIII receptors CD16 and CD14.[9] Classically, the 2 main subpopulations are usually described according to CD16 expression: $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$.[10] These cells display different chemokine-receptor expression profiles, potentially reflecting distinct recruitment properties. The $CD14^{+}CD16^{-}$ monocytes express a high level of chemokine (C-C motif) receptor (CCR)-2 (chemokine ligand-2 receptor) and low levels of CCR5 (chemokine ligand-3 receptor) and CX3

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chemokine receptor-1 (the fractalkine receptor). On the contrary, the $CD16^+$ subpopulation is CCR2 negative but expresses high levels of CX3 chemokine receptor-1 and CCR5 receptors.[11] These $CD16^+$ cells exhibit a macrophage-like phenotype with enhanced antigen-presenting capacities and higher endothelial affinity, and they are potent producers of proinflammatory cytokines. They express higher levels of tumor necrosis factor-α and major histocompatibility class II and lower levels of interleukin-10, as compared with $CD16^-$ monocytes. The $CD16^+$ monocyte population is increased in inflammatory situations, such as sepsis, rheumatoid arthritis, and infections.[11]

A significant increase in the $CD16^+$ subset has also been described in human chronic pathologies with low-grade inflammation components, such as in obesity[12,13] and related cardiovascular diseases.[14] $CD16^+$ monocyte frequency is related to intima-media thickness (IMT), a marker of subclinical atherosclerosis[15,16] in patients with chronic kidney disease characterized by high cardiovascular risk, and in healthy volunteers as well.[17] Based on these observations, it is considered that $CD16^+$ monocytes could be cellular players, mediating the pathophysiological relationships between metabolic and cardiovascular diseases.

The cell population of $CD16^+$ per se also exhibits phenotype heterogeneity, with 2 described subsets that express either low levels of CD14 ($CD14^{dim}CD16^+$) or high levels of CD14 ($CD14^+CD16^+$). These cell subsets display distinct phenotypic and functional properties.[18,19] Whether these monocyte subsets show different pathogenic roles in cardiometabolic diseases has not been clearly established. A recent study conducted in 622 healthy volunteers showed that the $CD14^{dim}CD16^+$ cell count was correlated with BMI.[13] The same team observed that in patients with chronic kidney diseases, $CD14^+CD16^+$ monocytes were independently associated with cardiovascular events.[17] Finally, Rothe et al showed that $CD14^{dim}CD16^+$ frequency correlated positively with atherogenic lipoproteins and negatively with high-density lipoprotein cholesterol, suggesting a role for this subpopulation in atherogenesis. This cellular population has not been deeply explored in the obesity context or, in particular, in weight loss, a situation well known to be associated with changes in systemic inflammation and in an increase in cardiovascular risk.[21,22]

In this study, we explored the hypothesis that both the $CD14^{dim}CD16^+$ and the $CD14^+CD16^+$ monocyte subsets could be modulated in obesity and during weight loss and could associate with metabolic phenotypes and subclinical atherosclerosis. Thus, we studied both $CD16^+$ subsets in different conditions of obesity and in response to different programs inducing fat mass variations, induced by either diet or surgery. In this latter condition, metabolic and inflammatory parameters and vascular phenotypes obtained in morbidly obese subjects were analyzed in parallel to $CD16^+$ monocyte subset frequencies. We demonstrate for the first time a relationship between changes in the $CD14^{dim}CD16^+$ subset and reduction of fat mass.

Materials and Methods

Subjects

Three groups of subjects were included in this study. The first population (the obese [OB] and obese diabetic [OB/D] groups) included 105 obese subjects involved in a gastric surgery program, prospectively recruited between 2008 and 2009 in the Department of Nutrition of Pitié-Salpêtrière Hospital (reference center for the medical and surgical care of obesity, Paris, France). Patients meeting the criteria for obesity surgery included those with a BMI $\geq$40 kg/m^2 or $\geq$35 kg/m^2 with at least 1 comorbidity (hypertension, type 2 diabetes, dyslipidemia, or obstructive sleep apnea syndrome). The preoperative evaluation included a detailed medical history and physical, nutritional, metabolic, cardiopulmonary, vascular, and psychological assessments. The weight of the included subjects had been stable (variation of less than $\pm$2 kg) for at least 3 months before surgery. Subjects did not demonstrate evidence of acute or chronic inflammatory disease, infectious diseases, cancer, or known alcohol consumption (>20 g per day). Patients displaying surgical complications during the first year after surgery were subsequently excluded. They did not take any antiinflammatory drugs. Thirty-eight subjects were classified as having type 2 diabetes by registering a fasting glycemia of greater than 7 mmol/L or by their use of an antidiabetic drug. These 38 subjects (the OB/D group) were treated with metformin and hypolipemic drugs (either fibrates or statins). Nine subjects were also treated with insulin. An oral glucose tolerance test was performed before Roux-en-Y gastric bypass (RYGB) and confirmed that all patients in the nondiabetic obese group (the OB group) had glucose levels of less than 11 mmol/L (200 mg/dL) in the 2 hours following a 75-g oral glucose challenge. Clinical and biological parameters and monocyte subpopulations were assessed before RYGB surgery (n=105) and 3 and 6 months (n=36) after surgery.

A second population (the Diet group) included 39 overweight and moderately obese subjects undergoing a weight loss program (1200 kcal daily over 6 weeks).[23] Clinical and biological parameters and monocyte subpopulations were assessed before diet intervention (n=39) and after 6 weeks of caloric restriction (n=20).

The third population (the control [C] group) included 32 lean, healthy, white volunteers living in the same area as the obese subjects.

Total body fat mass was determined by DXA scanning (GE Lunar Prodigy Corp, Madison, WI). The ethics committee of the Hôtel-Dieu Hospital approved the clinical investigations for both obese and lean individuals. All subjects gave written informed consent. The study was conducted in accordance with the Helsinki Declaration and was registered in the ClinicalTrials.gov registry.

Metabolic and Inflammatory Parameters

Venous blood samples collected in the fasting state were used to assess lipid, insulin, and glucose values (enabling the determination of insulin-sensitivity parameters) and many others factors, outlined in Poitou et al.[21] Homeostasis model assessment (HOMA) insulin resistance (IR) was determined using the HOMA Calculator version 2.2.2 (http://www.dtu.ox.ac.uk/).[24]

Carotid and Femoral Artery IMT Measurement

Carotid and femoral B-mode ultrasound imaging was performed using Sequoia 512 ultrasound mainframes (Acuson, Mountain View, CA). A 7-MHz linear array transducer was used for clearly displaying both the blood-intima and media-adventitia boundaries on the far wall of the arteries. The lumen of the arteries was maximized with gain settings to optimize the image quality.

The protocol for measuring carotid IMT (CIMT) consisted of scanning the right and left common carotid arteries longitudinally in the segment 5 to 20 mm proximal to the carotid bulb and on a site free of plaques. Similarly, measurements of femoral IMT (FIMT) were obtained from longitudinal scans of the right and left common femoral arteries in the segment 5 to 20 mm proximal to the bifurcation and on a site free of plaques. IMT measurements were performed offline on a personal computer, and automated edge-detection software (M'Ath, ICN-METRIS) was used to locate the lumen-intima and media-adventitia echographic boundaries. All scans and IMT measurements were performed by a single experienced physician trained in vascular ultrasound, and the intraobserver coefficient of variation for CIMT was <3%.

Table 1. Bioclinical Characteristics in the Different Cohorts: Lean Controls (C), Overweight and Moderately Obese Subjects (Diet), Obese (OB), and Obese Diabetic (OB/D).

	C	Diet	OB	OB/D	Overall *P* Value
No.	32	39	67	38	
Sex ratio, F/M	25/7	31/8	57/10	26/12	χ^2 NS
Age, y	$33.9\pm1.6^{B*}$	43.1 ± 1.9^{C}	37.6 ± 1.5^{B}	49.8 ± 1.8^{A}	$<10^{-4}$
Weight, kg	58.9 ± 1.3^{C}	90.1 ± 2.4^{B}	127.2 ± 2.4^{A}	135.0 ± 3.9^{A}	$<10^{-4}$
BMI, kg/m^2	21.5 ± 0.2^{C}	32.3 ± 0.6^{B}	46.4 ± 0.9^{A}	48.8 ± 1.3^{A}	$<10^{-4}$
Fat mass, kg	14.7 ± 1.3^{C}	33.2 ± 1.5^{B}	58.5 ± 1.7^{A}	61.3 ± 2.6^{A}	$<10^{-4}$
Fat mass, %	25.1 ± 1.9^{C}	38.1 ± 1.2^{B}	46.8 ± 0.6^{A}	46.3 ± 0.9^{A}	$<10^{-4}$
Glycemia, mmol/L	4.7 ± 0.1^{B}	5.1 ± 0.1^{B}	5.1 ± 0.06^{B}	8.1 ± 0.3^{A}	$<10^{-4}$
HbA1c, %	ND	ND	5.7 ± 0.04^{B}	7.5 ± 0.3^{A}	ND
Insulinemia, μU/mL	6.0 ± 0.9^{C}	9.6 ± 0.8^{C}	17.2 ± 1.5^{B}	24.8 ± 3.5^{A}	$<10^{-4}$
HOMA- IR, %	0.62 ± 0.08^{C}	1.08 ± 0.09^{C}	1.90 ± 0.16^{B}	2.95 ± 0.40^{A}	$<10^{-4}$
Total cholesterol, mmol/L	4.6 ± 0.2	5.3 ± 0.4	5.0 ± 0.1	4.7 ± 0.1	NS
Triglycerides, mmol/L	1.03 ± 0.10	$1.44\pm0.14^{B,C}$	1.41 ± 0.08^{B}	2.04 ± 0.15^{A}	$<10^{-4}$
HDL-c, mmol/L	1.30 ± 0.07^{B}	1.33 ± 0.07^{B}	1.18 ± 0.04^{A}	1.11 ± 0.07^{A}	0.05
Leptin, ng/mL	ND	43.1 ± 3.8	46.3 ± 2.1	39.8 ± 3.7	NS
Adiponectin, μg/mL	ND	13.7 ± 1.3^{B}	6.8 ± 0.5^{A}	5.8 ± 0.4^{A}	$<10^{-4}$
CRP, mg/L	2.3 ± 0.4^{B}	4.4 ± 0.7^{B}	10.4 ± 1.0^{A}	8.1 ± 0.9^{A}	$<10^{-4}$
IL6, pg/mL	ND	ND	3.46 ± 0.22	4.22 ± 0.74	NS

All values are expressed as mean±SEM. Data are expressed as mean±SEM. Overall *P* values were obtained by 1-way ANOVA on log-transformed data adjusted for age. When the ANOVA procedure revealed significant differences, Bonferroni multiple tests were used for post hoc comparisons. BMI indicates body mass index; CRP, C-reactive protein; F, female; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment insulin resistance; IL, interleukin; M, male; ND, not determined; NS, nonsignificant data.

*Data not sharing the same letter (A, B, or C) within a horizontal line are significantly different ($P<0.05$).

Peripheral Blood Mononuclear Cell Isolation and Flow Cytometry Analysis

Peripheral blood mononuclear cells were isolated from blood by centrifugation on a Ficoll/Hypaque gradient (PAA Laboratories), and were counted by trypan blue exclusion for each patient. Single-cell suspensions were analyzed by 3-color flow cytometry. Cells were incubated with fluorescein isothiocyanate–conjugated anti-CD14 (clone MOP9 from BD Biosciences), phycoerythrin-conjugated anti-CD16 (clone NKP15 from Becton Dickinson), and Alexa Fluor 647–conjugated anti-CCR2 (clone 48607 from Becton Dickinson) or isotypic controls for 20 minutes at 4°C and analyzed with an FACSCalibur cytometer (BD Biosciences). Flow cytometry data were analyzed using Cellquest Pro software (BD Biosciences). Monocyte cells were first gated according to their forward- and side-scatter profiles and then defined as $CD14^+$ cells.

Statistics

The normal distribution of the data were tested using the Shapiro-Wilk test. Data were log-transformed when required. Quantitative variables, including clinical and biological parameters as well as monocyte subset percentages, were expressed as mean±SEM values. All analyses were adjusted for age. ANOVA was used to assess the statistical significance of the differences in clinical and biological parameters, as well as in monocyte subsets between the different groups at baseline. When the ANOVA procedure revealed significant differences, Bonferroni multiple tests were used for post hoc comparisons. Relationships between percentages of monocyte subpopulations and continuous variables were assessed by nonparametric Spearman rank correlations. Multiple regression analyses were used to assess the independent associations and contributions of bioclinical variables (age, gender, BMI, fat mass, insulin, glycemia, HOMA-IR, TG, high-density lipoprotein cholesterol, leptin, adiponectin, and high-sensitivity CRP [hsCRP]) at baseline, with $CD14^{dim}CD16^+$ or $CD14^+CD16^+$ percentages as the dependent variables. Multivariate ANOVA was used to explore global variations of the analyzed parameters throughout the follow-up after surgery. Paired Wilcoxon rank sum tests were performed to analyze changes in clinical outcomes and biological marker levels among various time points after surgery. probability values were Bonferroni-corrected. The significance of the strongest dynamic associations involving clinical-biological parameters and $CD16^+$ monocyte subset frequencies was further evaluated by building linear mixed-effects (LME) models to test for intervariable redundancies and to adjust for potential confounding factors. All LME models were fit by maximizing the restricted log-likelihood of their estimated coefficients. LME modeling was performed by relying on functions available in the NLME package, with subjects' identification as the random variable. Age, gender, and diabetic status were systematically considered as confounding covariates in the LME models. Statistical analyses were performed using the R program (http: /www.r-project.org) or JMP Start Statistics (SAS, Cary, NC). A 2-sided significance level was fixed at 5%.

Results

$CD14^{dim}CD16^+$ and $CD14^+CD16^+$ Monocyte Distributions in Obesity and Diabetes

Table 1 presents the bioclinical characteristics of lean subjects (the C group, BMI range 17.6 to 23.8 kg/m^2), overweight and moderately obese subjects from the Diet group (BMI range 25.3 to 35.5 kg/m^2), obese subjects (the OB group) (BMI range 35.4 to 66.1 kg/m^2), and diabetic obese subjects (the OB/D group) (BMI range 35.3 to 68.6 kg/m^2) before RYGB. As expected, obese subjects in the Diet, OB, and OB/D groups showed higher fat mass; deterioration of metabolic parameters, such as glucose, insulin, and lipid

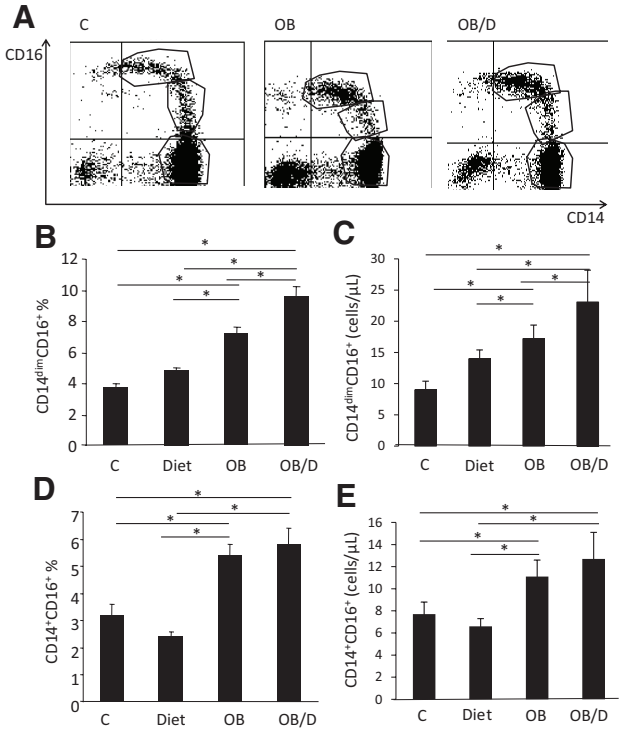

Figure 1. Differences in $CD16^+$ monocyte subsets in lean control subjects (C) and obese subjects in the diet (Diet) and surgery (obese [OB] and obese diabetic [OB/D]) groups. A, Representative staining patterns of the expression of CD14 and CD16 in the C, OB, and OB/D groups. B and D, $CD14^{dim}CD16^+$ and $CD14^+CD16^+$ monocyte subsets frequencies expressed as percentages of total $CD14^+$ cells. C and E, $CD14^{dim}CD16^+$ and $CD14^+CD16^+$ absolute monocyte subset counts. All values are expressed as mean±SEM. Comparison between groups was performed using a Wilcoxon rank sum test adjusted for age. The Tukey test was used for post hoc comparisons between groups. *$P<0.05$.

values; and changes in leptin and adiponectin concentrations. The hsCRP levels were significantly higher in obese subjects, in agreement with obesity-associated low-grade inflammation.

The absolute count for peripheral blood mononuclear cells was not different among the 4 groups ($2.94\times10^3\pm0.96$, $3.14\times10^3\pm1.20$, $2.96\times10^3\pm1.01$, and $3.22\times10^3\pm1.84$ cells/μL for the C, Diet, OB, and OB/D groups, respectively). The distribution of $CD14^{dim}CD16^+$ and $CD14^+CD16^+$ monocytes was determined by flow cytometry. We observed 2 $CD16^+$ monocyte subpopulations, according to CD14, CD16 and CCR2 level expression (Supplemental Figure IA, available online at http://atvb.ahajournals.org). We confirmed the presence of a first population characterized by a low expression of CD14, high expression of CD16, and no or low expression of CCR2 ($CD14^{dim}CD16^+CCR2$) and of a second one characterized by high expression of CD14 and CD16 and moderate expression of CCR2 ($CD14^+CD16^+CCR2+$).[19]

A representative staining pattern of the surface expression of CD14 and CD16 on monocytes from patients of the C, OB, and OB/D groups is shown in Figure 1A.

Having observed some differences in the mean age of the groups, we adjusted all analyses for age. The mean percentage of $CD14^{dim}CD16^+$ was significantly different among the 4 groups (Figure 1B, 3.7±0.3%, 4.8±0.2%, 7.2±0.4%, and 9.6±0.6% for the C, Diet, OB, and OB/D groups, respectively, $P<10^{-4}$). This cell population was 2-fold increased in the OB group compared with lean subjects with significant additional effects of diabetes. Similar findings were observed with absolute counts of $CD14^{dim}CD16^+$ (Figure 1C). The mean percentage of $CD14^+CD16^+$ was higher in the OB and OB/D groups, compared with the Diet and C groups, but without additional effects of diabetes (Figure 1D; 3.2±0.4%, 2.4±0.2%, 5.4±0.4% and 5.8±0.6% for the C, Diet, OB, and OB/D groups, respectively, $P<10^{-4}$). Similar findings were observed with absolute counts of $CD14^{dim}CD16^+$ (Figure 1E). Conversely, the mean of $CD14^+CD16^-$ subset was significantly different among the 4 groups, with a decrease of $CD14^+CD16^-$ frequencies in the obese subjects (93.1±0.6%, 92.8±0.4%, 87.4±0.6%, and 85.0±1.1%, for the C, Diet, OB, and OB/D groups, respectively, $P<10^{-4}$). Similar findings were observed with absolute counts of $CD14^+CD16^-$. Obesity is thus associated with an increase in both $CD16^+$ monocyte subsets, with a selective and significant enrichment of the $CD14^{dim}CD16^+$ subset in obese patients with type 2 diabetes.

Table 2. Correlations Between $CD16^+$ Monocyte Subpopulations and Bioclinical Data in the Whole Cohort

	$CD14^{dim}CD16^+$		$CD14^+CD16^+$	
	r	*P* Value	*r*	*P* Value
Age	0.12	0.02	NS	NS
BMI	0.52	$<10^{-4}$	0.37	$<10^{-4}$
Fat Mass (kg)	0.47	$<10^{-4}$	0.44	$<10^{-4}$
Fat Mass (%)	0.35	$<10^{-4}$	0.35	$<10^{-4}$
Glycemia	0.42	$<10^{-4}$	0.20	0.01
HbA1c*	0.32	8.10^{-4}	0.18	0.05
Insulin	0.45	$<10^{-4}$	0.24	4.10^{-3}
HOMA IR	0.46	$<10^{-4}$	0.24	4.10^{-3}
Triglycerides	0.23	2.10^{-3}	NS	NS
HDL-c	−0.19	0.03	NS	NS
Adiponectin	−0.33	2.10^{-4}	−0.35	$<10^{-4}$
CRP	0.30	5.10^{-4}	0.18	0.04

n=166 subjects. Data show Spearman correlation coefficient. BMI indicates body mass index; CRP, C-reactive protein HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment insulin resistance; NS, nonsignificant data.

*HbA1c was only measured in the obese and obese diabetic groups.

$CD16^+$ Monocyte Subsets and Obesity-Associated Phenotypes

To evaluate the clinical relevance of increased $CD16^+$ monocytes, we searched for associations between the $CD14^{dim}CD16^+$ and $CD14^+CD16^+$ subsets and phenotypes related to corpulence and metabolic status in 166 subjects (the C, Diet, OB, and OB/D groups). We found strong associations between $CD14^{dim}CD16^+$ and $CD14^+CD16^+$ subpopulations and parameters of corpulence (BMI, fat mass), glucose tolerance and insulin sensitivity (glycemia, insulin, HOMA-IR), and inflammation markers, such as hsCRP (Table 2). In the Diet, OB, and OB/D groups,

in which adiponectin was measured, high percentages of $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ subpopulations were associated with lower adiponectin concentrations. In the OB and OB/D groups, in which glycosylated hemoglobin (HbA1c) was systematically determined, a positive association was found with monocyte subset frequencies. Furthermore, the percentage of $CD14^{dim}CD16^{+}$ monocytes was positively correlated with age and TG and negatively with high-density lipoprotein cholesterol (Table 2), unlike the $CD14^{+}CD16^{+}$ subset.

In multivariate analysis, taking into account age, gender, BMI, fat mass (kg), HOMA-IR, TG, and glycemia, only BMI (or fat mass) and fasting glycemia were independently associated with the percentage of $CD14^{dim}CD16^{+}$ monocytes (β=0.11, $P<10^{-4}$, and β=0.67, $P<10^{-4}$, respectively). On the other hand, the relationships among $CD14^{+}CD16^{+}$ percentage and other quantitative variables (Table 2) were not independent of BMI (or fat mass) because BMI was the only significant regressor determined from multivariate regression analysis (β=0.2, $P=8.10^{-4}$).

These results strongly suggest that the $CD14^{dim}CD16^{+}$ population is linked to glycemia modifications, an observation not made with the $CD14^{+}CD16^{+}$ population, which is mainly linked with corpulence parameters. We further examined the changes of these monocyte subsets in nutritional situations known to modulate fat mass and related changes in inflammatory and metabolic parameters differently.

$CD16^{+}$ Monocytes Subsets and Diet-Induced Weight Changes

The dietary intervention consisted of a hypocaloric diet over 6 weeks, leading to a mean decrease of 5.9±0.6% in body weight and BMI (Supplemental Table I). As anticipated, caloric restriction led to a decrease in fat mass (−8.3%±1.3) and improvement of metabolic parameters, such as circulating insulin, HOMA-IR (−37.4%±3.8), and lipid profile, whereas it had no significant impact on inflammatory parameters (hsCRP and interleukin-6).

Despite significant improvements in fat mass and insulin sensitivity, the dietary challenge was not accompanied by a significant modification of $CD16^{+}$ monocyte populations. Indeed, $CD14^{dim}CD16^{+}$ percentages were 4.3±0.3% and 3.9±0.3%, and $CD14^{+}CD16^{+}$ percentages were 2.3±0.2% and 2.5±0.3% at baseline and after the dietary intervention, respectively. Similar findings were observed with absolute counts of $CD14^{dim}CD16^{+}$ (14.7±2.9 versus 13.4±1.4 cells/μL) and $CD14^{+}CD16^{+}$ (6.8±0.8 versus 7.8±2.7 cells/μL). However, in 11 of 20 patients, a significant decrease in $CD14^{dim}CD16^{+}$ monocytes could be observed after the diet compared with baseline. To explore the relationships between the kinetic variations of $CD14^{dim}CD16^{+}$ monocytes and changes in bioclinical markers, we performed an LME model (n=20). In a multivariate analysis associating age, BMI or fat mass (kg), TG, and glycemia as fixed effects in a combined LME model, we found a positive relationship between the changes in $CD14^{dim}CD16^{+}$ frequencies and the variations in fat mass (P=0.04).

$CD16^{+}$ Monocyte Subsets and RYGB-Induced Weight Loss

We further examined the kinetic evolution of $CD16^{+}$ monocyte subsets in 36 obese subjects before and after RYGB. Of these subjects, 15 were diabetic. RYGB resulted in significant decreases in BMI (−16.8±0.9% and −23.9±1.2% from baseline at 3 and 6 months, respectively) and in fat mass (kg) (−19.0±0.9% and −32.4±1.1% from baseline at 3 and 6 months, respectively) (Table 3). As expected, this RYGB-induced weight loss was associated with major improvements in blood lipids and glucose homeostasis, with a 49% decrease in the insulin resistance surrogate HOMA-IR and a reduction in low-grade inflammation (−32.9±0.8% diminution of hsCRP) at 3 months. These metabolic and inflammatory changes were also observed in both the OB and OB/D groups when considered separately (Supplemental Figure II).

RYGB-induced weight loss was associated with a drastic reduction of both $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes, with a sharper decrease in $CD14^{dim}CD16^{+}$ 3 months (−36±4% versus −17±6% for $CD14^{+}CD16^{+}$) (Table 3 and Figure 2A). At 6 months postsurgery, the percentages of both monocyte subsets increased slightly but remained significantly lower than presurgical levels (Figure 2A). The kinetic profiles were similar in the OB and OB/D groups, with a greater decrease at 3 months in OB/D patients (Figure 2B and 2C). Considering the total monocyte counts in whole blood, similar changes in the absolute numbers of $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ were observed (Table 3).

The major improvement in corpulence-related parameters and blood TG was associated with variations in the $CD16^{+}$ subpopulation following RYGB. Through univariate analyses, we observed a positive correlation between BMI, fat mass (kg), and TG changes and variations in the percentages of $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes ($P<0.05$). On the contrary, we found no significant association with blood glucose and insulin-resistance markers. A trend in correlation was only found between $CD14^{dim}CD16^{+}$ and HbA1c (P=0.09).

The multivariate models confirmed that variations of monocyte subsets after surgery were mainly related to variations in adiposity and blood lipids but were not independent of glucose tolerance and insulin-sensitivity markers. Indeed, the analysis associating diabetic status, age, gender, BMI (or fat mass [kg]), TG, and HbA1c as fixed effects in a combined LME model confirmed the positive relationship between changes in $CD14^{dim}CD16^{+}$ or $CD14^{+}CD16^{+}$ monocyte percentages and changes in BMI (or fat mass) (P=0.05) and TG (P=0.01).

We examined the individual responses of subjects to RYGB and separated the 36 subjects into 2 groups based on BMI reduction at 6 months after RYGB (Figure 2D). Before surgery, no significant difference was found in anthropometric, metabolic, or inflammatory variables or in the concentrations of serum factors between the 2 groups (data not shown). The subjects with higher weight loss displayed greater decreases in $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ subsets at 6 months (Figure 2E).

Combining values of fat mass variations from the diet and surgery groups, we showed that changes in fat mass were

Table 3. Changes in Bioclinical Characteristics and $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ Monocyte Subpopulations in 36 Subjects Following RYGB

	Preoperative Baseline	3 Mo	6 Mo	Overall *P* Value
Weight, kg	133.9±4.2	111.6±3.7*	101.9±3.5*†	$<10^{-4}$
BMI, kg/m^2	49.1±1.4	40.9±1.2*	37.3±1.2*†	$<10^{-4}$
Fat mass, kg	61.7±2.6	49.9±2.2*	41.6±2.0*†	$<10^{-4}$
Fat mass, %	47.5±0.7	44.5±0.7*	41.0±0.8* †	$<10^{-4}$
Glycemia, mmol/L	6.4±0.3	5.6±0.2*	5.1±0.1*†	$<10^{-4}$
HbA1c, %	6.4±0.2	5.9±0.1*	5.8±0.1*	6.10^{-4}
Insulinemia, μU/mL	20.1±2.2	11.6±2.0*	10.7±2.2*	$<10^{-4}$
HOMA-IR, %	2.32±0.23	1.36±0.23*	1.18±0.24*	2.10^{-4}
Total cholesterol, mmol/L	4.9±0.2	4.4±0.2*	4.2±0.1*	8.10^{-4}
Triglycerides, mmol/L	1.6±0.11	1.4±0.07*	1.2±0.08*	2.10^{-3}
HDL-c, mmol/L	1.24±0.07	1.17±0.06	1.22±0.06	NS
Leptin, ng/mL	44.1±3.1	24.7±2.3*	21.7±2.3*†	$<10^{-4}$
Adiponectin, μg/mL	5.9±0.4	6.7±0.6	6.3±0.6	NS
CRP, mg/L	10.6±1.5	6.7±1.0*	5.8±1.4*	$<10^{-4}$
IL6, pg/mL	4.2±0.7	4.7±0.8	3.2±0.4	NS
PBMC, 10^3 cells/μL	3.0±0.3	2.5±0.3	3.0±0.6	NS
$CD14^{dim}CD16^{+}$, %	8.3±0.6	5.0±0.3*	5.3±0.6*	$<10^{-4}$
$CD14^{dim}CD16^{+}$, cells/μL	20.7±2.9	12.4±1.7*	10.9±2.8*	$<10^{-4}$
$CD14^{+}CD16^{+}$, %	5.6±0.8	4.7±0.5*	4.8±0.5*	6.10^{-3}
$CD14^{+}CD16^{+}$, cells/μL	15.1±2.1	10.8±1.7*	7.9±4.2*	3.10^{-3}

Data are shown as mean±SEM. Overall *P* values were obtained using repeated-measures multivariate ANOVA. Comparisons between preoperative baseline and each time point after gastric surgery were obtained by paired Wilcoxon test. *P* values were Bonferroni corrected. BMI indicates body mass index; CRP, C-reactive protein; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment insulin resistance; IL, interleukin; NS, not significant; PBMC, peripheral blood mononuclear cell; RYGB, Roux-en-Y gastric bypass.

*$P<0.050$ compared with preoperative value.

†$P<0.050$ compared with 3 mo value.

strongly related to variations in $CD14^{dim}CD16^{+}$ (Figure 3A). Results show that a decrease of at least 5% in fat mass is necessary to observe a significant reduction in the percentages of $CD14^{dim}CD16^{+}$ monocyte subpopulation. However, variations in $CD14^{+}CD16^{+}$ subset percentage were not significantly correlated with changes in fat mass ($R=0.22$, $P=0.1$) (Figure 3B).

The results of this series of analysis confirmed the strong dependence between the amount of adiposity reduction and changes in $CD16^{+}$ monocyte subpopulations, as well as the association with blood TG, but they did not show strong evidence of a link with the improvement of glucose metabolism or with insulin sensitivity.

Improvement of Vascular Phenotype Associated With Variation of $CD14^{+}CD16^{+}$ Monocytes Subsets During Weight Loss

The data suggested that $CD16^{+}$ monocytes could be important cellular actors in atherosclerosis development.[14–17,20,25–27]

We evaluated whether a relationships exists between $CD16^{+}$ monocyte subsets and subclinical indicators of early atherosclerosis. We measured FIMT and CIMT in OB and OB/D patients who had never experienced cardiovascular events. No differences in FIMT and CIMT were found between the 2 groups (0.51±0.04 versus 0.54±0.04 mm and 0.63±0.05 versus 0.66±0.05 mm in the OB group versus the OB/D group for FIMT and CIMT, respectively). At baseline, neither $CD14^{dim}CD16^{+}$ nor $CD14^{+}CD16^{+}$ percentages were correlated with FIMT or CIMT in the univariate analysis.

We found that age, BMI, fat mass, leptin, and CRP were significantly correlated with FIMT and CIMT in the OB and OB/D groups together (data not shown). In the multivariate analysis, taking into account age, gender, BMI, fat mass (kg), leptin, and CRP, only BMI and fat mass were independently associated with CIMT ($P=0.02$ and $P=5.10^{-3}$, respectively, for BMI and fat mass), whereas the only significant relationship with age persisted with FIMT. Thus, in this population of morbidly obese subjects, no statistical link was found between the $CD16^{+}$ monocyte subset and subclinical atherosclerosis.

We further analyzed the variations of subclinical atherosclerosis indicators 3 months after RYGB. Mean FIMT decreased from 0.51±0.01 mm to 0.48±0.01 mm ($P=0.08$), whereas CIMT decreased from 0.62±0.02 to 0.59±0.02 mm ($P=0.07$) at 3 months.

In the univariate analysis, variations in $CD14^{+}CD16^{+}$ monocytes were correlated with variations in CIMT ($P=0.02$), with a trend toward a similar relationship with FIMT ($P=0.08$). However, no relationship was found with changes in $CD14^{dim}CD16^{+}$ monocytes. In the multivariate

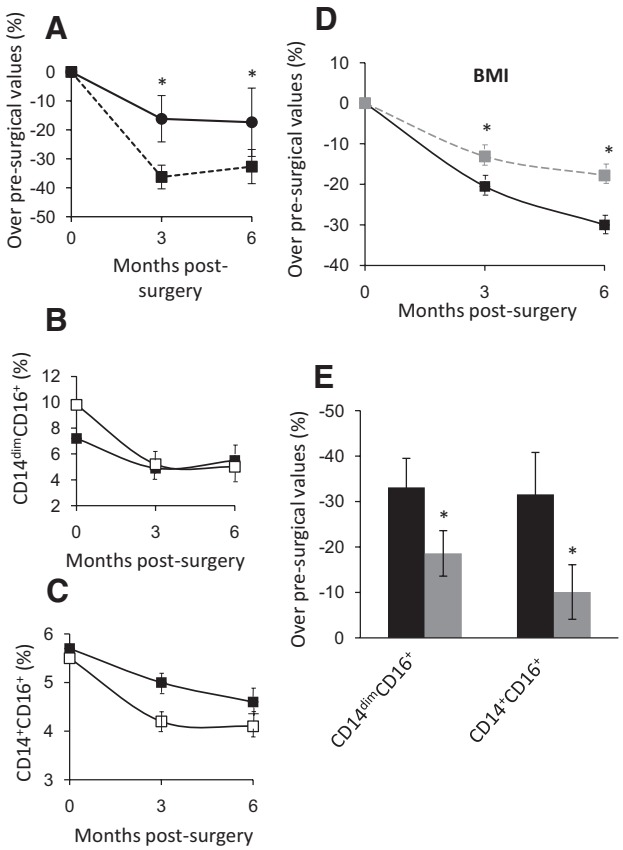

Figure 2. Kinetic variations in monocyte subpopulations $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ at 3 and 6 months following Roux-en-Y gastric bypass (RYGB) and the effect of a distinct amount of BMI reduction on $CD16^{+}$ monocyte subsets at 6 months after RYGB. A, Variations in $CD14^{+}CD16^{+}$ (solid line) and in $CD14^{dim}CD16^{+}$ (dotted line) monocyte subpopulations in the whole population in 36 obese subjects. Data are expressed as percentages over presurgical values and are shown as mean±SEM. Comparisons were performed using a Wilcoxon rank test adjusted for age; *$P<0.0002$ compared with preoperative values. B and C, Percentages of $CD14^{dim}CD16^{+}$ (B) and $CD14^{+}CD16^{+}$ (C) monocytes in 21 obese (solid line) and in 15 obese diabetic subjects (dotted line). Data are expressed as mean±SEM at baseline, 3 months, and 6 months. D Variations in body mass index (BMI) at 3 and 6 months after RYGB are presented for 2 groups of patients defined according to the median (−24.5%) of BMI loss at 6 months. The solid black line represents patients with BMI loss higher than the median (group A), and the dotted gray line represents patients with BMI loss less than the median (group B). E, Changes in monocyte subsets at 6 months in groups A (black bars) and B (gray bars). Data are expressed as percentages over presurgical values and are shown as mean±SEM. Comparisons between groups was performed using a Wilcoxon rank test; *$P<0.05$.

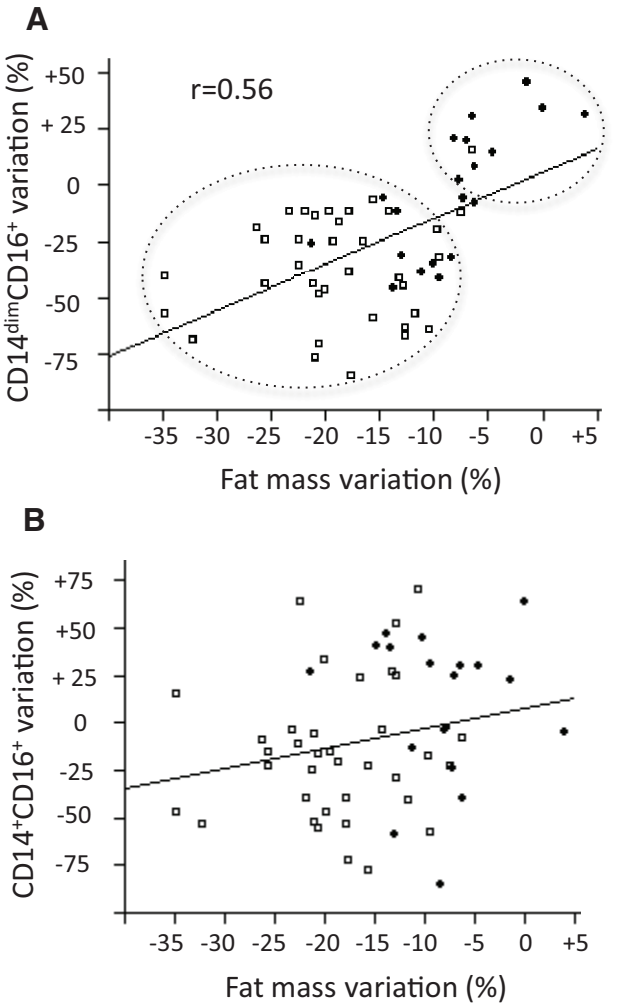

Figure 3. Correlations between percentages of fat mass variation and $CD14^{dim}CD16^{+}$ (A) and $CD14^{+}CD16^{+}$ (B) subpopulations during weight loss. Black circles represent values from patients after 6 weeks of diet intervention. White squares represent values from patients 3 months after gastric surgery. The regression coefficient was obtained by a simple linear regression analysis. ANOVA was used to test the significance of regression.

analysis, taking into account age, gender, variations in BMI (or fat mass [kg]) and variations in $CD16^{+}$ subsets, the $CD14^{+}CD16^{+}$ monocyte count was not independently associated with IMT measurements, confirming a strong dependence with the level of corpulence.

Discussion

In the present study, combining subjects with different levels of corpulence (from moderate to severe obesity) and 2 clinical intervention studies inducing weight loss, we showed strong links between fat mass and the frequencies of $CD14^{dim}CD16^{+}$ monocytes in the population. Indeed, we observed an increase of about twice the percentage of $CD16^{+}$ monocyte subsets in obesity and a reduction of these cell populations by drastic fat mass loss. A fat mass decrease of at least 5% was sufficient to observe a reduction in the $CD14^{dim}CD16^{+}$ subpopulation. On the contrary, we could not demonstrate a convincing link with glucose homeostasis in patients involved in clinical trials improving insulin sensitivity. In this context, the only association found with metabolic parameters was with fasting TG.

In healthy humans, 3 monocyte subpopulations have been described ($CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$, and $CD14^{dim}CD16^{+}$), differing in phenotype and function.[19] Human obesity is characterized by a significant increase in the $CD16^{+}$ subset.[12,13] In our study, we demonstrated an increase in the 2 $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocyte subtypes in obese subjects. Furthermore, we also observed that diabetes is associated with an increased frequency of $CD14^{dim}CD16^{+}$ cells, a subtype also

correlated with fasting glycemia. This feature suggests that at least in morbid obesity, increased glycemia could be a parameter regulating $CD14^{dim}CD16^{+}$ population.

Our study raises the question of the association between monocyte phenotypes and insulin-resistance states. We approached this issue using 2 clinical procedures, inducing a moderate change or a more important change in insulin sensitivity, ie, either diet- or surgery-induced weight loss. RYGB is a well-established procedure to reduce body fat mass, to ameliorate metabolic status, and to reduce low-grade inflammation in severe obesity.[22] Weight loss also reduces the inflammatory activation of peripheral mononuclear cells in obese subjects.[28–31] Here, we observed that the percentages of $CD16^{+}$ subsets decreased with surgery-induced weight loss. Patients displaying a higher diminution of fat mass or BMI had greater decreases in $CD16^{+}$ monocyte subsets. A moderate weight reduction (ie, <5%) did not affect monocyte subset frequencies.

Furthermore, we found that a quantitative variation in fat mass during weight loss was strongly correlated with changes in $CD14^{dim}CD16^{+}$ monocytes. It is now well established that the enlarged fat mass characterizing obesity is associated with macrophage accumulation and with alteration of adipose-tissue secretions[32], which are also potent modulators of monocyte phenotypes. We have observed a significant correlation between the number of macrophages in visceral adipose tissue and the percentages of $CD14^{dim}CD16^{+}$ monocytes (Dalmas C, Tordjman J, Clement K, Veyrie N, Guerre-Millo M, Poitou C, personal communication), but an extended analysis is required to study the impact of adipose-tissue secretion on monocyte phenotypes in obesity and during weight loss to find the factors involved in the differentiation of $CD16^{+}$ monocytes.

Importantly, although both moderate and drastic weight-loss procedures unambiguously improve insulin-sensitivity surrogates, no kinetic association was found with changes in the percentages of $CD16^{+}$ monocytes. We nevertheless highlighted the association between the frequency of monocyte subsets and TG changes independent of BMI decrease. After RYGB, changes in TG are either related to variations in free fatty acid flux provided from adipose tissue lipolysis or to very-low-density lipoprotein production by the liver in relation to the improvement of insulin resistance. We did not observe any correlation with HOMA-IR or changes in $CD16^{+}$ subsets, suggesting that a decrease in $CD16^{+}$ monocytes could not be attributed to an improvement in insulin resistance. The association between macrophages in human adipose tissue and insulin resistance is also debated. Previous studies have shown a negative correlation between whole-body insulin sensitivity and the expression of the macrophage marker CD68 in subcutaneous adipose depots.[33,34] Preferential macrophage accumulation into visceral adipose tissue has been observed mainly in subjects with impaired glucose homeostasis,[35] and obese subjects with more crown-like structures of macrophages in adipose tissue have been shown to be more insulin-resistant than those subjects lacking these cell aggregates.[36] On the contrary and in agreement with this present work on circulating monocytes, no correlation in morbid obesity has been found between adipose tissue macrophages in visceral depots and blood-derived parameters of insulin resistance,[37,38] whereas an association was found with fasting TG.[39] An overfeeding challenge rapidly installed an insulin-resistant state in healthy subjects, despite the fact that no significant change occurred in the total macrophage accumulation in the adipose tissue and that there was no change in the number of circulating cells.[40] Additionally, we found that irrespective of the degree of insulin resistance in morbid obesity, macrophage accumulation in omental adipose tissue was associated with the severity of liver fibroinflammation, a well-known and severe complication of obesity.[38] Although the potential link between adipose tissue macrophages and cardiovascular complications in obesity has not been explored yet, several reports have indicated that there is a link between increases in $CD16^{+}$ monocyte subsets and the development of cardiovascular events[14–17] and coronary fibrous cap thickness in patients with unstable angina pectoris.[27] In morbidly obese subjects, we failed to find any relationship between $CD16^{+}$ monocyte subsets and subclinical atherosclerosis evaluated by IMT measurement after adjustment with other risk factors, such as BMI, diabetic status, or lipid parameters. This finding does not exclude that these monocyte subsets might be associated with more advanced stages of cardiovascular disease in obese individuals. However, improvement in IMT after surgery-induced weight loss was associated with a decrease in $CD14^{+}CD16^{+}$ monocyte frequency, but this association depended on BMI variation. This observation stimulates the need to explore in depth the relationships between monocyte heterogeneity and the biological events associated with fat mass loss, which is known to reduce cardiovascular risks.

In conclusion, our study highlighted, for the first time, the links between the $CD14^{dim}CD16^{+}$ monocyte subset and fat mass variation and between the $CD14^{+}CD16^{+}$ subset and vascular phenotype during weight loss. Further studies are required to characterize the functional properties of $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ subsets and to establish their specific roles in the development of atherosclerosis in obese subjects.

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Disclosures

None.

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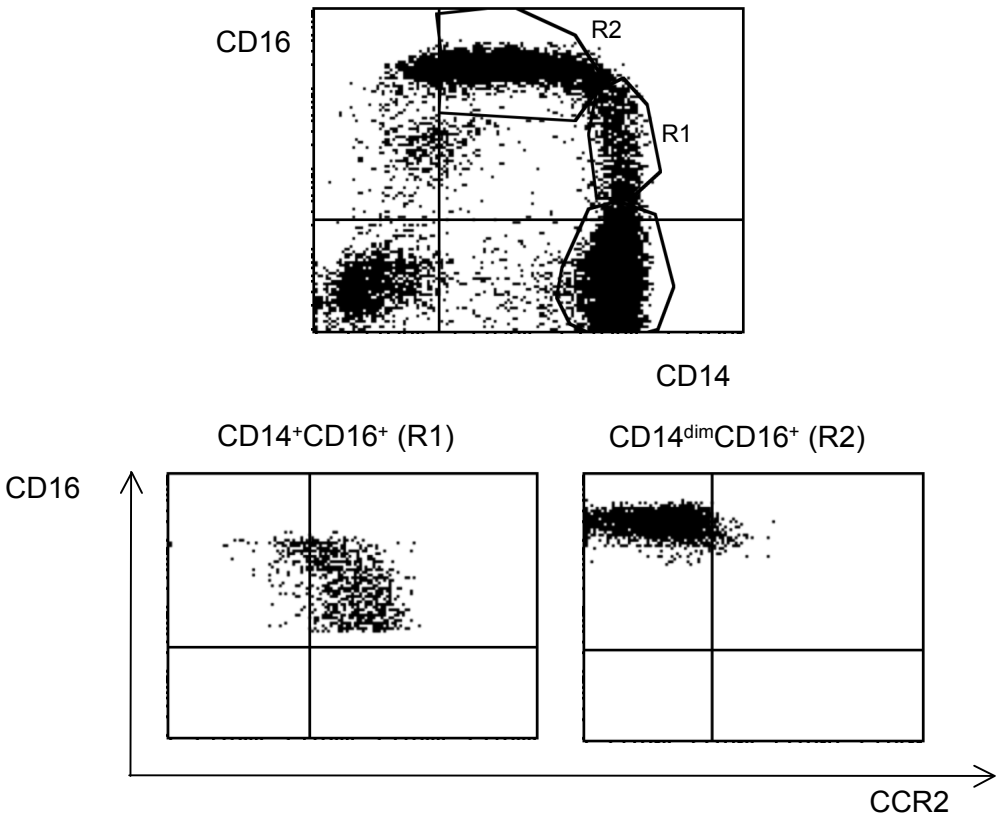
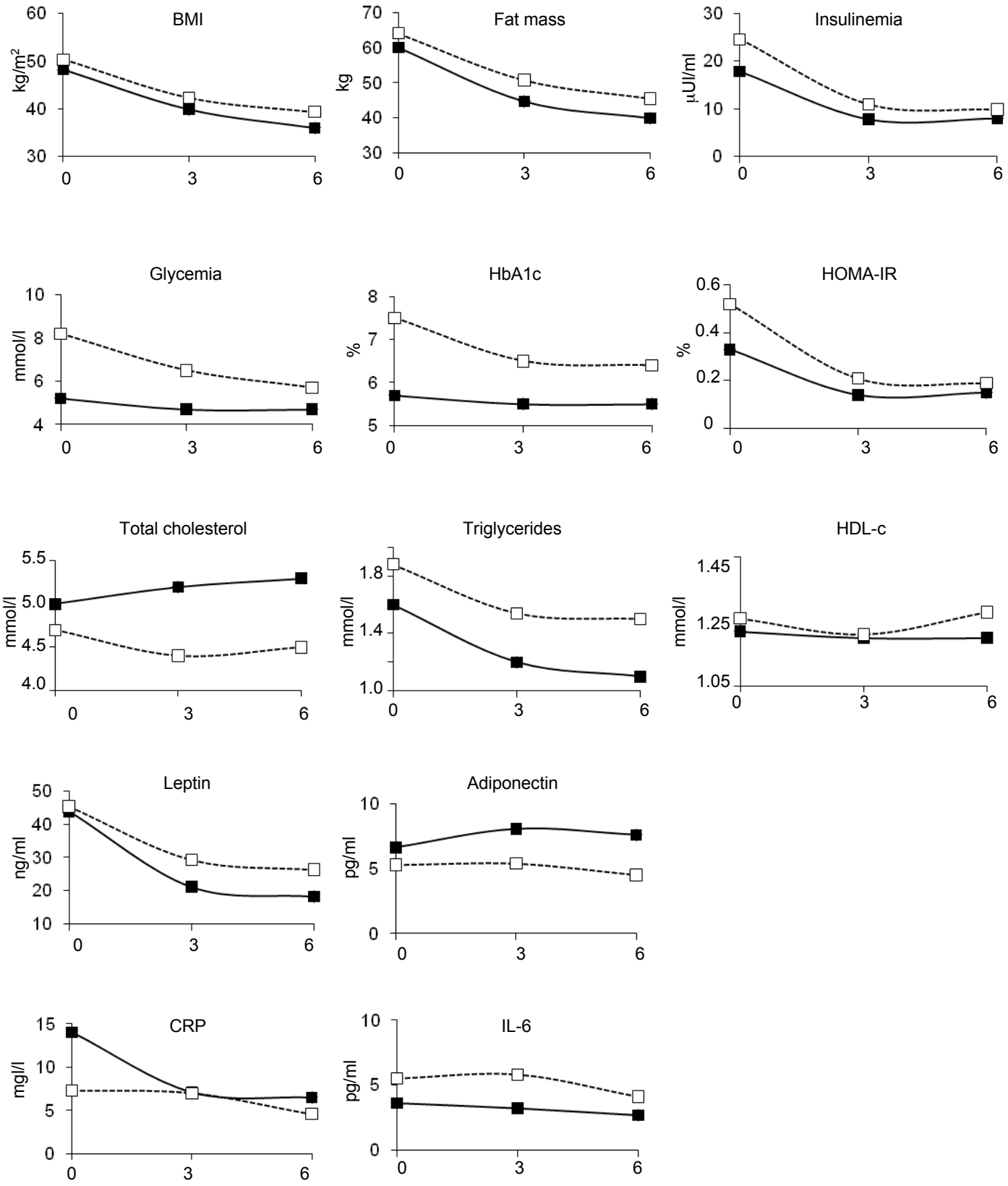


Supplemental Figure I



Supplemental Figure II

3. Discussion of study 1

Gastric bypass surgery is a very efficient way to massively lose weight and to improve metabolic parameters. While the acute phase protein, CRP, is markedly reduced during weight loss, we observed in article 1 that other circulating inflammatory mediators might not be readily down-regulated. We showed that IL-8, IL-9, IL-10, Eotoxin, MCP-1, MIP-1β, RANTES and VEGF followed a biphasic kinetic pattern at month 3 and 6 after surgery. All factors were ultimately down-regulated at later time points. Interestingly, in article 2, variations in both $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ percentages displayed a similar profil during weight loss. Monocyte subsets were markedly decreased at month 3 after surgery but tended to slightly increase back at month 6, although percentages over presurgical values remained significant. Thus, both circulating inflammatory mediators, with the exception of CRP, and monocytes are not linearly down-regulated in the early phase of surgery-induced weight loss and may not be directly modulated by fat mass loss. Supporting this idea, recent studies also report non-linear variations of circulating or adipose tissue inflammatory factors in response to very low calorie diet (Siklova-Vitkova et al., 2012)(Capel et al., 2009). Thus, weight loss might represent a physiological stress to the organism that reacts through inflammatory responses to, on one hand, drastic caloric restriction and, on the other hand, qualitative change in macronutrient intake. Kosteli *et al* showed that mice undergoing caloric restriction had increased macrophage accumulation in response to weight loss-induced lipolysis and subsequent up-regulation of circulating FFAs. Though, no increased in inflammatory mediators was observed in adipose tissue neither at the systemic level (Kosteli et al., 2010). Adipocyte « emptiness » and release of FFAs might not be the only explanation for inflammation oscillations during weight loss. In conclusion, these results show that weight loss is a complex phenomenon whose mechanisms are not yet completely understood in humans.

Besides, it is thought that obesity-associated systemic inflammation partly originates from circulating leukocytes. Indeed, PBMC were shown to be in an activated state in obese patients, capable of pro-inflammatory cytokine production such as TNF-α, IL-1β, IL-6 and IFN-γ and enhanced NF-κB pathway activation (Bories et al., 2011; Ghanim et al., 2004). It is very likely that macronutrient intake can also influence inflammatory responses in circulating leukocytes, thus contributing to systemic infammation. After gastric surgery, patients slowly get back into their food habits and up-regulate their calorie intake. In article #1, we show that carbohydrates appeared to be the only macronutrient class to increase back at month 6

compared to month 3 after surgery. Yet, carbohydrates are known to activate PBMC. In a comparative study, effects of consumption of glucose, orange juice, saturated fat as cream and water were assessed in circulating PBMC inflammatory status. Both glucose and saturated fat induced significant pro-inflammatory cytokines gene expression in PBMC few hours after consumption (Deopurkar et al., 2010). In the same way, high-fat high-carbohydrate diet induced increased oxidative and inflammatory stress in PBMC, that lasted more in obese compared to lean subjects (Patel et al., 2007). It is known that hyperglycemic conditions significantly regulate TLR-2 and TLR-4 production as well as NF-kB activity in monocytes within few hours and that these effects can last for 2-3 days (Shanmugan et al., 2003)(Dasu et al., 2008). Although, most *in vitro* studies used non physiological glucose levels, it is likely that consumption of high glycemic index carbohydrates, that induce high postprandial glucose, or poor glycemic controls that can occur in diabetic subjects, could stimulate circulating leukocytes on a daily basis, with potential additive effects. Food composition is thus very likely to influence inflammatory responses of circulating immune cells, contributing to systemic infammation.

D. STUDY 2: Adipose tissue inflammation in obesity and during gastric surgery-induced weight loss

Based on the study of morbid obese patients undergoing gastric surgery, IL-1β and IL-17 were identified as two key adipose tissue-derived cytokines that are up-regulated with obesity and obesity-induced T2D, while being down-regulated during surgery-induced weight loss. Since Th17 lymphocytes and related IL-17 production were already breifly presented in the introductory section B, IL-1β and its major regulator, that is the NLRP3 inflammasome, will be introduced here.

1. Introducting the inflammasome

1.1. IL-1β signalling

Since its discovery, IL-1 instigated the birth of cytokine biology and was considered as a "master molecule" capable of multiple biological activities and virtually affected all cell types. IL-1 family includes 11 cytokines with IL-1β, IL-1a and IL-18 being the most studied. In this manuscript, special attention will be given to IL-1β. Considering that IL-1β is a major pro-inflammatory cytokine and because its repector is widely expressed on different cell types, its signalling is a tightly regulated process, mastered by the production of antagonist and decoy receptors as illustrated in Figure 18. This regulation also applies to IL-1α, while IL-18 binds to its specific IL-18 receptor (Dinarello et al., 2010).

IL-1α/β signalling in mediating through type 1 receptor, IL-1R1, the only « active » receptor. Upon binding of IL1 to IL-1R1, an IL-1RAcP is recruited to form a high affinity IL-1R1-IL-1RAcP heterodimeric receptor, which initiates a pro-inflammatory downstream cascade. Through tumor necrosis factor-associated factor 6 (TRAF6), IL-1 leads to the activation of NF-κB, JNK and p38 MAPK pathways and subsequent nuclear tranlocation of NF-κB and AP-1, inducing gene expression of various pro-inflammatory cytokines, including IL-1β itself.

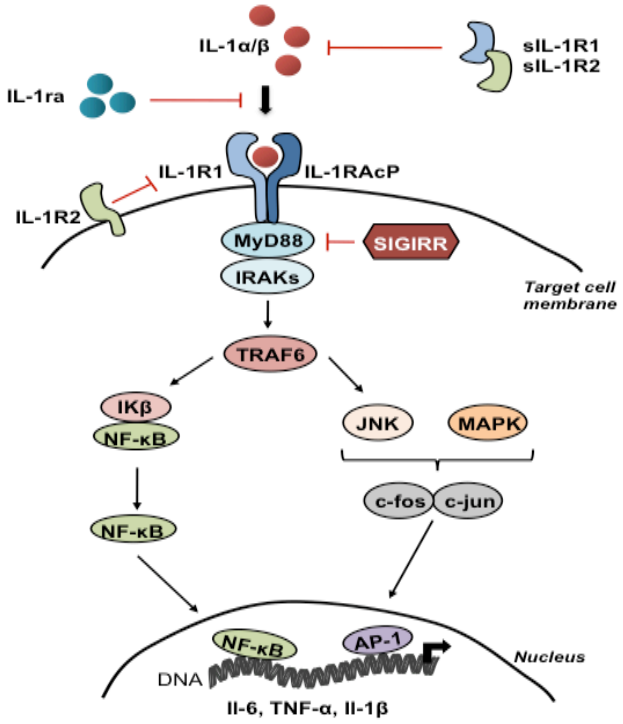


Figure 18 | IL-1 regulation and signalling pathway.

IL-1 signalling is highly regulated by the presence of multiple negative regulators of its active receptor. IL-1 also binds to the surface of IL-1R2 that is not signaling transducing and acts as a decoy receptor. Both IL-1R1 and IL-1R2 are secreted and these soluble truncated IL-1 receptors neutralize bioactive IL-1 in the circulation. IL-1ra, strongly binds to the IL-1R1 with a higher affinity than do IL-1α and IL-1β without signal induction. Finally, single Ig IL-1-related receptor (SIGIRR) is a negative regulator of IL-1 and interacts with MyD88/TRAF6, although exact mechanisms are unclear.

1.2. IL-1β secretion through NLRP3 inflammasome

Beside signalling regulation, IL-1β is highly controlled at the production level that requires two signals, underlying complex and unclear mechanisms, as summarized in Figure 17. The first signal involves sensing of PAMPs, DAMPs or exogenous molecules by PRRs, mainly *via* the TLR pathway, or cytokine receptors including IL-1R1 itself. Signal 1 ensues accumulation of intracellular stores of IL-1β inactive pro-form that is pro-IL-1β, through transcription regulation mechanisms involving the NF-kB pathway. In contrast, there is a constitutive intracellular pool of pro-IL-1α and pro-IL-18 and LPS-stimulation has little effect of IL-18 gene

expression (Dinarello et al., 2010)(Netea et al., 2006).

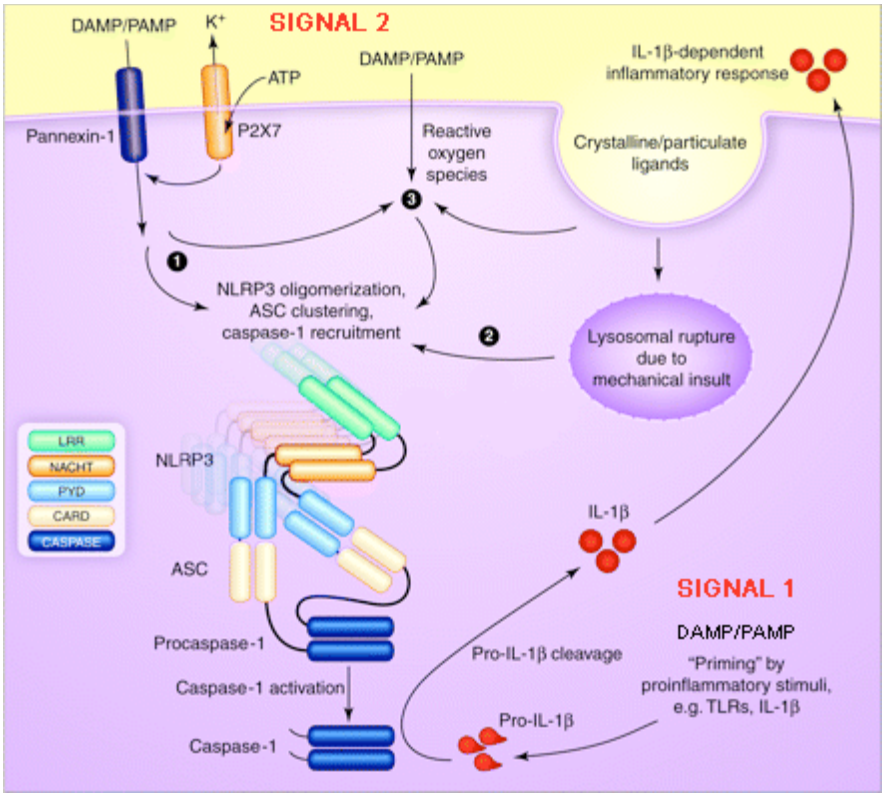


Figure 19 | IL-1β secretion mechanisms, involving NLRP3 inflammasome activation. SIGNAL 1 ensues production of pro-IL-1β through the recognition of DAMPs/PAMPs by TLRs or cytokine receptors. SIGNAL 2 induces NLRP3 activation that subsequently leads to caspase-1 activation that cleaves the proform into mature and secreted IL-1β. Considering NLRP3-ligand diversity, they might shared common mechanisms and to date, three mechanisms have been proposed: potassium efflux via P2X7 (1), lyzozyme rupture after phagocytosis of large crystalline or particulate structures, such as monosodium urate (2) and, generation of mitochondrial-derived ROS (3) (adapted from(Schroder and Tschopp, 2010)).

The second signal involves the NLRP3 inflammasome complex discovered by late Jürg Tschopp in 2002. Structurally, inflammasomes are cytoplasmic multiprotein complexes. The NLRP3 inflammasome contains NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD, also termed ASC or PYCARD, and the effector cysteine protease caspase-1. Following detection of cellular stress, NLRP3 oligomerizes through homotypic protein-protein interactions between NACHT, PYD and CARD domains to ultimately recruit pro-caspase-1. Pro-caspase-1 clustering on oligomerized NLRP3 results in caspase-1 auto-activation and caspase-1-dependent processing of cytoplasmic targets, including pro-IL-1β. The mature, biologically active cytokines are released from the cell by yet undefined secretion pathways. Interestingly, discrepancies exist between cell types and notably, monocytes constitutively express active caspase-1, allowing direct IL-1β secretion upon unique signal 1. NLRP3 is activated by numerous signals of mainly of endogenous origin.

There is no evidence that NLRP3 binds directly to any of its known activators since the diversity of ligand structures does not allowed a potential NLRP3 binding as shown in Figure 19 (Schroder and Tschopp, 2010). Of note, signal 1 is also a prerequisite for inflammasome activation, inducing up-regulation of NLRP3 gene expression. A very recent study showed that, upon TLR agonists, NF-kB binding elements were identifed in NLRP3 promoter (Qiao et al., 2012). Other NLR-containing inflammasomes such as NLRP1, NLRP7 and NLRC4 were also described to process IL-1β maturation.

1.3. NLRP3, IL-1β and pathophysiology

Over the years, IL-1β was shown to play a role in many inflammatory diseases and more pathology discoveries are yet to come. Notably, Il-1β was specifically associated with growing occurrence of autoinflammatory diseases defined as « self-directed tissue inflammation, where local factors at disease-prone sites determine activation of the innate immune system, including macrophages, with relative lack of evidence for adaptive immunity » (Dinarello et al., 2010). Most of these manifestations involve gene mutations of NLRP3 inflammasome components or genes upstream/downstream of the inflammasome, ensuing over production of IL-1β. Therefore, autoinflammation is also defined by the fact that targeting IL-1β is clinically relevant. Such pathologies include Familial Mediterranean fever, Cryopyrin-associated periodic syndromes (CAPS), Muckle-Wells Syndrom (MWS) or Systemic onset juvenile idiopathic arthritis (SoJIA). Other auto-inflammatory diseases are characterized by increased NLRP3 inflammasome activation by distinct or unclear endogenous ligands. Experimental and clinical evidences causally link IL-1β to the development of metabolic complications that are, most of the time, associated with obesity (De Nardo and Latz, 2011; Dinarello et al., 2010; Donath and Shoelson, 2011; Schroder et al., 2010). Major pharmaceutical firms have seized the opportunity to clinically develop numerous anti-IL-1β antibodies. However, the underlying molecular mechanisms that result in tissue-specific inflammasome activation in the context of obesity have only just begun to be uncovered. Recently, obese adipose tissue was found to produce significant amount of IL-1β via NLRP3 activation in link with insulin resistance status (Stienstra et al., 2011; Vandanmagsar et al., 2011). Human studies are especially needed to define the true role of adipose tissue-derived IL-1β in obesity and obesity-induced metabolic disturbances.

2. Article #3: IL-1β and IL-17 mediate a NLRP3-dependent pro-inflammatory crosstalk between macrophages and lymphocytes in human adipose tissue contributing to obesity-induced type 2 diabetes

Adipose tissue is infiltrated by both macrophages and lymphocytes that together contribute to an inflammatory microenvironment that alters adipocyte physiology during obesity. Less is known about the possible cross-talk between immune cells within adipose tissue during obesity. We hypothesized that macrophages and lymphocytes might develop special interactions to support adipose tissue inflammation. We showed that macrophage-derived IL-1β, through the NLRP3 activation, and lymphocyte-derived IL-17 were part of a paracrine inflammatory crosstalk within human adipose tissue.

As illustrated in figure 18, human subcutaneous or visceral adipose tissue, obtained during gastric surgical procedures, were minced into small pieces and incubated for 24h at 37°C for secretion to obtain explant conditioned media.

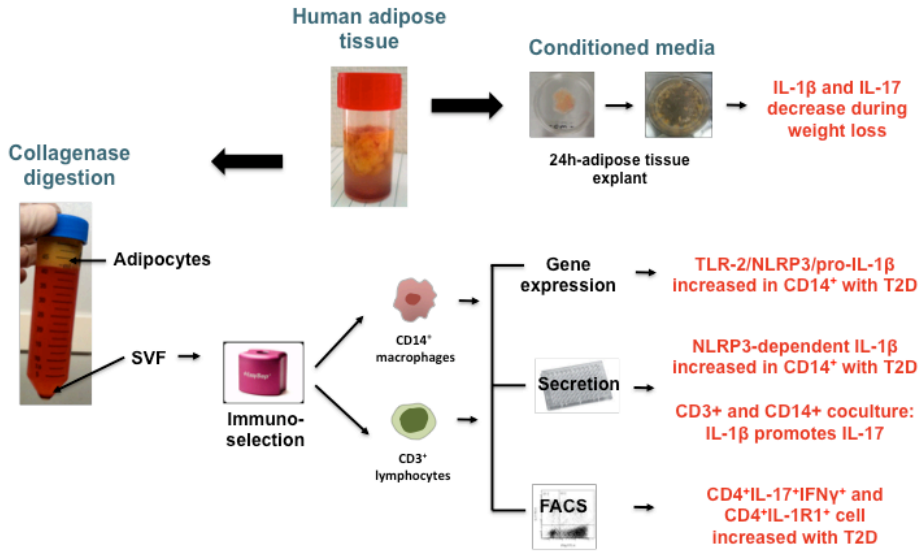


Figure 20 | Methodology of human adipose tissue study and main results.

Besides, when fat samples were big enough, adipose tissue was fractionated into adipocytes and stroma vascular cells (SVF) upon enzymatic digestion. Cells of the SVF were then immunoselectioned with antibody coupled-magnetic beads targeted towards $CD14^+$ macrophages and $CD3^+$ lymphocytes. Freshly isolated cells were used for gene expression, secretion and flow cytometry (FACS) analyses. Main results obtained through the different experimental techniques are highlighted in red in figure 20.

$CD14^+$ and $CD3^+$ co-culture experiments were of major importance as they allowed us to identify that adipose tissue macrophage-derived IL-1β promotes IL-17 production by adipose tissue lymphocytes, and vice-versa, as illustrated in the pictures below.

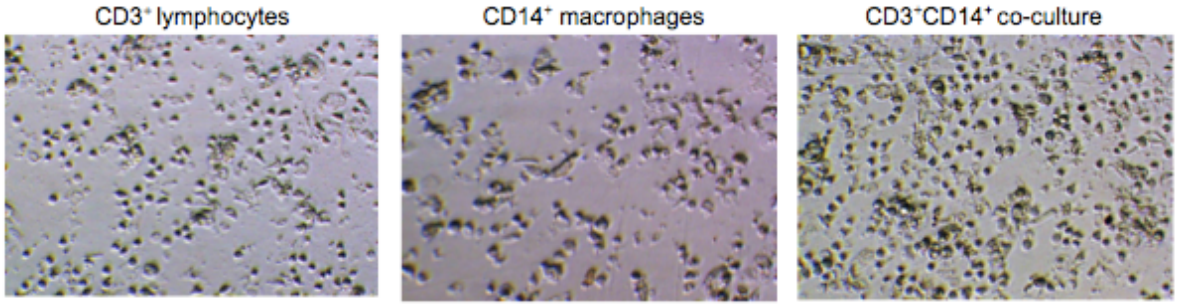


Figure 21 | Pictures of $CD3^+$ lymphocytes and $CD14^+$ macrophages, freshly isolated from obese visceral adipose tissue, cultured alone or together at a 1:1 ratio.

IL-1β and IL-17 mediate NLRP3-dependent pro-inflammatory cross-talk between macrophages and lymphocytes in human adipose tissue: relation with obesity-induced type 2 diabetes

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Abstract

Accumulation of immune cells in adipose tissue is a key component of chronic low-grade inflammation in obesity. Little is known about the contribution of potential cross-talks among adipose tissue immune cells to obesity co-morbidities. Here, while exploring adipose tissue cytokine signature in response to weight loss, we found that IL-1β and IL-17 were coordinately down regulated. We identified adipose tissue $CD14^+$ cells as the major source of IL-1β, which secreted the cytokine through activation of NLRP3 inflammasome. This process was down-regulated by weight loss but overactivated in type 2 diabetic patients. Adipose-derived secreted factors reproduced these regulations in monocytes-derived macrophages. Both $CD68^+$ cell content and IL-1β release in adipose tissue increased with patient's altered glycemic status assessed by elevation of Hb1Ac and fasting insulin levels. In adipose tissue, IL-17 producing $CD4^+$ T cells were more frequent in obese diabetic patients. This increase was driven by specific enrichment of double positive $IL\text{-}17^+IFN\text{-}\gamma^+$ T cells. The abundance of these double producers was significantly correlated with Hb1Ac in obese subjects. In primary cell co-culture experiments, adipose tissue $CD14^+$ cell-derived IL-1β markedly enhanced IL-17 production by adipose tissue $CD3^+$ T cells, while IL-17 reciprocally induced IL-1β secretion by $CD14^+$ cells. Thus, we identified IL-1β and IL-17 as two key cytokines mediating a new paracrine inflammatory cross-talk between adipose tissue macrophages and T cells that might contribute to the deterioration of glycemic status in human obesity.

Introduction

Obesity, defined as an excess of white adipose tissue, is considered a low-grade inflammatory state that might be the instigator of obesity-related metabolic and cardiovascular complications. In obesity, the most devastating complication is type-2 non-insulin dependent diabetes (T2D), which accounts for over 90 % of the diagnosed cases of diabetes and affects about 300 million people worldwide. It is now well accepted that the adipose tissue itself is a site of chronic inflammation, where local secretion of pro-inflammatory cytokines by accumulated immune cells might participate into the development and/or aggravation of insulin resistance (review in (1, 2)). Based on clinical and experimental evidences, visceral adipose tissue (VAT) accumulation is considered a major risk factor for T2D, while subcutaneous adipose tissue (SAT) may also contribute to whole body insulin resistance. Yet, the cellular and molecular mechanisms that initiate and perpetuate inflammation in adipose tissue remain to be fully deciphered.

The presence of immune cells in the human adipose tissue was first reported in 2000 (3). Thereafter, macrophages were shown to infiltrate adipose tissue in mouse models of genetic and diet-induced obesity, with a typical organization in crown like structures (CLS) surrounding single adipocytes (4, 5). A causal relationship between the abundance of adipose tissue macrophages and systemic insulin resistance has been clearly established in mouse studies, in which macrophage accumulation was manipulated through diet, genetic or pharmacological interventions (4, 6-8). In clinical studies, increased adipose tissue macrophage content has been associated with impaired glucose homeostasis (9-11). Also, in the setting of weight loss induced by bariatric surgery or dietary intervention, adipose tissue macrophage infiltration decreased along with amelioration of glycemic status (12, 13). Besides, the phenotype of adipose tissue macrophages has emerged as a valuable determinant to be considered in insulin resistance (review in (1)).

The molecular actors linking macrophage accumulation in adipose tissue to insulin resistance are not all identified. One cytokine in particular, IL1β, appears to be a key mediator of T2D. Locally, IL-1β is known to impair insulin signaling, increase fatty acid release and down regulate the production of the insulin-sensitizing hormone adiponectin in adipocytes (14-16). Experimental and clinical studies have stressed the pathological implication of IL-1β in obesity-induced alteration of glycemic status. Treatment with anti-IL-1β neutralizing antibody or deletion of the signaling IL-1RI receptor reduced local adipose tissue inflammation and improved insulin-resistance in diet induced obese mice (17-19). In humans, detectable circulating level of IL-1β is a risk factor for developing T2D and blockade of IL-1β with a recombinant IL-1 receptor antagonist (anakinra) reduces systemic inflammation and improves glycemic status in T2D patients (20, 21).

Secretion of IL-1β is tightly controlled through the combination of two distinct triggers. Signal 1 typically involves triggering by a Toll-like receptor (TLR) ligand that leads to accumulation of cytosolic pro-IL-1β. Signal 2 involves a series of events, including the assembly of inflammasome complex NLRP3 / ASC / caspase-1 that activates cleavage of pro-IL-1β into bioactive IL-1β (22). All these components, including TLR-2 and TLR-4, have been shown to play critical roles in the pathogenesis of obesity-induced insulin resistance in mice (23-28). In human obesity, pro-IL-1β and NLRP3 gene expression was positively associated with insulin resistance and decreased by weight loss (27, 29, 30).

Besides, growing attention has been given to adaptive immune cells in the adipose tissue (31-33). Recent papers

demonstrated that T cells, whose proportions vary with obesity, contribute to adipose tissue inflammation (32, 34-38). In adipose tissue of lean mice, $CD4^+$ cells with anti-inflammatory phenotype, such as Th2 and regulatory T cells (Tregs) predominate, whereas pro-inflammatory $CD4^+$ Th1 and $CD8^+$ T cells accumulate with obesity. Furthermore, adipose tissue macrophages could be activated by $CD4^+$ Th1-derived IFN-γ since this cytokine is classically known to activate macrophages. More recently, the presence of IL-17-producing cells has been reported in mice and human adipose tissue (35, 39, 40). However, whether or not obesity influences their abundance remains elusive. Th17 cells represent a distinct lineage of $CD4^+$ T cells and are associated with the pathogenesis of several autoimmune diseases (review in (41)). Notably, obesity was shown to exacerbate autoimmune experimental diseases through selective expansion of the Th17 cell lineage (42).

Interestingly, IL-1β is a master regulator of Th17 expansion and IL-17 production in human T lymphocytes (41). However, such a link has not been addressed in the adipose tissue and in the context of obesity. In the present study, while exploring the adipose tissue cytokine signatures during weight loss, we noticed that IL-1β and IL17 were coordinately down regulated. This prompted us to determine the cell sources of these cytokines and their interactions. Our data identify IL-1β and IL-17 as two pivotal cytokines mediating a new paracrine inflammatory cross-talk between adipose tissue macrophages and T cells that might contribute to the deterioration of glycemic status in human obesity.

Results

IL-1β and IL-17 releases are coordinately regulated in human adipose tissue

We measured a series of cytokines released by SAT biopsies obtained in obese subjects who undergone laparoscopic Roux-en-Y gastric bypass (RYGB) surgery (group 1): at the time of surgery and at months 3, 6 and 12 post-surgery. The serum concentrations of two major adipocyte-produced adipokines, leptin and adiponectin, were either reduced (leptin) or remained stable (adiponectin) after surgery (Table 1). Mirroring these changes in the circulation, leptin release by SAT explants dropped markedly, while adiponectin release was only slightly decreased upon weight loss (Fig 1A). Several pro-inflammatory cytokines, TNF-α, IL-6, IL-12 and IFN-γ, were stable or minimally regulated after surgery. In the same SAT explants, IL-1β and IL-17 were released significantly at lower rates at each time point post-RYGB. Interestingly, surgery-induced changes in IL-1β and IL-17 secretion rates were significantly correlated (Fig 1B). Positive correlations were also found between the rates of IL-1β and IL-17 release in both SAT and VAT of obese subjects (Fig 1C). In line with increased pro-inflammatory status of the visceral depot, IL-1β and IL-17 to a lesser extent were produced in higher amounts in VAT than in paired SAT (Fig 1D, E). These initial observations strengthen the hypothesis that IL-1β mediates IL-17 production in human adipose tissue.

Adipose tissue IL-1β release is related to macrophage content and glycemic status

Next, we addressed the cellular source of IL-1β in adipose tissue. IL-1β release was significantly correlated with accumulation of cells labeled in immunochemistry experiments by anti-CD68 antibody (Fig 2A). This antibody detects macrophages isolated or organized in CLS in human adipose tissue. Patient's biopsies with macrophage crowning (CLS^+) secreted significantly higher amounts of IL-1β than adipose tissue without such structure (CLS^-) (Fig 2B). This suggests that IL-1β is produced by adipose tissue macrophages with a greater contribution from those disposed in CLS. Of note, the number of

$CD68^+$ cells and CLS^+ biopsies decreased with weight loss (Table 1), in line with reduced rates of IL-1β secretion (Fig 1A). $CD68^+$ cell content in SAT increased in relation to patient's altered glycemic status as assessed by elevation of Hb1Ac and fasting insulin levels (Fig 2C, D). IL-1β released by the same biopsies increased in parallel (Fig 2E, F), suggesting a crucial role for macrophage-derived IL-1β in obesity-linked deterioration of glucose homeostasis.

IL-1β production by adipose tissue $CD14^+$ cells increased with T2D

To further explore the impact of T2D on adipose tissue macrophage IL-1β, we recruited a new group of 41 severely obese subjects (Table 1, group 2), including 18 T2D patients. VAT was fractionated into adipocytes and cells of the stroma vascular fraction (SVF). $CD14^+$, $CD3^+$ and $CD14^-$ $CD3^-$ (Neg) cells were immunoselected from SVF. The $CD14^+$ cell-enriched fraction, which includes macrophages and potentially monocytes and dendritic cells, expressed 15-fold more pro-IL-1β mRNA than any other adipose tissue cell type (Fig 3A), supporting macrophages as a major source of IL-1β in human adipose tissue. To assess the effect of obesity *per se*, a control group of 5 non-obese (BMI < 30 kg/m²) subjects was investigated (Table 1, group 3). We found that pro-IL-1β gene expression and release of IL-1β by $CD14^+$ cells were significantly higher in T2D patients *versus* non-obese and obese subjects (Fig 3B, C). By contrast, pro-IL-18 gene expression and amounts of IL-18 secretion were similar in all three groups (Fig 3B, D). In line with the data on $CD14^+$ cells isolated from adipose tissue, the stimulation of healthy-donors' monocytes-derived macrophages (MoDM) with adipose tissue-conditioned media (Cm) from obese and obese T2D subjects induced pro-IL-1β gene expression (Fig 3E). Again, pro-IL-18 gene expression was not significantly changed in this experimental setting. Additionally, Il-1β secretion by MoDM tended to increase in response to CmSAT from obese and obese T2D patients compared to non-obese subjects (Fig 3F). Conversely, CmSAT obtained 3 months after RYGP was significantly less potent to induce IL-1β release by MoDM than pre-surgery CmSAT (Fig 3G). In further support of a link with altered glycemic status already shown in SAT (Fig 2), the rates of IL-1β release by $CD14^+$ macrophages increased with HbA1c (Fig 3H). The inverse relationship found with serum concentrations of insulin-sensitizing adiponectin reinforces the relationship between adipose tissue macrophage-derived IL-1β and insulin resistant state in human obesity (Fig 3I).

IL-1β release by adipose tissue $CD14^+$ cells is NLRP3/caspase-1 dependent

Next, we sought to determine the impact of obesity and/or T2D on the molecular sensors required for IL-1β secretion by VAT $CD14^+$ macrophages. We first observed that the gene expression of TLR2 and TLR4, albeit to a lesser extent, was increased in the cells isolated from obese and obese T2D patients (Fig 4A). Flow cytometry analysis further confirmed increased expression of TLR-2 on the surface of VAT $CD14^+$ macrophages from these patients (Fig 4B). In MoDM, obese adipose tissue Cm increased both TLR2 and TLR4 gene expression (Fig 4C). Importantly, LPS was not detected in the adipose tissue Cm (data not shown), weakening a potential role for this prototype ligand of TLR4. Then, we determined that NLRP3, similar to pro-IL-1β, was expressed at a 15-fold higher level in $CD14^+$ macrophages than in other VAT cell fractions (Fig 4D). Immunochemistry targeted against NLRP3 detected the presence of the protein in CLS in obese adipose tissue (Fig 4E). NLRP3 gene expression was increased in VAT $CD14^+$ cells of T2D obese patients (Fig 4F). This up-regulation was confirmed in MoDM cultured in presence of adipose tissue Cm

(Fig 4G). In contrast, gene expression of other inflammasome components, ASC and caspase-1, was unchanged or down-regulated. Despite no change in gene expression, caspase-1 activity assessed by flow cytometry was enhanced in VAT $CD14^+$ cells of obese T2D subjects (Fig 4H). To confirm the role of NLRP3 inflammasome in IL-1β release, VAT $CD14^+$ macrophages were cultured in presence of the sulfonylurea glyburide, a potent NLRP3 inhibitor (43). Glyburide dose-dependently inhibited IL-1β release, while it has no significant effect on inflammasome-independent TNF-α release (Fig 4I). In MoDM, obese CmSAT induced caspase-1 activation and IL-1β secretion to at least one third of the stimulation obtained with LPS plus ATP (Fig 4J, K). In this experimental setting, glyburide also markedly reduced IL-1β release and down regulated caspase-1 activity. Additionally, IL-1β release in CmSAT-stimulated MoDM was reduced, although not significantly, by Z-VAD-fmk caspase-1 inhibition. Thus, we show here for the first time that adipose tissue-derived $CD14^+$ macrophages secrete IL-1β through a NLRP3-dependent mechanism, to which TLR2 ligands might contribute. Moreover, our data suggest that NLRP3 inflammasome is over-activated in the adipose tissue of obese patients with T2D.

VAT of T2D patients is enriched with $IL\text{-}17^+IFN\text{-}\gamma^+$ CD4+ T cells

Considering that IL-1β is a Th17-expanding cytokine, we next explored VAT T cell populations. The number of $CD3^+$ T cells was positively correlated with the number of $CD14^+$ macrophages in the same biopsy, supporting virtual interplay between the adaptive and innate immune compartments in the human adipose tissue (Fig 5A). The vast majority of obese VAT $CD3^+$ T cells displayed a memory phenotype, as assessed by CD45RO positivity in 96 and 81 % of $CD4^+$ and $CD8^+$ cells, respectively (n = 5). As expected, in VAT cell fractions, IL-17 and IFN-γ were almost exclusively expressed in $CD3^+$ cells (Fig 5B). In this cell population, the percentage of $CD4^+$ and $CD8^+$ IFN-γ producing T cells did not change with obesity and T2D (Table 2). With reference to IL-17, $CD4^+$ cells represent the major producers in VAT, which are specifically enriched in obese T2D patients (Fig 5C and Table 2). VAT IL-17 production seems to be driven by double positive $IL\text{-}17^+IFN\text{-}\gamma^+$ $CD4^+$ T cells, as this subset was significantly increased both in percentage and absolute number in these patients (Fig 5D, E). Strikingly, the abundance of the double producers was significantly correlated with Hb1Ac (Fig 5F). These data identify for the first time the presence of $IL\text{-}17^+IFN\text{-}\gamma^+$ $CD4^+$T cells in human adipose tissue and suggest their contribution to obesity-induced T2D.

Paracrine interaction between adipose tissue $CD14^+$ and $CD3^+$ T cells

To substantiate a potential effect of IL-1β on adipose tissue T cells, we measured the abundance of IL-1RI, the signaling receptor of IL-1β, on VAT $CD3^+$ T cells. In flow cytometry analyses, IL-1RI was overexpressed on VAT $CD4^+$ cells from obese T2D patients (Fig 6A, B). Next, we found that when VAT $CD3^+$ T cells were co-cultured with $CD14^+$ macrophage counterparts with anti-CD3 and anti-CD28 polyclonal stimulation, they produce three times more IL-17 than when cultured alone (Fig 6C). In co-cultured cells, IL-17 secretion was drastically impaired upon treatment with anti-human IL-1β blocking antibody or with anakinra (Fig 6 D, E). Besides, stimulation of IL-17 production was similar with and without addition of anti-CD28 factor (Fig 6C). This suggests that VAT $CD14^+$ macrophages deliver a strong co-stimulation to $CD3^+$ cells in addition to providing the cytokine source for Th17 expansion (IL-1β). In line with this possibility, $CD14^+$ cells expressed CD40, CD80 and CD86 co-stimulatory molecules, with an enhancing effect of obesity (Fig 6F, G). Notably, IL-23, an

additional cytokine involved in Th17 expansion, was not detected in VAT $CD14^+$ cell supernatant (data not shown). Since co-culture experiments revealed that macrophage-derived IL-1β increases IL-17 production, we explored a potential reciprocal effect of IL-17 on macrophages. We found that VAT $CD14^+$ cells expressed an IL-17 signaling receptor, IL-17RA, to a slightly higher level in obese than in non-obese subjects (Fig 6H). Accordingly, IL-1β secretion by $CD14^+$ cells was significantly enhanced in presence of $CD3^+$ cells and was hampered by addition of anti-IL-17 neutralizing antibody (Fig 6I, J). These findings argue for an unexpected role for IL-17 to promote IL-1β release by adipose tissue macrophages. We thus identified a new a pro-inflammatory cross-talk between macrophages and T cells that might sustain adipose tissue inflammation in human obesity (Fig 7).

Discussion

In the 10 past years, much has been learnt on obesity-induced changes in the cellular composition of adipose tissue characterized by adjustment in the numbers and the phenotypes of multiple immune cell subsets. While adipose tissue macrophages were initially considered major effectors, the contribution of T cells to adipose tissue inflammation has recently garnered a lot of interest. Immune cells might contribute to a network of local interactions that alter microenvironment and drastically impact the biology of adipose tissue. These interacting actors might be differentially involved in mouse and human conditions. In this original study performed with human samples, we show for the first time that adipose tissue macrophages interact with IL-17 producing T cells *via* a paracrine loop, where IL-1β and IL-17 reciprocally stimulate each other secretion. This is likely to create a pro-inflammatory feed forward cross-talk between the two cell types. To what extent this novel aspect of adipose tissue inflammation participates in the obesity-related metabolic co-morbidities remains to be established. The two clinical conditions explored in this study, i.e. T2D and weight loss, are characterized respectively by insulin resistance and amelioration of insulin sensitivity. Since they provide opposite responses with respect to IL-1β and IL-17 production, this strengthens the implication of these cytokines and their deleterious interplay in obesity-induced alteration of glucose homeostasis.

We identified NLRP3 inflammasome-activated macrophages as the major cellular source of IL-1β in human adipose tissue. In line with preferential accumulation of macrophages in VAT (9, 44), IL-1β release was higher in VAT than in SAT. Importantly, VAT macrophage-derived IL-1β secretion was markedly increased in T2D patients, along with significant up-regulation of TLR2 gene expression and NLRP3 inflammasome activation. Significant correlations between macrophage-derived IL-1β and blood-derived surrogates of insulin resistance stress the pathogenic relevance of adipose tissue IL-1β production. Conversely, the steady decrease of IL-1β release by adipose tissue production might contribute to RYGB-induced amelioration of glycemic status. In line, a recent study showed that IL-1β expression and macrophage accumulation were increased in VAT of insulin-resistant *versus* insulin-sensitive obese subjects (45). On the contrary, unlike IL-1β that was up-regulated in T2D subjects, VAT $CD14^+$ cell-derived IL-18 was not associated with T2D status in massively obese subjects. This does not preclude a role for increased circulating IL-18 levels in metabolic complications in distinct populations (46).

The effect of T2D on IL-1β production in VAT $CD14^+$ cells was faithfully reproduced in MoDM in response to adipose tissue-derived factors. Yet, we do not know what the specific mediators are that elicit IL-1β secretion in

macrophages. Lipid species, such as saturated fatty acids or ceramides that were both shown to activate inflammasome (27, 47, 48) could be implicated. Indeed, explanted human VAT and SAT release significant amounts of free fatty acid in the culture media (data not shown). The contribution of endogenous danger signals arising from damaged adipocytes surrounded by CLS of macrophages might also be implicated (49). Another potential trigger is serum amyloid A (SAA) that is highly secreted by hypertrophied adipocytes, as shown by our group (50) and was recently found to induce NLRP3-dependent IL-1β release in myeloid cells in the context of allergy (51, 52). Since obesity is associated with alterations in gut microbiota and permeability, microbiota-derived PAMPs could participate into TLRs and NLRP3 activation in adipose tissue (53, 54). Considering the abundance and variety of TLRs ligands and NLRP3 activators (22, 55), several biomolecules could collectively activate this pathway in adipose tissue.

IL-1β and IL-17 were coordinately regulated in human adipose tissue. Given the key role of IL-1β to expand Th17 cells, we thus addressed the question of the cell source of adipose IL-17. In mice adipose tissue, IL-17 is predominantly produced by innate γδ T lymphocytes (39), but this specific T cell subset is virtually absent of human adipose tissue ((56) and data not shown). One recent study reported the presence of IL-17 producing cells in adipose tissue $CD3^+$ T cells (40). Here, we show that these cells originate from the $CD4^+$ T cell population and that they are more frequently found in the adipose tissue of obese T2D patients. Strikingly, this increase was driven by specific enrichment of double positive IL-17^+IFN-γ^+ T cells. Based on a mouse model designed to map the fate of IL-17-producing T cells, it has been suggested that chronic inflammatory conditions favor the release of alternative cytokines by Th17 cells (57). Thus, this suggests that VAT IL-17^+IFN-γ^+ cells were originally IL-17-single positive, highlighting the plasticity of $CD4^+$ T cells in obese adipose tissue. IL-1β has been shown to induce IL-17 and IFN-γ producing cells in the $CD45RO^+$ memory T cell subset (58). In line, our results support a role for this cytokine to drive IL-17^+IFN-γ^+ memory T cell accumulation in adipose tissue in the context of obesity-linked T2D. In our population of massively obese subjects, IL-17^+IFN-γ^+ cell proportion was significantly correlated with deterioration of glucose homeostasis reflected by increased HbA1c. Such double producers were already reported in coronary artery of patients with atherosclerosis, where IFN-γ and IL-17 may act synergistically to induce vascular smooth muscle cell inflammation (59). In adipose tissue, IL-17 and IFN-γ, both known to alter metabolic pathways and insulin signaling in adipocytes (39, 56, 60), might cooperate to trigger and perpetuate inflammation and insulin resistance.

Primary cell co-culture experiments revealed reciprocal interactions between adipose tissue $CD14^+$ and $CD3^+$cells, in which IL-1β and IL-17 stimulated each other secretion through their respective cognate receptor. IL-17-producing cells represent a small proportion of adipose tissue $CD3^+$ T cells, while the vast majority produced IFN-γ. Although the contribution of IFN-γ cannot be ruled out, neutralization experiment supports a role for IL-17 to mediate IL-1β secretion by adipose tissue macrophages. In line with this idea, IL-17 has been previously shown to stimulate the secretion of pro-inflammatory cytokines, including IL-1β, in human monocytes (61) and murine macrophages (62). Finally, our data suggest that T2D exacerbates the cross-talk IL-1β and IL-17, since IL-1RI and, to a lesser extent, IL-17RA are increased in adipose tissue $CD4^+$ and $CD14^+$ cells, respectively, in T2D patients.

In several inflammatory conditions, secretion of IL-1β by antigen-presenting

cells is a critical step for inducing IL-17 producers. For instance, gain-of-function NLRP3 mutations lead to excess IL-1β secretion and subsequent Th17 cell expansion in human autoinflammatory diseases (63, 64). Conversely, inhibition of IL-1β action through targeted deletion of NLRP3, caspase-1 or IL-1RI results in reduced Th17 cell abundance and attenuation of experimental autoimmune disease severity (65-67). In the context of metabolic disorders, clinical trials with anakinra or neutralizing IL-1β antibody have been proven efficient to improve glucose homeostasis and alleviate T2D (21, 68), potentially by acting in part on adipose tissue-derived IL-1β. In our hands, VAT $CD14^+$ cell release of IL-1β was potently inhibited by glyburide. However, it is unlikely that the clinically relevant dose of this antidiabetic drug would reach the concentration required for this experimental effect. New molecules that could alleviate adipose tissue NLRP3-dependent IL-1β release will probably be therapeutic candidates. In contrast, no published study has been done assessing anti-IL-17 antibody treatment in obesity, although clinical trials have provided promising results in patients with rheumatoid arthritis or psoriasis (69, 70). The identification of a deleterious relationship between both cytokines in obese adipose tissue opens novel avenues to explore the clinical relevance of their individual or concomitant inhibition to ameliorate glycemic status in human obesity.

Collectively, our data show that adipose tissue macrophages secrete IL-1β in a NLRP3-dependent way that is up-regulated in adipose tissue of obese T2D patients. Besides, we identified a specific enrichment in $CD4^+$ $IL\text{-}17^+IFN\text{-}\gamma^+$ double producers in VAT of these subjects. We further showed that IL-1β and IL-17 mediate a paracrine inflammatory cross-talk between macrophages and T cells in human adipose tissue, where they are likely to exacerbate local inflammation and contribute to adipocyte insulin resistance. We propose that adipose tissue IL-1β creates a potent Th17-expanding microenvironment that is sustained by a reciprocal effect of IL-17 on macrophages. Altogether these findings provide an additional rationale for targeting IL-1β in obesity and T2D.

Material & Methods

Study populations

For this study, we enrolled 3 groups of participants (Table 1): two groups of morbidly obese subjects eligible for laparoscopic Roux-en-Y gastric bypass (RYGP) surgery (groups 1 and 2) and one group of 5 non-obese individuals (NO) involved in programmed surgery for hernia (n = 3), nissen fundoplication or gallbladder ablation (group 3). Clinical and biological variables were assessed at the time of surgery (baseline) in all subjects. NO participants were selected without dyslipidemia, T2D, or chronic inflammatory or infectious diseases. The obese subjects were included in a larger population previously described in details (71). The effect of weight loss was investigated in 20 obese subjects (OB-WL) at 3, 6 and 12 months after the surgery (group 1). In this group, 5 T2D patients were treated with metformin, including 1 patient treated with additional insulin. Four out of 5 T2D subjects were normoglycemic and discontinued their treatment after the surgery. In group 2, obese subjects were segregated in obese (OB) and T2D obese patients (OB/D). The 18 OB/D patients of group 2 were all treated with metformin, including 5 patients with additional insulin. Paired subcutaneous (SAT) and visceral adipose tissue (VAT) biopsies were sampled during surgery in all subjects. In group 1, SAT biopsies were collected by incision of the skin under local anesthesia at month 3, 6 and 12 months after RYGP surgery. The study was conducted in accordance with the Helsinki Declaration and was registered in a public trial registry. The Ethic Committee (CPP Ile-de-France)

approved the clinical investigations for both obese and non-obese individuals. All subjects provided written informed consent.

Adipose tissue explants

SAT and VAT tissue biopsies (0.1g) were minced and incubated in 1 mL of endothelial cell basal medium (Promocell) 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).

Adipose cell isolation and culture

Adipose tissue was digested using collagenase (Roche) as previously described (72) to obtain adipocytes and cells of the stroma vascular fraction (SVF). To avoid contamination by blood leukocytes, the biopsies were extensively washed in PBS and vessels carefully discarded before digestion. SVF cells were resuspended in endotoxin-free PBS supplemented with 2% FCS and 1 mM EDTA. Subsequent isolation of $CD14^+$ and $CD3^+$ cells was performed using positive selection magnetic beads (Stemcell Technologies), according to the manufacturer's instructions. The $CD14^-$ $CD3^-$ cells (Neg) were also recovered. Cells were immediately used for RNA extraction, *in vitro* culture or flow cytometry analyses. $CD14^+$ cell supernatant (10^6 cells/mL) was obtained after 24h of culture in RPMI 1640 medium (Lonza) supplemented with 10% FCS (HyClone). When indicated, $CD14^+$ cells were cultured with 10, 50 or 100 μM glyburide (Sigma-Aldrich). For co-culture experiments, $CD14^+$ cells and $CD3^+$ cells were cultured for 48h at 1/1 ratio in RPMI 1640 medium supplemented with 10% FCS, anti-CD3 (2.5 μg/mL) and anti-CD28 (1 μg/mL) antibodies (R&D Systems) and, when indicated, with IgG1 isotype control (2.5 μg/mL), anti-IL-1β (2.5 μg/mL) or anti-IL-17 (30 ng/mL) neutralizing antibodies (eBioscience), or anakinra (100 ng/mL, Swedish Ophan Biovitrum).

Cell surface marker and intracellular cytokine staining

For analysis of cell surface proteins, Pacific Blue-labeled anti-CD4 (RPA-T4), Alexa -700-labeled anti-CD8 (RPA-T8), FITC-labeled anti-CD45RO (UCHL1), FITC-labeled anti-CD86 (FUN-1) (all from BD Biosciences), PerCP-Cy5.5-labeled anti-CD45 (HI30), Alexa-700-labeled TLR-2 (TL2.1) (all from eBioscience), PE-labeled anti-IL1-RI and control PE-labeled mouse IgG1 (from R&D Systems) were used to characterize freshly immunoselected adipose tissue $CD14^+$ or $CD3^+$ cells. Cells were treated with human 10% AB serum for 15 min, washed, and then suspended in FACS buffer (PBS with 2% AB serum) and stained with appropriate antibodies or isotype controls for 30 min at 4°C in the dark. Caspase-1 activity was assessed in $CD14^+$ cells or in MoDM using FAM-FLICA caspase-1 kit (Eurovision), according to the manufacturer's instructions. Intracellular cytokine analysis of $CD3^+$ cells was performed in cells stimulated with phorbol-12-myristate-13-acetate (30 ng/mL, Sigma-Aldrich) and ionomycin (1μg/mL, Alexis Biochemicals) at 37°C for 6 hours with GolgiStop (BD Biosciences) for the last 3 hours. Cells were then permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and incubated for 40 min at room temperature in the dark with PE-labeled anti-IL-17 (ebio64DEC17, eBioscience) and FITC-labeled anti-IFN-γ (4S.B3, BD Biosciences). Data were acquired on a FACSCanto II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences) and were analyzed with FACSDiva or FlowJo 9.4 software (Treestar) with doublet exclusion based on forward and side scatter.

Monocyte-derived macrophages
Human PBMC were isolated from enriched buffy coats of healthy volunteer donors (Etablissement français du sang, Hôtel-Dieu hospital) by Ficoll density gradient centrifugation (PAA). Monocytes were purified by negative selection using Monocyte Isolation kit II (Miltenyi Biotec) and differentiated (5.10^5 cells/mL) for 6 days in RPMI 1640 medium supplemented with 10% FCS and 100 ng/mL of M-CSF (Miltenyi Biotec) to obtain monocyte-derived macrophages (MoDM). MoDM were cultured in RPMI 1640 medium supplemented with adipose tissue conditioned media (1/10) for 18h, with addition of Z-VAD-fmk caspase inhibitor (10 µg/mL, Invivogen) or glyburide (50 µM, Sigma-Aldrich) when indicated. The cells were then either directly processed for RNA extraction or cultured for 24h in fresh RPMI to assess IL-1β secretion. As positive control, MoDM were treated with LPS (100 ng/mL; Sigma-Aldrich) for 18h and ATP (5mM, Invivogen) for 30 minutes.

Cytokine measurement
Adipose tissue conditioned media were analyzed for cytokines using 27 Bio-Plex Pro Human Cytokine (Bio-Rad). LPS contamination was assessed with ToxinSensor chromogenic LAL endotoxin assay kit (GenScript). IL-1β, IL-6, TNF-α, IL-18 and IL-17 were analyzed by specific ELISA in immunoisolated cells and MoDM culture supernatants (eBioscience and R&D Systems).

Real-Time PCR
Adipose tissue freshly isolated cells and MoDM were processed for RNA extraction using the RNeasy RNA Mini Kit (Invitrogen). RNA quality was monitored using Agilent 2100 bioanalyzer. Complementary DNAs were synthesized from and prepared with Supercript II reverse transcriptase (Invitrogen). SYBR green primers were designed by manufacturer (Roche or Qiagen) and used for qRT-PCR using the 7300 real time PCR system (Applied Biosystem). 18S was used for normalization and for relative quantification of gene expression.

Immunochemistry
Immunohistochemical stains were performed on human adipose tissue paraffin-embedded sections using antibodies targeted to CD68 (KP1; DAKO) or NLRP3 (Sigma-Aldrich). Slides were counterstained with Mayer's hematoxylin. Protocol was performed as previously described in details elsewhere (73) Section images were taken by a Zeiss 20 Axiostar Plus microscope (Zeiss), acquired by a TRI CCD camera (Sony) and processed using Perfect Image V7.7. Adipocyte diameters were determined with computer image analysis of histological sections.

Statistical analyses
Data are shown as mean ± SEM. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Sotware Inc.). Differences between groups were determined by non parametric paired Wilcoxon or unpaired Mann Whitney comparative tests. Correlations were assessed by non parametric Spearman's test. For visual purposes, straight lines were added based on linear regression analysis. Differences were considered significant when $p < 0.05$.

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Figure 1

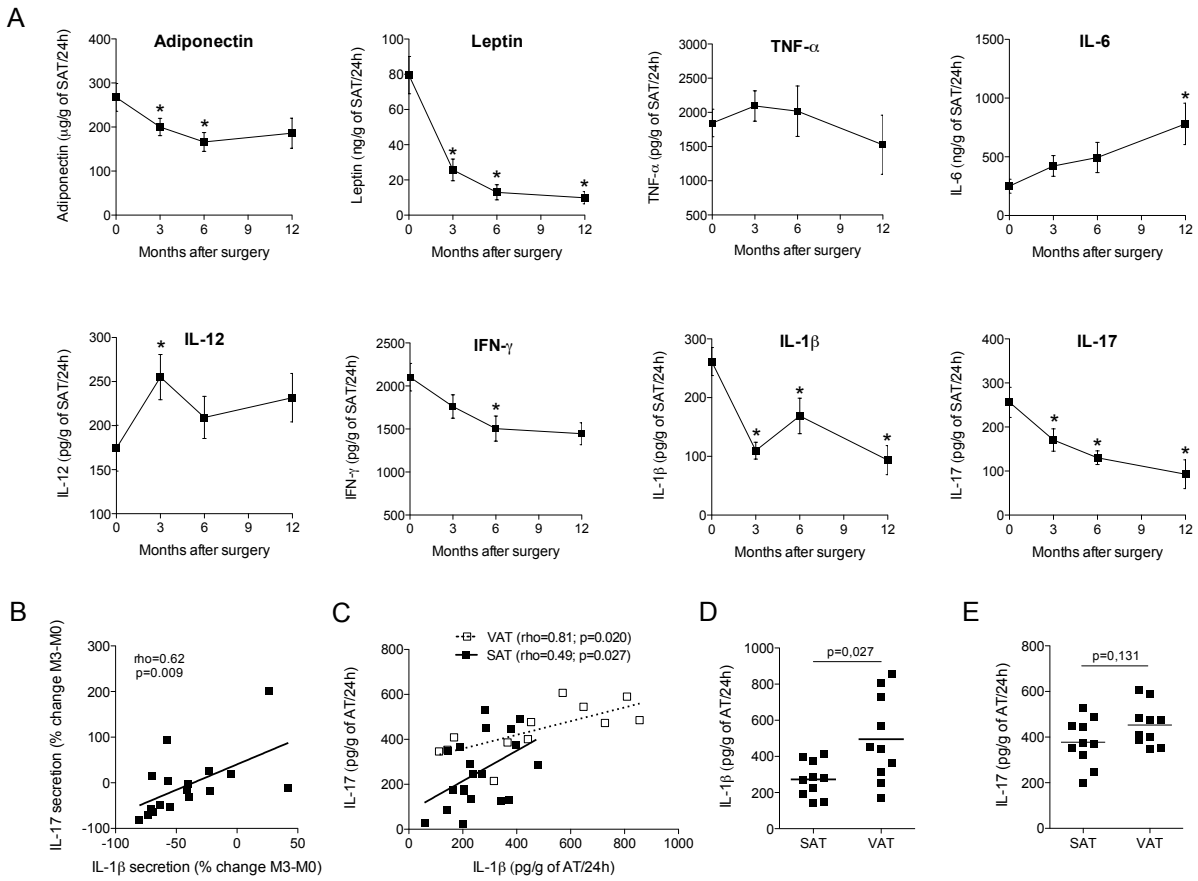
A
Adiponectin
Adiponectin (µg/g of SAT/24h)
Leptin
Leptin (ng/g of SAT/24h)
TNF-α
TNF-α (pg/g of SAT/24h)
IL-6
IL-6 (ng/g of SAT/24h)
IL-12
IL-12 (pg/g of SAT/24h)
IFN-γ
IFN-γ (pg/g of SAT/24h)
IL-1β
IL-1β (pg/g of SAT/24h)
IL-17
IL-17 (pg/g of SAT/24h)
Months after surgery
B
rho=0.62
p=0.009
IL-17 secretion (% change M3-M0)
IL-1β secretion (% change M3-M0)
C
VAT (rho=0.81; p=0.020)
SAT (rho=0.49; p=0.027)
IL-17 (pg/g of AT/24h)
IL-1β (pg/g of AT/24h)
D
p=0,027
IL-1β (pg/g of AT/24h)
SAT
VAT
E
p=0,131
IL-17 (pg/g of AT/24h)
SAT
VAT

Figure 2

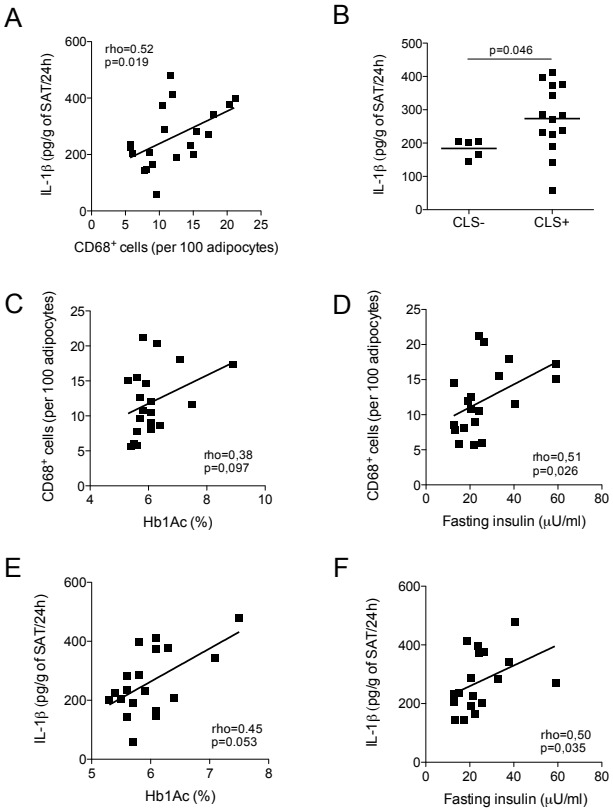
A
rho=0.52
p=0.019
IL-1β (pg/g of SAT/24h)
CD68+ cells (per 100 adipocytes)
B
p=0.046
IL-1β (pg/g of SAT/24h)
CLS-
CLS+
C
CD68+ cells (per 100 adipocytes)
rho=0,38
p=0,097
Hb1Ac (%)
D
CD68+ cells (per 100 adipocytes)
rho=0,51
p=0,026
Fasting insulin (µU/ml)
E
IL-1β (pg/g of SAT/24h)
rho=0.45
p=0.053
Hb1Ac (%)
F
IL-1β (pg/g of SAT/24h)
rho=0,50
p=0,035
Fasting insulin (µU/ml)

Figure 3

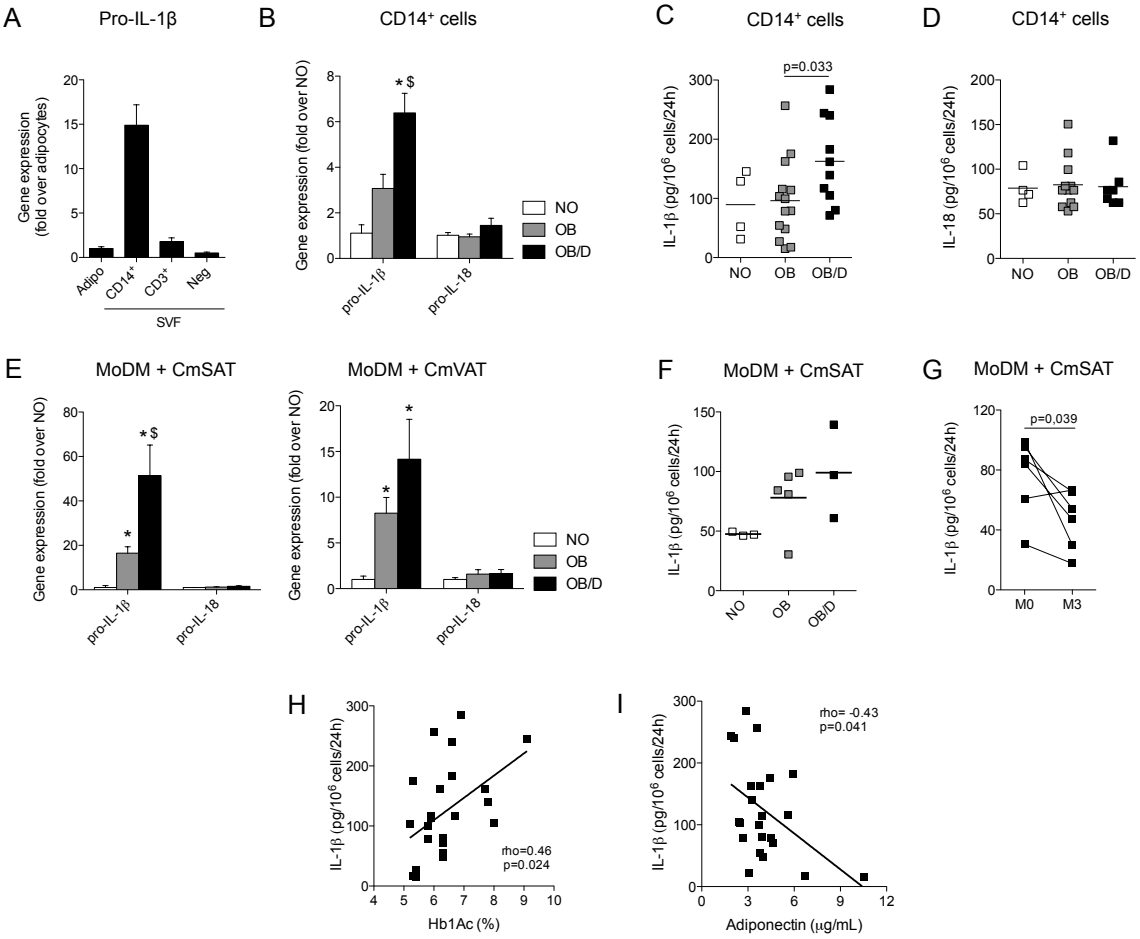
A
Pro-IL-1β
Gene expression (fold over adipocytes)
Adipo
CD14+
CD3+
Neg
SVF
B
CD14+ cells
Gene expression (fold over NO)
pro-IL-1β
pro-IL-18
NO
OB
OB/D
C
CD14+ cells
p=0.033
IL-1β (pg/10^6 cells/24h)
NO
OB
OB/D
D
CD14+ cells
IL-18 (pg/10^6 cells/24h)
NO
OB
OB/D
E
MoDM + CmSAT
MoDM + CmVAT
Gene expression (fold over NO)
pro-IL-1β
pro-IL-18
NO
OB
OB/D
F
MoDM + CmSAT
IL-1β (pg/10^6 cells/24h)
NO
OB
OB/D
G
MoDM + CmSAT
p=0,039
IL-1β (pg/10^6 cells/24h)
M0
M3
H
IL-1β (pg/10^6 cells/24h)
rho=0.46
p=0.024
Hb1Ac (%)
I
IL-1β (pg/10^6 cells/24h)
rho= -0.43
p=0.041
Adiponectin (μg/mL)

Figure 4

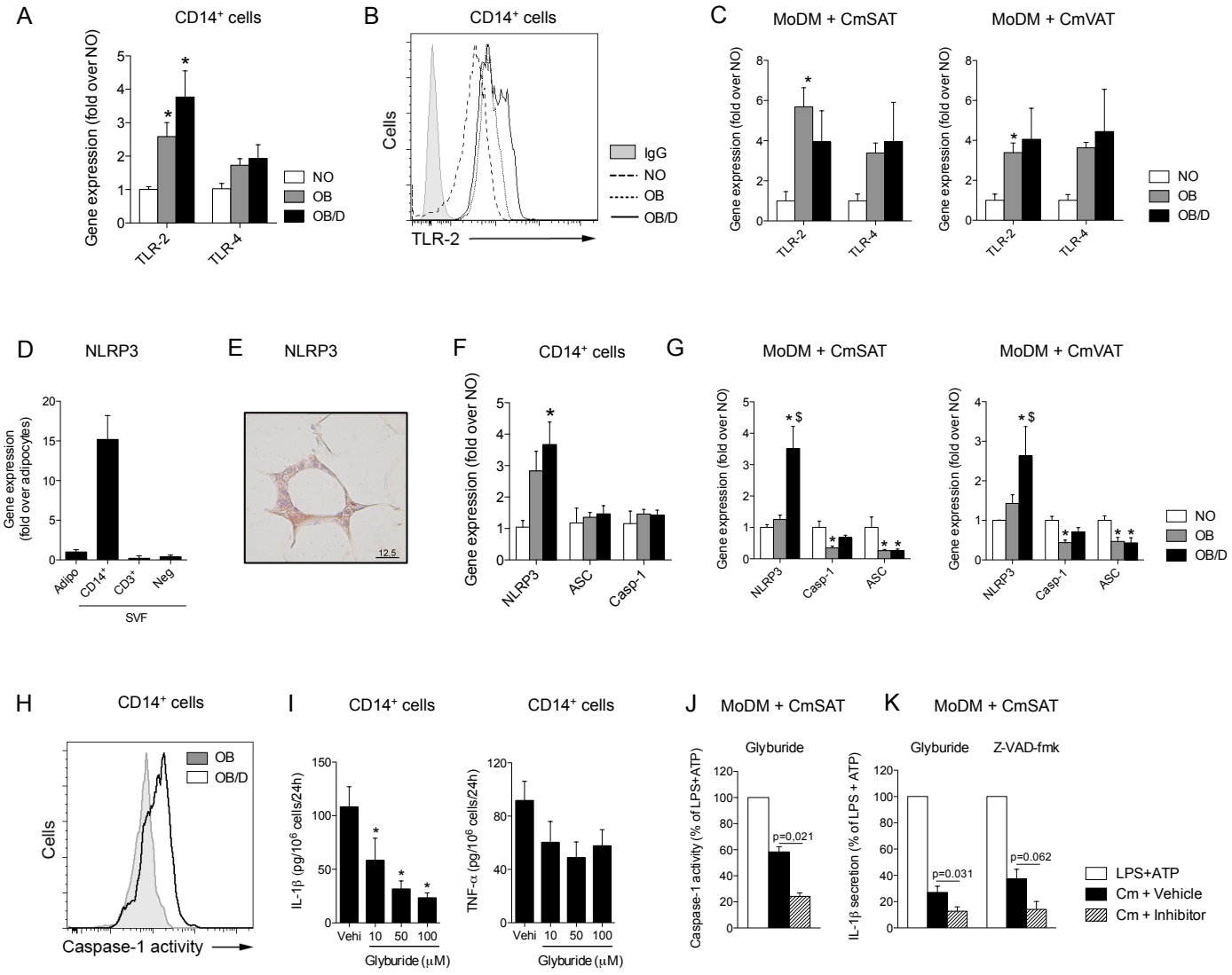
A
CD14+ cells
Gene expression (fold over NO)
TLR-2
TLR-4
NO
OB
OB/D
B
CD14+ cells
Cells
TLR-2
IgG
NO
OB
OB/D
C
MoDM + CmSAT
MoDM + CmVAT
Gene expression (fold over NO)
TLR-2
TLR-4
NO
OB
OB/D
D
NLRP3
Gene expression (fold over adipocytes)
Adipo
CD14+
CD3+
Neg
SVF
E
NLRP3
12.5
F
CD14+ cells
Gene expression (fold over NO)
NLRP3
ASC
Casp-1
G
MoDM + CmSAT
MoDM + CmVAT
Gene expression (fold over NO)
NLRP3
Casp-1
ASC
NO
OB
OB/D
H
CD14+ cells
Cells
OB
OB/D
Caspase-1 activity
I
CD14+ cells
IL-1β (pg/10^6 cells/24h)
Vehi
10
50
100
Glyburide (μM)
CD14+ cells
TNF-α (pg/10^6 cells/24h)
Vehi
10
50
100
Glyburide (μM)
J
MoDM + CmSAT
Glyburide
Caspase-1 activity (% of LPS+ATP)
p=0.021
K
MoDM + CmSAT
Glyburide
Z-VAD-fmk
IL-1β secretion (% of LPS + ATP)
p=0.031
p=0.062
LPS+ATP
Cm + Vehicle
Cm + Inhibitor

Figure 5

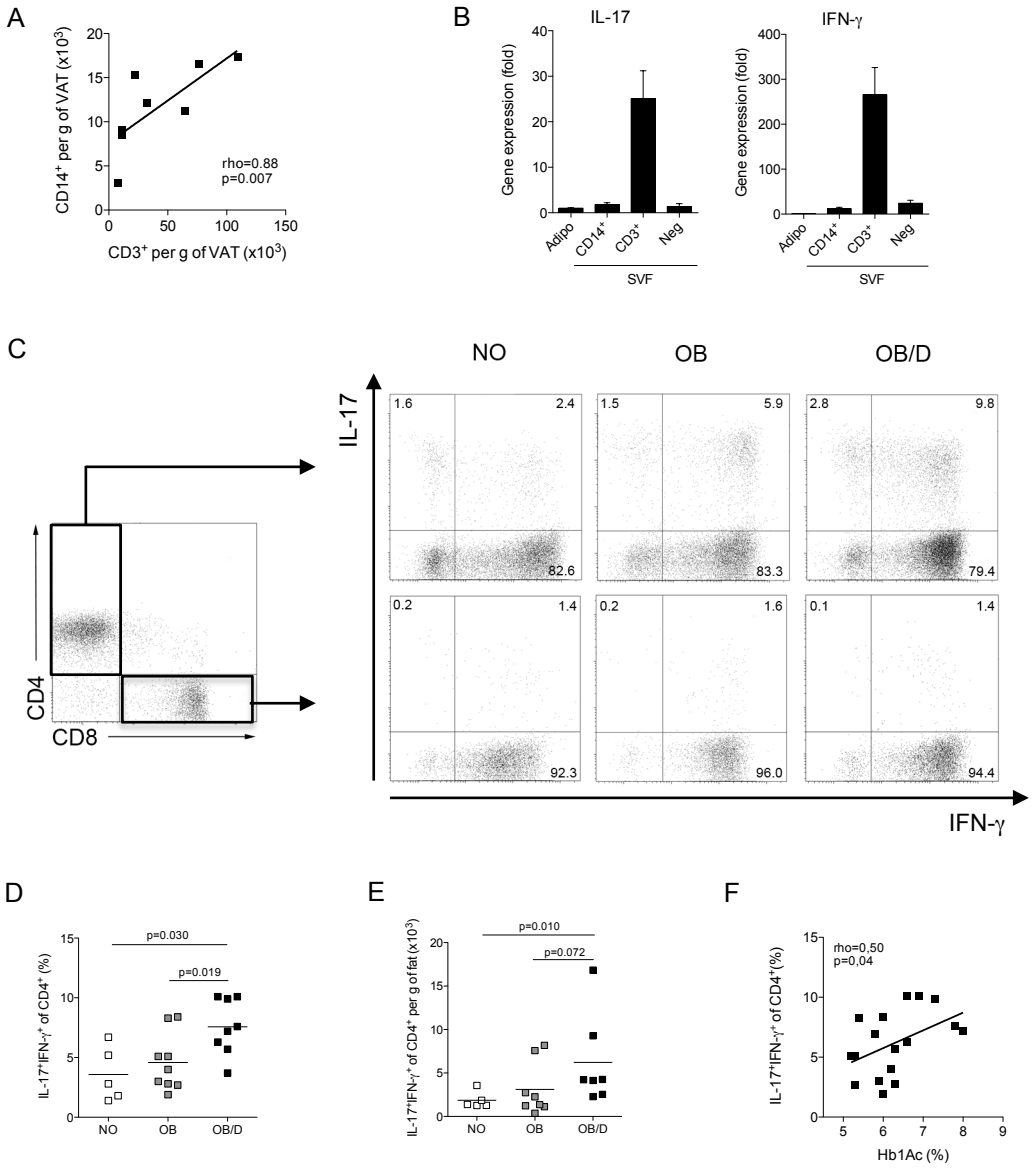
A
CD14+ per g of VAT (x10³)
CD3+ per g of VAT (x10³)
rho=0.88
p=0.007
B
IL-17
IFN-γ
Gene expression (fold)
Adipo
CD14+
CD3+
Neg
SVF
C
NO
OB
OB/D
IL-17
IFN-γ
CD4
CD8
1.6
2.4
82.6
1.5
5.9
83.3
2.8
9.8
79.4
0.2
1.4
92.3
0.2
1.6
96.0
0.1
1.4
94.4
D
IL-17+IFN-γ+ of CD4+ (%)
p=0.030
p=0.019
NO
OB
OB/D
E
IL-17+IFN-γ+ of CD4+ per g of fat (x10³)
p=0.010
p=0.072
F
IL-17+IFN-γ+ of CD4+(%)
rho=0,50
p=0,04
Hb1Ac (%)

Figure 6

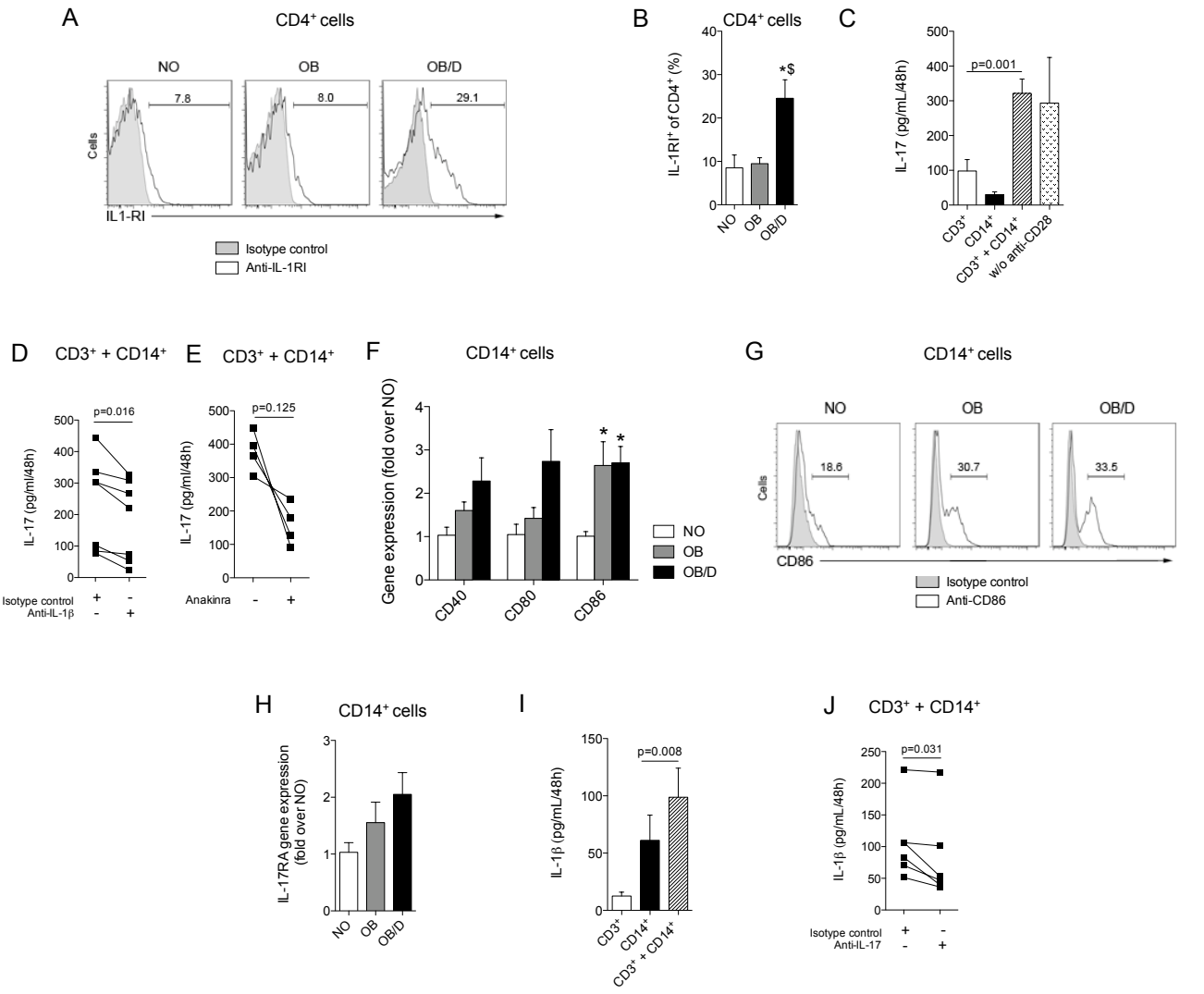
A
CD4+ cells
NO
OB
OB/D
7.8
8.0
29.1
Cells
IL1-RI
Isotype control
Anti-IL-1RI
B
CD4+ cells
IL-1RI+ of CD4+ (%)
*$
NO
OB
OB/D
C
IL-17 (pg/mL/48h)
p=0.001
CD3+
CD14+
CD3+ + CD14+
w/o anti-CD28
D
CD3+ + CD14+
IL-17 (pg/ml/48h)
p=0.016
Isotype control
Anti-IL-1β
E
CD3+ + CD14+
IL-17 (pg/ml/48h)
p=0.125
Anakinra
F
CD14+ cells
Gene expression (fold over NO)
CD40
CD80
CD86
NO
OB
OB/D
G
CD14+ cells
NO
OB
OB/D
18.6
30.7
33.5
Cells
CD86
Isotype control
Anti-CD86
H
CD14+ cells
IL-17RA gene expression (fold over NO)
NO
OB
OB/D
I
IL-1β (pg/mL/48h)
p=0.008
CD3+
CD14+
CD3+ + CD14+
J
CD3+ + CD14+
IL-1β (pg/mL/48h)
p=0.031
Isotype control
Anti-IL-17

Figure 7

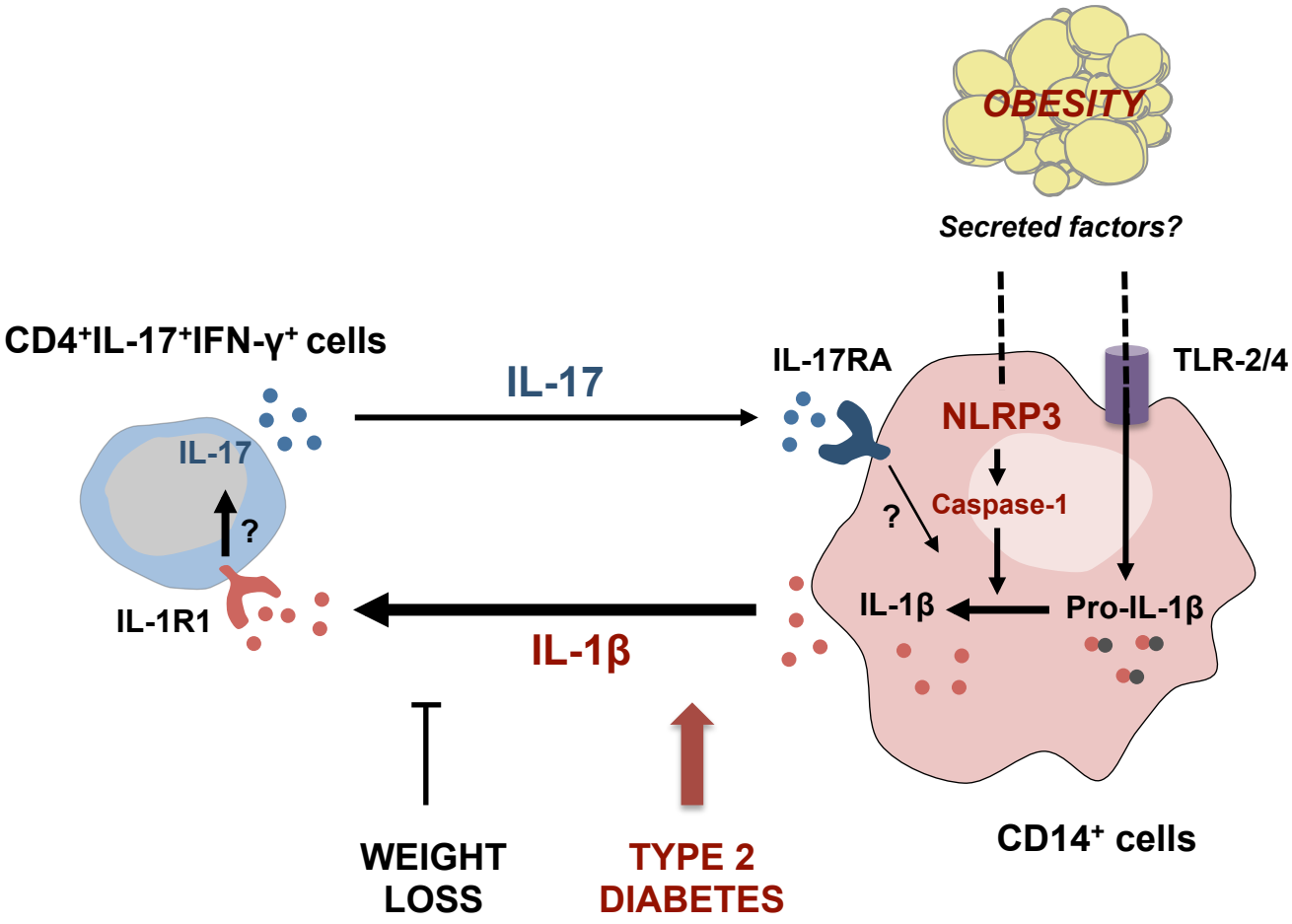
OBESITY
Secreted factors?
CD4+IL-17+IFN-γ+ cells
IL-17
IL-17RA
TLR-2/4
NLRP3
IL-17
?
Caspase-1
?
IL-1β
Pro-IL-1β
IL-1R1
IL-1β
WEIGHT LOSS
TYPE 2 DIABETES
CD14+ cells

Figure legends

Figure 1: Effect of weight loss on cytokine and adipokine release by human adipose tissue

A Release of adiponectin, leptin, TNF-α, IL-6, IL-12, IFN-γ, IL-1β and IL-17 by subcutaneous adipose tissue (SAT) in obese subjects of group 1 (months 0, 3, 6: n = 20; month 12: n = 10). * $p < 0.05$ *versus* month 0. **B** Correlation between changes in IL-1β and IL-17 release between month 0 (M0) and month 3 (M3) post-surgery (n = 20). **C** Correlations between IL-1β and IL-17 release in SAT (n = 20) and VAT (n = 12) of obese subjects at month 0. **D, E** Comparison of IL-1β (D) and IL-17 (E) release by SAT and visceral adipose tissue (VAT) of obese subjects at month 0 (n = 10).

Figure 2: IL-1β release by subcutaneous adipose tissue (SAT): relation with $CD68^+$ cell accumulation and diabetic status

A Correlation between SAT IL-1β release and the number of $CD68^+$ cells in SAT paraffin sections of group 1 obese subjects (n= 20). **B** IL-1β release by SAT biopsies with macrophage crown-like structures (CLS+) or without (CLS-) (n = 20). **C, D** Correlations between SAT $CD68^+$ cell number and Hb1Ac (C) or fasting insulin (D) in group 1 obese subjects (n = 20). **E, F** Correlations between SAT IL-1β release and Hb1Ac (E) or fasting insulin (F) in group 1 obese subjects (n = 20).

Figure 3: Effect of obesity and type 2 diabetes on IL-1β production by adipose tissue $CD14^+$ macrophages

A Pro-IL-1β gene expression in visceral adipose tissue (VAT) cell fractions of group 2 obese subjects (n = 10). Adipo: adipocytes; SVF: stroma vascular fraction. Neg: $CD14^-CD3^-$ cells. **B** pro-IL-1β and pro-IL-18 gene expression in $CD14^+$ cells immunoisolated from VAT of 4 non obese (NO) subjects and 16 (OB) and 12 diabetic (OB/D) obese subjects of group 2. * $p < 0.05$ *versus* NO; $ $p < 0.05$ *versus* OB. **C, D** IL-1β (C) and IL-18 (D) secretion by VAT $CD14^+$ cells of NO (n = 4), OB (n = 14) and OB/D (n = 10) subjects. $ $p < 0.05$ *versus* OB. **E** pro-IL-1β and pro-IL-18 gene expression in monocytes-derived macrophages (MoDM) in response to conditioned media of SAT (CmSAT) or VAT (CmVAT) from NO (n = 3), OB (n = 11) and OB/D (n = 5) subjects of group 1 and 3.* $p < 0.05$ *versus* NO; $ $p < 0.05$ *versus* OB. **F** IL-1β release by MoDM in response to CmSAT from NO (n = 3), OB (n = 5) and OB/D (n = 3) subjects. **G** IL-1β release by MoDM in response to CmSAT obtained in 5 obese subjects of group 1, before (M0) and 3 months (M3) after RYGB surgery. **H, I** Correlations between VAT $CD14^+$ cell-derived IL-1β and Hb1Ac (H) or circulating adiponectin (I) in OB (n = 14) and OB/D (n = 10) subjects of group.

Figure 4: IL-1β release by VAT $CD14^+$ cells is NLRP3/caspase-1-dependent

A Gene expression of TLR-2 and TLR-4 in $CD14^+$ cells immunoisolated from visceral adipose tissue (VAT) of 4 non obese (NO) subjects and 16 (OB) and 12 diabetic (OB/D) obese subjects of group 2. * $p < 0.05$ versus NO. **B** $CD14^+$ macrophages immunoisolated from VAT of NO, OB and OB/D subjects were analyzed for TLR-2 expression by flow cytometry. **C** Gene expression of TLR-2 and TLR-4 in monocyte-derived macrophages (MoDM) in response to conditioned media of SAT (CmSAT) and VAT (Cm VAT) from NO (n = 3), OB (n = 11) and OB/D (n = 5) subjects. * $p < 0.05$ *versus* NO. **D** NLRP3 gene expression in VAT cell fractions in group 2 obese subjects (n = 10). Adipo: adipocytes; SVF: stroma vascular fraction. Neg: $CD14^-CD3^-$ cells. **E** Representative immunostaining targeted to NLRP3 in obese VAT. **F** Gene expression of NLRP3, ASC and caspase-1 (casp-1) in VAT $CD14^+$ cells of NO (n = 4), OB (n = 16) and OB/D (n = 12) subjects. * $p < 0.05$ *versus* NO. **G** Gene expression of NLRP3, ASC and caspase-1 (casp-1) in MoDM in response to CmSAT and Cm VAT from NO (n = 3), OB (n = 11) and OB/D (n = 5) subjects. * $p < 0.05$ versus NO; $ $p < 0.05$ *versus* OB. **H** Representative profile of caspase-1 activity assessed by FLICA fluorescent staining in VAT $CD14^+$ cells of 3 OB and 2 OB/D subjects. **I** IL-1β and TNF-α

release by VAT $CD14^+$ macrophages treated with incremental doses of glyburide (n=5 separate culture experiments). * $p < 0.05$ *versus* vehicle (Vehi). **J** Caspase-1 activity in MoDM treated with LPS+ATP (open bar), CmSAT (black bar) or CmSAT + 50 µM glyburide (hatched bar) in 3 independent culture experiments. **K** IL-1β release by MoDM in response to LPS+ATP (open bar), CmSAT (black bar) or CmSAT + glyburide (50 µM) or Z-VAD-fmk (20 µM) as indicated (hatched bar) in 6 independent culture experiments.

Figure 5: IL-17^+IFN-γ^+ double producing CD4+ T cells in human adipose tissue: effect of type 2 diabetes

A Correlation between $CD14^+$ and $CD3^+$ cell number in visceral adipose tissue (VAT) stroma vascular fraction of NO (n = 3) and OB (n = 5) subjects. **B** IL-17 and IFN-γ gene expression in VAT cell fractions in group 2 obese subjects (n = 10). Adipo: adipocytes; SVF: stroma vascular fraction. Neg: $CD14^-$ $CD3^-$ cells. **C** $CD3^+$ T cells immunoisolated from VAT of NO, OB and OB/D subjects were analyzed by flow cytometry. Samples were gated for CD4 and CD8 and examined for intracellular production of IL-17 and IFN-γ. **D, E** Percentage of VAT IL-17^+IFN-γ^+ cells among $CD4^+$ cells (D) and number of VAT IL-17^+IFN-γ^+ cells of $CD4^+$ per gram of fat (E) in 5 NO, 8-9 OB and 7-8 OB/D. **F** Correlation between the percentage of VAT IL-17^+IFN-γ^+ cells among $CD4^+$ cells and Hb1Ac in 9 OB and 8 OB/D of group 2 obese subjects.

Figure 6: Paracrine loop between $CD14^+$ and $CD3^+$ cells in adipose tissue

A $CD3^+$ T cells immunoisolated from VAT of NO, OB and OB/D subjects were gated for CD4 and examined for IL-1RI expression. **B** Percentage of $CD4^+$IL-$1RI^+$ cells in VAT of NO (n = 3), OB (n = 4) and OB/D (n = 5). * $p < 0.05$ *versus* NO; $^{\$}$ $p < 0.05$ *versus* OB. **C** IL-17 release by VAT $CD3^+$ cells co-cultured with VAT $CD14^+$ cells upon anti-CD3 activation and in the presence (n = 11) or absence (n = 3) of anti-CD28 co-stimulation. **D, E** IL-17 release by VAT $CD3^+$ and $CD14^+$ cell co-culture upon anti-CD3 and anti-CD28 stimulation, in the presence of isotype control, anti-IL-1β blocking antibody (D, n = 7) or anakinra (E, n = 4). **F** Gene expression of CD40, CD80 and CD86 in VAT $CD14^+$ cells of NO (n = 4), OB (n = 16) and OB/D (n = 12) subjects. * $p < 0.05$ *versus* NO. **G** $CD14^+$ cells immunoisolated from VAT of NO, OB and OB/D subjects were analyzed for CD86 expression by flow cytometry. **H** Gene expression of IL-17RA in VAT $CD14^+$ cells of NO (n = 4), OB (n = 16) and OB/D (n = 12) subjects. * $p < 0.05$ *versus* NO. **I** IL-1β release by VAT $CD14^+$ cells co-cultured with VAT $CD3^+$ cells upon anti-CD3 and anti-CD28 stimulation. **J** IL-1β release by VAT $CD3^+$ and $CD14^+$ cell co-culture upon anti-CD3 and anti-CD28 stimulation and in the presence of isotype control or anti-IL-17 blocking antibody (n = 6).

Figure 7: Hypothetical model of macrophage-derived IL-1β and lymphocyte-derived IL-17 interplay in obese adipose tissue. In the presence of yet unknown adipose tissue secreted factors, $CD14^+$ macrophages produced IL-1β in a NLRP3-dependent manner. IL-1β targets $CD4^+$ T cells (defined as $CD4^+$IL-17^+IFN-γ^+) to secrete IL-17 that, in turn, sustains a pro-inflammatory feed-forward cascade in obesity. This adipose tissue cross-talk is worsened in the context of obesity-induced T2D and tempered during weight loss.

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

	Group 1				Group 2		Group 3
	OB-M0	OB-M3	OB-M6	OB-M12	OB	OB/D	NO
Months post-surgery	0	3	6	12	0	0	0
Number of subjects	20	20	20	20	23	18	5
Sex (F/M)	16/4				20/3	11/7	3/2
Age (years)	41.9 ± 2.9				44.7 ± 2.3	46.1 ± 2.8	60.2 ± 7.3
BMI (kg/m^2)	50.7 ± 2.2	42.0 ± 1.8*	38.4 ± 1.7*	34.8 ± 1.7*	47.7 ± 1.5$^{\#}$	50.1 ± 1.7$^{\#}$	27.6 ± 0.7
Number of diabetic (T2D) subjects	4	1	1	0	0	18	0
Glycemia (mmol/L)	5.9 ± 0.2	5.1 ± 0.2*	4.7 ± 0.1*	4.8 ± 0.3*	5.4 ± 0.1	7.5 ± 0.9$^{\# \$}$	5.0 ± 0.4
Insulinemia (µU/mL)	27.1 ± 3.3	10.9 ± 0.9*	10.4 ± 1.3*	10.8 ± 1.1*	23.0 ± 1.6	18.3 ± 2.6	
Hb1Ac (%)	6.1 ± 0.2	5.6 ± 0.1*	5.6 ± 0.1*	5.7 ± 0.2*	5.8 ± 0.1	7.3 ± 0.3$^{\$}$	
Leptin (ng/mL)	49.6 ± 6.0	26.5 ± 3.3*	20.7 ± 2.3*	23.3 ± 2.9*	84.2 ± 6.4	72.5 ± 7.7	
Adiponectin (µg/mL)	5.1 ± 0.5	5.1 ± 0.5	5.1 ± 0.5	4.9 ± 0.5	4.8 ± 0.4	3.4 ± 0.3$^{\$}$	
Adipocyte ∅ (µm)$^{\&}$	86.7 ± 2.2	76.3 ± 2.3*	66.4 ± 2.3*	64.5 ± 2.0* ^{a}			
$CD68^{+}$ cells (per 100 adipocytes)$^{\&}$	12.1 ± 1.0	11.7 ± 1.5	8.7 ± 0.9*	11.4 ± 1.6 ^{a}			
Number of subjects with CLS$^{\&}$	17	8	8	3 ^{a}			

BMI: body mass index; T2D: type-2 diabetes; ∅ : diameter; CLS: crown-like structure. $^{\&}$ in subcutaneous adipose tissue (SAT). Data are shown as mean ± SEM. * $p < 0.05$ *versus* OB-M0; $^{\#}$ $p < 0.05$ *versus* NO; $^{\$}$ $p < 0.05$ *versus* OB; ^{a} assessed in 10 women.

Table 2: Proportions of IFN-γ and IL-17 producing cells in CD3+ T cells of visceral adipose tissue.

VAT $CD3^+$ T cells		**NO**	**OB**	**OB/D**
Number of subjects		5	9	8
$IFN\text{-}\gamma^+$ cells	$CD4^+$ cells	87.8 ± 3.4	82.3 ± 3.5	88.7 ± 1.2
	$CD8^+$ cells	94.1 ± 1.4	91.6 ± 1.7	95.7 ± 0.9
$IL\text{-}17^+$ cells	$CD4^+$ cells	5.1 ± 1.9	6.7 ± 1.1	9.5 ± 0.9*****
	$CD8^+$ cells	2.2 ± 1.1	1.7 ± 0.3	1.9 ± 0.4

Data show the percentage of IFN-γ and IL-17 producing cells in immunoselected VAT $CD3^+$ T cells gated on $CD4^+$ or $CD8^+$ populations. Visceral adipose tissue (VAT) was obtained from the indicated number of subjects included in group 2 and 3 of study participants. Data are shown as mean ± SEM. ***** $p < 0.05$ *versus* NO.

3. Complementary results & comments

3.1. Regional fat differences in IL-1β/IL-1ra production

In article #3, we confirmed the inflammatory status of visceral adipose tissue with increased adipose tissue-derved IL-1β compared to subcutaneous counterpart. One could wonder whether IL-1 receptor antagonist was also very much increased in visceral fat, suggesting strong regulation of local IL-1β signaling. As illustrated in figure 22, IL-1ra release was not increased in visceral compared to subcutaneous depots and the IL-1ra/IL-1β ratio tended to decrease in the visceral depot. These observations implie that visceral adipose tisse is associated with both increased IL-1β production and potentially, enhanced IL-1β signaling compared to subcutaneous depot.

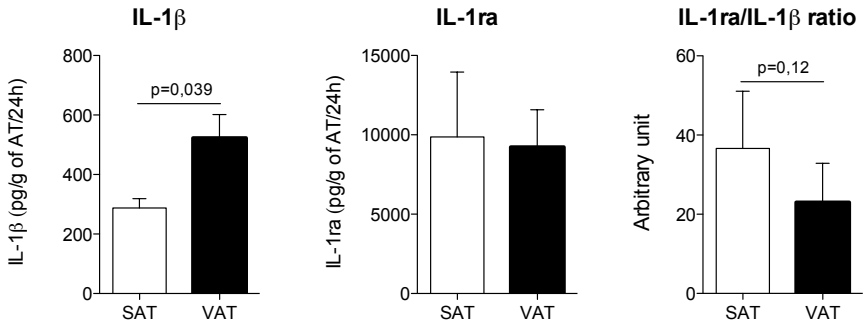

Figure 22 | Release of IL-1β and IL-1ra by subcutaneous (SAT) and visceral (VAT) adipose tissue. Secretion rates were measured in 9 paired fat biopsies from obese subjects.

3.2. Additional inflammatory cytokines secreted by subcutaneous adipose tissue during weight loss

During article #3, we measured several inflammatory cytokines in adipose tissue conditioned media obtained from culture explants during weight loss. Of note, it is interesting to note that kinetic profile of IL-1β release by adipose tissue explants resembles the variation pattern observed with circulating factors in article #1 and $CD14^{dim}CD16^{+}$ monocytes of article #2, suggesting common regulatory processes.

As illustrated in figure 23, we also assessed anti-inflammatory cytokines. We observed that

IL-1ra and IL-10 were significantly decreased after surgery. Production of IL-1ra is induced by many of the stimuli that usually induce IL-1β in the same cell (Juge-Aubry et al., 2003). Similarly, macrophages are known to secrete IL-10 after activation with various ligands, mainly of the TLRs (Sabat et al., 2010). Hence, so-called anti-inflammatory cytokines might be regulators of inflammation to control the production of inflammatory mediators and prevent exacerbated tissue damage (see also Table 2). Drops in IL-10 and IL-1ra are likely to be considered as the hallmarks of decreased inflammation in adipose tissue during weight loss.

On the other hand, while IL-4 remained roughly stable, IL-13 was the only factor to be significantly up-regulated in adipose tissue during weight loss. Both IL-4 and IL-13 are protypical Th2 lymphocyte cytokines. Number of Th2 lymphocytes in adipose tissue is likelyto be reduced with obesity, although results are contradictory (see Introduction section). Perhaps, up-regulation of IL-13 secretion might reflect an infiltration of Th2 cells within adipose tissue upon weight loss. IL-13 could induce M2-like macrophage polarization and promotes local regulation of adipose tissue inflammation (Mosser and Edwards, 2008). Consistently, publication from the laboratory showed that subcutaneous adipose tissue macrophages are of a (M1-like) pro-inflammatory phenotype at the obese state, whereas it might shift towards a less pro-inflammatory (M2-like) profil during weight loss (Aron-Wisnewsky et al., 2009).

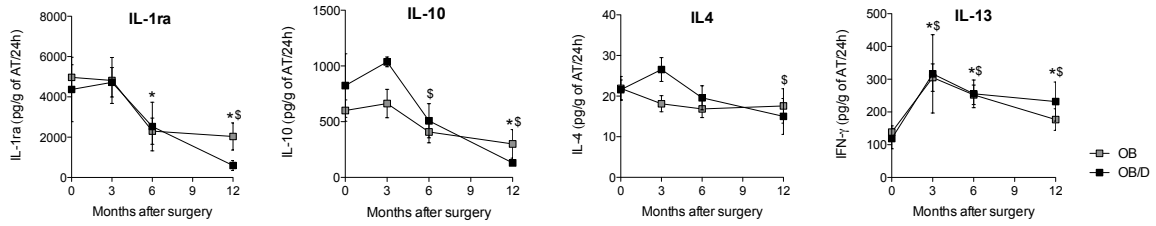

Figure 23 | Release of anti-inflammatory factors by adipose tissue during surgery-induced weight loss. Secretion rates were measured in subcutaneous adipose tissue during gastric surgery-induced weight loss in 16 obese (NO) and 4 diabetic obese (OB/D) subjects (*, p<0,005 versus baseline for OB; $, p<0,005 versus basline for OB/D; Wilcoxon comparative test).

3.3. Pro-angiogenic factors secreted by subcutaneous adipose tissue during weight loss

Considering the crucial role of adipose tissue vascularization and related hypoxia in obesity, we measured fat secretion rates of three major angiogenic factors: VEGF, bFGF and PDGF-

BB in the same conditioned media obtained after surgery. To our surprise, all factors drastically increased during weight loss as shown in figure 24.

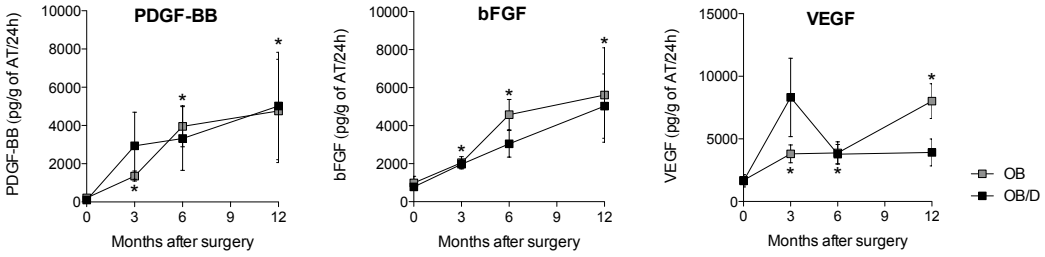

Figure 24 | Release of pro-angiogenic growth factors by adipose tissue during surgery-induced weight loss. Secretion rates were measured in subcutaneous adipose tissue during gastric surgery-induced weight loss in 16 obese (NO) and 4 diabetic obese (OB/D) subjects (*, p<0,005 versus baseline for OB; $, p<0,005 versus basline for OB/D; Wilcoxon comparative test).

On one hand, VEGF, whose function has been extensively studied, is a major inducer of endothelial cells proliferation and migration and it seems to play a key role in adipose tissue angiogenessis in obesity (Ledoux et al., 2008; Neufeld et al., 1999). On the other hand, experimental study showed that PDGF-BB and bFGF may synergize to establish stable and functional vascular network, while single angiogenic factor could not (Cao et al., 2003).

These results suggest that vascularization should be increased in adipose tissue during weight loss. In that way, we tried to quantify vessels in subcutaneous adipose tissue. To do so, immunohistochemistry targeted for Von Willebrand Factor (VWF), a useful marker for endothelial cells, was performed in 5 obese subjects at the time of surgery and 3 and 6 months after the surgery. Two analyses were performed. First, surface of VWF staining (μm^2) was quantified relative to 100 adipocytes using ImageJ software. Then, number of capillaries was counted per 100 adipocytes. A capillary was defined as one-cell-thick endothelium surrounding a lumen. Both surface of VWF per 100 adipocytes and number of capillaries per 100 adipocytes tended to increase in subcutaneous adipose tissue during weight loss as illustrated in Figure 25.

One can speculate that weight loss is associated with increased adipose tissue vascularization. This is supported by the fact that subcutaneous adipose tissue showed a higher number of blood vessels in ex-obese subjects compared to control patients with equivalent BMI but no history of weight fluctuations (Baptista et al., 2009). Although, it

remains to know whether this neovasculature orginates from the growth of pre-existing vessels (angiogenesis) or spontaneous blood-vessel formation (vasculogenesis).

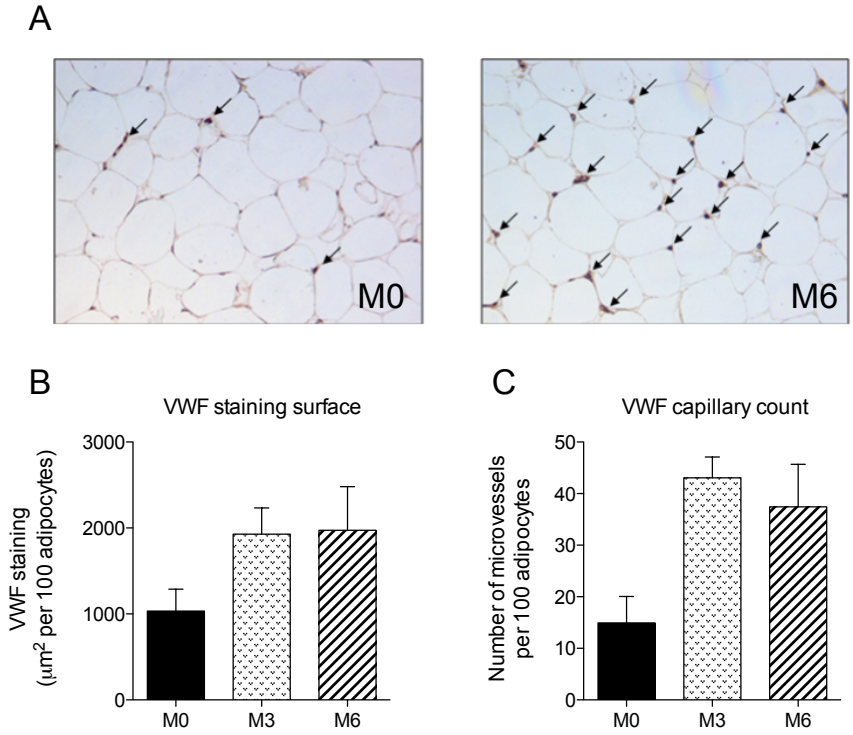

Figure 25 | Quantification of capillaries in adipose tissue during weight loss. (A) VWF staining in subcutaneous adipose tisse at the time of surgery (M0) and 6 month after surgery (M6). (B) Surface of VWF staining in μm^2 per 100 adipocytes and (C) count of VWF positive capillaries per 100 adipocytes at the time of surgery and 3 and 6 months after the surgery.

These are very preliminary resuls. Vessel quantification was performed using Van Willebrand factor that is not specific for endothelial cells. It is known to be produced by macrophages and I typically observed VWF staining in crown-like structures. Although, VWF staining appears to be increased during weight loss, macrophage CD68 staining was reduced in the same biopsies. More work is needed in order to evaluate the vascularization on adipose tissue during weight loss. It would be interesting to evaluate the angiogenic potency of adipose tissue conditioned media obtained after surgery using the chick chorioallantoic membrane assay.

3.4. Circulating monocytes and adipose tisse macrophages: a link?

Since macrophages accumulate in adipose tissue during obesity, it is relevant to wonder if any connection exits with their precursor cells, that are, the circulating monocytes. In an attempt to answer these questions, we tried to link the different monocyte subsets presenting in Study 1 with count of $CD68^+$ macrophages in adipose tissue of the same 40 morbid obese

individuals. Among the monocyte subpopulations, we observed an unique and significant correlation between circulating numbers of $CD14^{dim}CD16^{+}$ monocytes and number of $CD68^{+}$ macrophages in visceral adipose tissue of the same patients, as shown in figure 26.

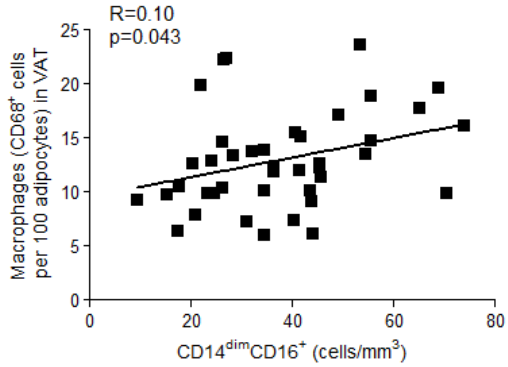

Figure 26 | Correlation between number of circulating $CD14^{dim}CD16^{+}$ monocytes and number of $CD68^{+}$ macrophages per 100 adipocytes in visceral adipose tissue. (Pearson' correlation test).

Then, we sought to define the phenotype of $CD14^{dim}CD16^{+}$ monocytes in obesity. It appears that they displayed a more pro-inflammatory phenotype in obese patients compared to control subjects with a significant increase in pro-IL-1β gene expression as shown in Figure 27. To further study a link between circulating monocytes and adipose tissue macrophages, we set up an *in vitro* assay during which untouched monocytes were isolated from lean, obese and diabetic obese patients and differentiated for 6 days into mature macrophages (MoDM), following the same protocol of Article #3. Cells were then stimulated with LPS and ATP to specifically induce IL-1β secretion. Age and BMI of obese subjects were not significantly different. Interestingly, we observed that MoDM from OB/D had increased IL-1β secretion upon stimulation, compared to lean and OB subjects, as shown in Figure 28.

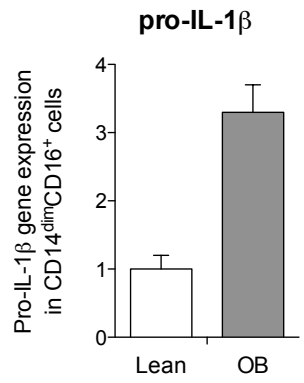

Figure 27 | Pro-IL-1β gene expression more in $CD14^{dim}CD16^{+}$ monocytes from lean and obese (OB) subjects.

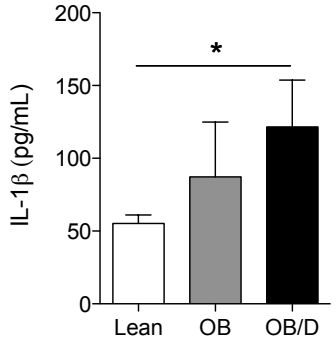

Figure 28 | IL-1β secretion from MoDM isolated from 6 lean, 6 obese (OB) and 6 diabetic obese (OB/D) patients upon LPS and ATP stimulation. Subjects were age-matched.

Overall, these results tackle a very important question that is to which extent blood monocytes play a crucial role in obesity in respect to adipose tissue macrophage accumulation. In other words, how do the phenotype of these circulating monocytes matter in the setting of adipose tissue inflammation? Are monocytes « pre-programmed » to infiltrate adipose tissue in obesity? Interestingly, we found an association between circulating numbers of $CD14^{dim}CD16^{+}$ monocytes and number of $CD68^{+}$ macrophages in visceral adipose tissue of the same patients. We also observed that $CD14^{dim}CD16^{+}$ monocytes showed increased pro-IL-1β gene expression in obese patients compared to control subjects. Although overly simplified, it is tempting to speculate that $CD14^{dim}CD16^{+}$ monocytes might be the precursors of fat macrophages in diabetic obese subjects, supporting that fat macrophages secrete more IL-1β because of their circulating phenotype. To further study this possible relationship, we described that *in vitro* MoDM from diabetic obese patients tend to secrete more IL-1β in response to LPS and ATP stimuli, than MoDM from lean and obese groups. Hence, this *in vitro* differentiation did not « reset » the cell phenotype. Since MoDM were prepared from untouched monocytes (*i.e.* including the three monocyte subsets), one explanation could be that MoDM from diabetic subjects secrete more IL-1β due to increased proportion of $CD14^{dim}CD16^{+}$ subset. These preliminary data raise numerous exciting questions. A recent study showed that MoDM from obese subjects were more refractory to differentiate towards an alternative M2-like phenotype upon IL-4 stimulation, compared to lean groups (Bories et al., 2011). These observations suggest that blood monocytes are programmed and maintained their phenotype once cultured *in vitro*, which could also occur *in*

vivo. Though, it is commonly thought that *in vivo* monocytes undergo diapedesis to reach a given tissue wherein they mature and acquire a phenotype in response to environmental cues. Actually, it is likely that monocyte phenotype and tissue microenvironment both cooperate to give the macrophage its differentiated state. Beside, functional *in vitro* studies usually follow monocyte differentiation protocols ranging from 5 to 10 days according to the litterature. But how long does it really take for a circulating monocyte to differentiate into a mature tissue macrophage *in vivo*? This differentiation pathway is relatively poorly studied *in vivo* and make the underlying mechanims very difficult to unravel.

To my knowledge, only one recent study addressed this question in obesity using a model of PKH26 fluorescent labeled monocytes (Oh et al., 2012). They showed that when fluorescent monocytes were injected into recipient mice, monocytes were cleared from the blood within several hours and that fluorescence was detected in adipose tissue 6h post-injection. This suggests that monocyte diapedesis and supposed differentiation within adipose tissue is really fast *in vivo*. Furthermore, using labeled monocytes from lean or obese mice, adipose tissue macrophage accumulation was greater in obese recipient mice compared with lean mice, regardless of the source of donor monocytes. Thus, they concluded that circulating monocytes were naive of their ultimate fate and that macrophage accumulation in fat is only dependent of adipose tissue signals (Oh et al., 2012). On the contrary, in other pathologies, it is thought that specific monocyte subset accumulate in inflammatory tissue. For instance, in mouse model of altherosclerosis, $GR1^{+}/LyC^{high}$ monocytes (resembling classical $CD14^{++}CD16^{-}$ subset in humans according to (Shi and Pamer, 2011)) were shown to preferentially infiltrate aortic lesions and become atherosclerotic macrophages (reviewed in (Woollard and Geissmann, 2010)). Thus, monocytes appear not to be simple precursors but potential effector cells likely to play a role in obesity-associated inflammation, up-stream of fat macrophages. Much more work is needed to define what role circulating monocytes play in respect to adipose tissue macrophage accumulation. Considering discrepancies in monocyte subset classification between mice and men, human studies are especially required.

GENERAL DISCUSSION AND PERSPECTIVES

E. Surgery-induced weight loss: a complex and dynamic phenomenon

During this PhD project, we followed a group of morbid obese subjects before and after gastric bypass surgery. This surgerical procedure allowed significant weight loss, improvement of glycemic parameters with a very high percentage of systemic inflammation and T2D resolutions. Despite general amelioration, we showed in Article #1 that most of circulating inflammatory mediators did not readily decrease after the surgery and followed a biphasic kinetic pattern. Interestingly, such profils were also observed for the proportion of circulating $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes and for secretion of IL-1β by adipose tissue explants as described in article #2 and #3, respectively. These observations support the idea that weight loss is not a straighforward regulator of systemic and local inflammation, potentially in relation with a major physiological stress for the organism.

Considering that weight loss mainly results from decrease in the volume of adipocytes, it appears consistent that drastic weight loss is also a time period during which adipose tissue undergoes dynamic remodeling. We showed that weight loss was associated with increased fat release of pro-angiogenic factors that are VEGF, PDGF-BB and bFGF, and our preliminary data suggest that number of vessels might be increased in subcutaneous adipose tissue following gastric surgery. Ex-obese subjects were shown to have increase number of vessels in their fat depots relative to controls (Baptista et al., 2009). Considering that one of the obesity theories lays hypoxia at the basis of adipose tissue inflammation, increased vascularization, and notably capillaries, would restore oxygen supply and subsequently, favors relief of inflammation in fat depots during weight loss. Studies focusing on mechanisms of vessel formation during weight loss would be of interest and it remains to assess whether this neo-vasculature is really functional.

Interestingly, VEGF, PDGF-BB and bFGF might also exert pro-fibrotic effects as shown in lung pathology (Chaudhary et al., 2007). Moreover, a review even highlights the growing connectivity between these two processes that are, angiogenesis and fibrosis (Kalluri and Sukhatme, 2000). Fibrosis, defined as deposition of ECM components, is mainly produced by activated fibroblasts (Wynn, 2007). Yet, this process becomes pathogenic when it persists uncontrolled, typically during chronic inflammatory conditions. Fibrosis, whether it is localized around adipocytes or in bundles, was found to be associated with obesity (Divoux et al., 2010). Considering secretion of potential pro-fibrotic factors by adipose tissue, weight loss might also be associated with ECM remodeling but in a non-inflammatory context, in opposition to weight gain. This is supported by the fact that IL-13 which is significantly up-regulated during weight loss, is also known to contribute to ECM deposition, by stimulating collagen production from fibroblasts (Barron and Wynn, 2011). More work is needed to quantify fibrosis during weight loss, identify cellular sources of these remodeling factors within adipose tissue and the potential role of weight loss-associated ECM deposition. Together, increased angiogenesis and ECM deposition along with decreased inflammation might be hallmarks of fat tissue repair during weight loss. However, these processes might also be seen as predictors of weight re-gain since absence of inflammation favors adipogenesis while angiogenesis supports tissue expansion. Thus, adipose tissue growth could be facilitated in case of new episodes of excess energy intake. Increased fibrosis in subcutaneous adipose tissue was shown to hamper fat mass loss after the surgery (Divoux et al., 2010). This would suggest that repeated cycles of weight loss/weight gain, the well-known « yo-yo » phenomenon, might lead to vicious circles of tissue remodeling during which it becomes easier to gain weight than to lose it.

F. IL-1β and NLRP3 inflammasome, key players in adipose tissue

In this work, I focused on visceral adipose tissue inflammation and immune cells. Special attention was given to macrophages whose accumulation in adipose tissue was shown to be associated with obesity-related metabolic complications. Although, different types of immune cells were described to infiltrate fat during obesity, macrophages appear to be the master regulators of fat inflammation. We propose that macrophage NLRP3/IL-1β pathways might be at stake in this process since we showed that macrophages secreted massive amount of IL-1β in a NLRP3-dependent mechanism in obese adipose tissue. Table 5 tries to compare and summarize possible ligand signals ensuing IL-1β secretion in the different target organs of obesity-associated metabolic complications. Alterations in pancreas, artery wall and adipose tissue are likely to occur concomitantly and make IL-1β a key pharmaceutical target

with additive beneficial effects. Interestingly, glyburide that is a potent NLRP3 inhibitor is also an anti-diabetic secretagogue drug from the sulfonylurea class. Although doses used in clinical practice are unlikely to be high enough, it would have been interesting to check whether adipose tissue inflammation was also down-regulated upon gluburyde treatment. Same thing could have been done during clinical trial with anakinra (Larsen et al., 2007).

Target Organ	**Pancreas**	**Artery wall**	**Adipose tissue**	**References**
Signal 1	FFAs, LDLs, hyperglycemia	FFAs, LDLs	FFAs **VAT secreted factors?**	(Masters et al., 2011; Nguyen et al., 2007; Shanmugam et al., 2003)
Signal 2	FFAs , IAPP, ROS, mitochondrial dysfunction	FFAs, Cholesterol crystals	FFAs, Ceramides, ROS, mitochondrial dysfunction, ATP, ER stress **VAT secreted factors?**	(Duewell et al., 2010; Masters et al., 2010; Menu et al., 2012; Vandanmagsar et al., 2011; Wen et al., 2011; Zhou et al., 2011)
Target cells	β cells	Intima media cells	Adipocytes	(Donath and Shoelson, 2011; Masters et al., 2011)
Metabolic consequences	insulin resistance, death	Foam cells ; Plaque formation	Insulin resistance, (death ?)	(Donath and Shoelson, 2011; Masters et al., 2011)
NLRP3$^{-/-}$ DIO models	Protection	No phenotype	Protection	(Vandanmagsar et al., 2011; Youm et al., 2011) (Menu et al., 2011)

Table 5 | Possible features related to myeloid NLRP3 inflammasome activation in target organs of metabolic complications. FFAs, fatty acids; LDLs, Low Density Lipoproteins; IAPP, islet amyloid polypeptides; ROS, Reactive Oxygen Species.

In our study, an important question that remains to be adressed is which factors specifically induce higher IL-1β secretion in adipose tissue macrophages from diabetic obese subjects compared to obese controls. We showed that *in vitro* adipose tissue secreted factors induce a pro-inflammatory phenotype in MoDM, similar to the one displayed by *ex vivo* adipose

tissue macrophages. It is thus likely that adipose tissue from diabetic subjects secrete specific up-regulated factors that induce both inflammasome-related signal 1 and signal 2 in macrophages. We ruled out an effect of LPS contamination but more work is definitely to be done to identify these molecules. Several ideas are to be developed. Indeed, it would be relevant to measure and define the nature of FFAs and lipid metabolites release by adipose tissue explants. In the same way, it should be checked the secretion of DAMPs that could be secreted by necrotic adipocytes for instance. Another relevant possibility would be to assess serum amyloid A (SAA) in those explants. It is true that SAA, a family of apolipoproteins, was shown to be highly expressed by adipocytes and up-regulated in adipose tisue during obesity (Poitou et al., 2009). Yet, SAA was found to activate NLRP3 inflammasome in monocyte-derived cells in the context of allergy (Ather et al., 2011; Niemi et al., 2011). Those observations are a strong incentive for further exploration of adipocyte-derived SAA on NLRP3 activity in the context on obesity. Finally, leptin whose secretion by adipocytes is also increased during obesity could be an interesting track since it was shown that leptin induced IL-1β secretion by human islets (Maedler et al., 2002). Macrophages are known to express leptin receptor, but it is currently unclear whether leptin specifically up-regulates IL-1β in such cells (Loffreda et al., 1998). Beside NLRP3 activation, our data also emphasize that adipose tissue secretions from obese subjects specifically increased TLR-2 gene expression in MoDM compared to non obese controls. This up-regulation in TLR-2 production was further confirmed in adipose tissue macrophages. This would suggest that macrophages would process pro-IL-1β through TLR-2 signalling, rather than TLR-4, in response to adipose tissue secreted factors. In line with our observations, Creely showed that TLR-2, but not TLR-4, gene expression was up-regulated in adipose tissue of diabetic obese subjects compared to controls (Creely et al., 2007). Considering that both TLR-2 and TLR-4 were shown to be involved in mice and human obesity, additional functional experiments are required to confirm that adipose tissue secretions preferentially signal through TLR-2 in macrophages and to unravel the nature of these ligands (Himes and Smith, 2010; Shi et al., 2006).

G. IL-17$^+$IFN-γ$^+$ cells, a new lymphocyte population in adipose tissue

In this work, we identified CD4$^+$IL-17$^+$/IFN-γ$^+$ cells in visceral adipose tissue. These IL-17/IFN-γ double producers are known to be enriched in the target organs of numerous autoimmune disease models and they appear to be particularly pathogenic in tissue inflammation (Peters et al., 2011). In agreement with a potential pathogenic relevance, we observed a significant

correlation between the percentage of $IL17^+IFN\text{-}\gamma^+$ among $CD4^+$ and Hb1Ac, suggesting a link with alterations of glycemic status in obese subjects. This discovery is quite intriguing. In line with our observations, a recent study reported the presence of IL17-producing cells in subcutaneous adipose tissue of obese subjects (Bertola et al., 2012). It remains to truly define the phenotype and the role of this $IL17^+IFN\text{-}\gamma^+$ population in adipose tissue and the reason why they are specifically accumulated in diabetic obese subejcts. It is usually thought that IFN-γ and IL-17 antagonize each other and that IFN-γ inhibits Th17 *in vitro* polarization (Zhu and Paul, 2008). Besides, it is known that Th17 cells have considerable plasticity and that probably, these double producing cells were originally polarized in Th17 cells and then, acquired the ability to produce IFN-γ (Hirota et al., 2011). One could wonder whether the production of IFN-γ by these cells is a protective mechanism in an ultimate attempt to limit IL-17-induced inflammation. Corroboring this hypothesis is the fact that we found a high percentage of cells, producing IFN-γ and these percentages were not different between non-obese, obese and diabetic obese subjects. Circulating levels of IFN-γ were also not found to be any different between lean and obese individuals (Sumarac-Dumanovic et al., 2009). Considering that IFN-γ is by definition an inflammatory cytokine with primary anti-bacterial properties, the question araises of why so many T cells secrete IFN-γ in adipose tissue and why they do not seem to be regulated by weight variations. What is even more intriguing is that IFN-γ is known to alter adipocyte biology (Duffaut et al., 2009b; Feingold et al., 1992; McGillicuddy et al., 2009; Rocha et al., 2008). An answer is likely to be found in the presence of anti-inflammatory T cell subsets, which are Th2 or Tregs that could counteract the effect of IFN-γ. Thus, studies focusing on deciphering the proportion of each lymphocyte subpopulation in lean and obese subjects are especially required.

H. Macrophage and lymphocyte dialogue within adipose tissue

Obesity is likely to affect each individual cell type present in adipose tissue, and distinct cells might also influence one another. During this PhD project, I decided to focus on fat immune cells. This has been already extensively studied but most research described effects of adipose tissue immune cells on adipocytes or preadipocytes. Less is known about potential interactions between immune cells to amplify adipose tissue inflammation. Thus, we identified a paracrine dialogue between macrophages and helper T lymphocytes within adipose tissue that appears to be a feature of diabetic obese subjects. Macrophage-derived IL-1β and lymphocyte-derived IL-17 reciprocally stimulate each other secretion and thus, aggravates inflammatory changes in adipose tissue. Interestingly, both cytokines have been shown to impair insulin signalling in adipocytes and disrupt adipogenesis (Lagathu et al.,

2006; Zuniga et al., 2010). It is likely that, during obesity, adipose tissue is characterized by the establisment of numerous inflammatory vicious circles between distinct cell types that together self-sustain and exacerbate adipose tissue alterations. Such a paracrine loop was already described between adipocyte-derived FFAs and macrophage-derived TNF-α (Suganami et al., 2005). In our study, co-culture experiments allowed us to highlight macrophage and lymphocyte interactions though a paracrine dialogue. Although, it would be interesting to get insights into how these cells actually cooperate in adipose tissue. In other words, do they need to develop cell contact to ensure auto-stimulation? We showed that adipose tissue macrophages expressed increased co-stimulatory molecules in obesity compared to non-obese groups. This suggests that macrophage could develop immunological synapses with lymphocytes. Thus, it would be interesting to further study those interactions with transwell experiments to assess whether cell contact is required for activation. In the same way, microscopy imaging of both cell type within adipose tissue would be of interest to see if they localized next to each other. It is currently unknown whether adipose tissue lymphocytes are activated by APCs within the adipose tissue or in regional lymphoid organs. In the first case, lymphocyte-macrophage synapses would be required for so-called antigen presentation in adipose tissue; in the second possibility, antigen recognition would be insured in lymphoid organs and simple « re-activation » of lymphocytes by paracrine cytokines would occur *in situ*. As mentionned in Box 4 of the introduction, whether or not lymphocytes respond to adipose tissue-associated antigens is currently unclear. An interesting theory is that macrophages and DCs present in adipose tissue would extend their pseudopods into the lumen of fat lymphatic vessels to import antigens transported into the lymph (abstract presented in the EMBO Worshop on Immunology and Metabolism reviewed in (Staels et al., 2011)). Now where would these antigens come from? Another theory suggests that gut microbiota and intestinal permeability are both altered in obesity allowing bacteral antigen translocations (Burcelin et al., 2012). In their elegant study, Flaveli's group showed that gut microbiota alterations induced translocations of microbial components, not whole bacteria, into the portal vein to induce hepatic alterations (Henao-Mejia et al., 2012).

I. How to disrupt the IL-1β and IL-17 paracrine loop?

Disrupting an inflammatory loop is likely to occur at several levels: blocking the stimuli of cytokine production, altering the transcription regulation of the cytokine secretion, neutralizing the cytokines themselves or their related receptors, or adding anti-inflammatory molecules. Coming back to weight loss, we showed that both IL-1β and IL-17 secretion rates were decreased in adipose tissue after the surgery, suggesting that the macrophage-

lymphocyte paracrine loop might be broken. Although the physiological mechanisms are unknown, this opens a window for pharmacological targetting of this deleterious dialogue. Indeed, clinical trials with anakinra or neutralizing antibody IL-1β have been and are still currently done to alleviate insulin resistance and T2D, potentially by acting in part on adipose tisue-derived IL-1β. On the other hand, no published study has been done assessing anti-IL-17 antibody treatment in obesity, although clinical trials with promising effects have been performed in patients suffering from psoriasis and rhumathoid arthritis (Genovese et al., 2010; Waisman, 2012). Another mechanism could rely on IL-13 anti-inflammatory properties. Strinkingly, in our hands, this is the only cytokine to be up-regulated in adipose tissue during weight loss. IL-13 is known to hamper IL-1β secretion in macrophages and to attentuate IL-17 production in Th17 cells (Newcomb et al., 2011; Scotton et al., 2005). Besides, Il-13 was also found to protect mice against experimental autoimmune myocarditis by regulating macrophage differentiation and IL-13-deficient mice showed exacerbated disease partly through increase caspase-1 and IL-1β production (Cihakova et al., 2008). Thus, up-regulation of IL-13 during weight loss could exert anti-inflammatory properties within adipose tissue and it would be relevant to further study these regulations, in our co-culture assay for instance.

Finally, IL-1β secretion could be impaired at the transciptional levels. Studies focusing on regulation of macrophage phenotype identified several transcription factors involved in either M1-like or M2-like polarization (reviewed in (Lawrence and Natoli, 2011)). Many publications showed that manipulation of transcription factors of macrophages could modulate mouse response to high fat feeding. During this PhD, a collaboration project was developed with the group of Mukesh Jain (Pennsylvania, USA) focusing on the role of Krüppel-like factor 4 (KLF4) in determining alternative M2-like polarization in macrophages (Annex 2). We showed that KLF4 gene expression was down-regulated in adipose tissue of obese subjects relative to controls and that DIO myeloid KLF4 deficient mice displayed worsened insulin resistance and adipose tissue inflammation compared to wildtype controls. Interestingly, DIO myeloid KLF4 deficient mice had increased pro-IL-1β gene expression in their adipose tissue compared to controls. In the meantime, another publication caught all of our attention. Interferon regulatory factor 5 (IRF5) was shown to be critical in M1-like macrophage polarization. IRF-5 overexpression in human macrophages up-regulated many pro-inflammatory genes, including pro-IL-1β and macrophages sorted from IRF5-deficient mice show decreased IL-1β secretion upon LPS challenge compared to controls (Krausgruber et al., 2011). These two studies suggest that targetting an up-regulation of KLF4 or a down-regulation of IRF5 could modulate IL-1β production in fat macrophages. What is even more

interesting is that the IRF5-deficient mice also showed impaired IL-17 production in response to LPS, supporting the idea that macrophages, partly via IL-1β, control the Th17 adaptive immune responses. To go on with this idea, a study is on-going in our team to assess the role of IRF5 in obesity, postulating that IRF5-deficient mice under high fat feeding would have lower IL-1β and IL-17 production and subsequently, improved insulin sensivity.

J. Taking a closer look to blood monocytes

Circulating monocytes were also shown to be regulated in obesity and during surgery-induced weight loss. We and another group showed that circulating monocytes from obese and diabetic obese subjects maintained their proinflammatory phenotype once differentiated *in vitro*. These observations raise many questions (Bories et al., 2011). On one side, results suggest that obesity might be associated with circulating monocytes that are prone to become highly inflammatory macrophages in adipose tissue, including increased IL-1β secretion. Beside, blood monocytes could be less suceptible to acquire alternative M2-like phenotype in adipose tissue, suggesting a failure of anti-inflammatory control mechanisms. Impaired M2 polarization is critical since resident adipose tissue macrophages were shown to be of a M2-like phenotype with protective properties (Lumeng et al., 2007). Besides, impairment of macrophages M2 polarization was shown to exacerbate obesity inflammation and insulin reisstance in DIO PPAR-γ deficient mice (Odegaard et al., 2007). If, at the circulating level, monocytes are already predispose to become pro-inflammatory macrophages, they become true effector cells in the pathogenesis of obesity. As already discussed, serum microenvironment, notably hyperglycemia or hyperlipidemia, is likely to influence blood leukocyte activation. However, such monocyte commitment towards proinflammatory phenotype in obesity also suggests the involvement of potential epigenetic mechanisms. Chromatin modifications have already been observed in both lymphocytes and monocytes of patients suffering from type 1 diabetes (Miao et al., 2012; Miao et al., 2008). It is likely that epigenetic changes might play an important role in the incidence of T2D and studies focusing on epigenetics in circulating leukocytes of diabetic obese subjects would be of particular interest. Thus, decreasing proportion of specific monocyte subsets or modulating their circulating phenotypes should be reconsidered in order to decrease fat macrophage accumulation or activation.

K. Conclusion

This PhD project focused on describing obesity-associated low-grade inflammation at the systemic level and within adipose tissue in obesity and during the dynamics of surgery induced weight loss. Figure 29 tries to summarize our main observations and hypotheses regarding IL-1β production by adipose tissue macrophages. The gastric bypass surgery, that is to date one of the best models of weight fluctuations in humans, is the common basis of our three articles. This model leads us to identify inflammatory mediators and effector cells that appear of major importance due to their down-regulation during weight loss. Furthermore, adipose tissue analysis allowed us to identify a paracrine dialogue between fat macrophages and lymphocytes, mediating by NLRP3-dependent IL-1β and IL-17 in close association with the diabetic status of subjects. We also discovered the enrichment of a peculiar IL-17/IFN-γ double producing $CD4^+$ in adipose tissue of diabetic obese subjects that is thought to be very pathogenic in tissue inflammation. Both IL-1β secretion and $IL\text{-}17^+IFN\text{-}\gamma^+$ cells were found to correlate with glycemic parameters of obese subjects, highlighting their potential role in metabolic complications. Considering the role of IL-1β in Th17 polarization, we propose a model according to which, in response to IL-1β/NLRP3 hyperactivation, a Th17 adaptive immunity might be subsequently promoted, contributing to IL-1β-associated pathophysiology. Thus, additional work is needed to decipher the actual mechanisms by which macrophage-derived IL-1β is induced and the specific roles of this $IL\text{-}17^+IFN\text{-}\gamma^+$ population in adipose tissue, in respect to glycemic parameters. Besides, it is likely that in the nearby future, other inflammasome complexes will be identified to play a role in obesity pathophysiology. Also, one growing dimension that will have to be taken into account is the gut microbiota that appears to be also regulated by inflammasomes and whose alterations are likely to directly influence metabolic organs (Henao-Mejia et al., 2012). Of note, a recent study by our team showed major changes in gut microflora during gastric surgery-induced weight loss (Furet et al., 2010). Finally, our data also suggest that circulating monocyte subsets are regulating by weight variations and could influence the recruitment and/or the phenotype of adipose tissue macrophages. Studies focusing on the link between fat macrophages and their precursor cells should be reconsidered.

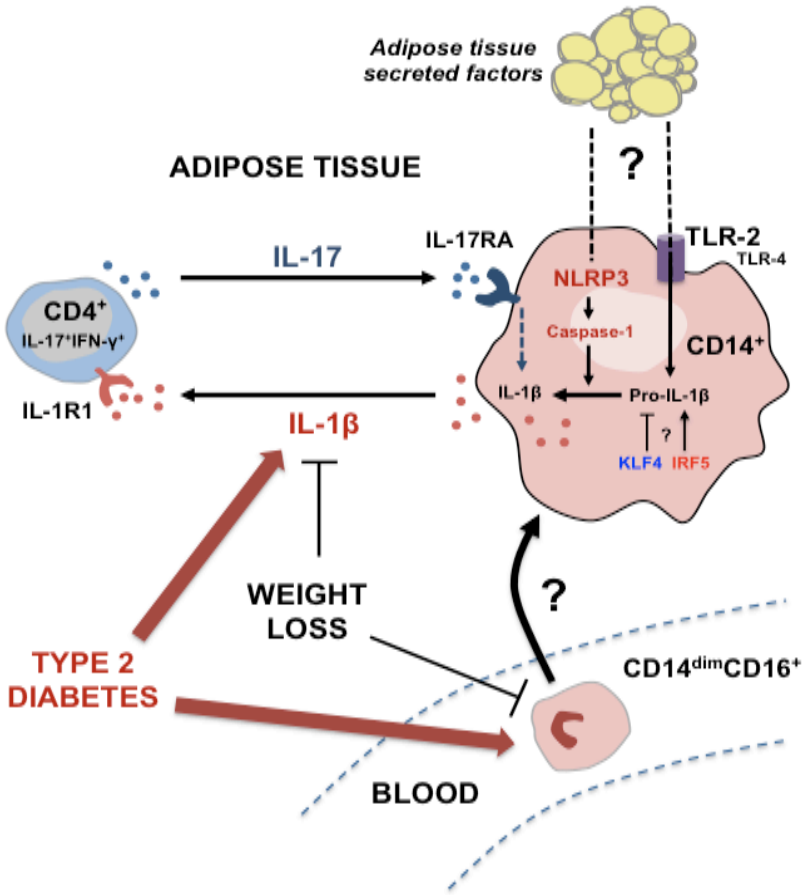

Figure 29 | Immune cell interactions in adipose tissue in obesity and during surgery induced-weight loss: summary of the main points.

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ANNEX 1

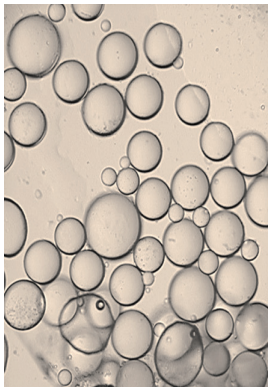
médecine/sciences 2011 ; 27 : 993-9

Le tissu adipeux

Un nouveau terrain de jeu pour les cellules immunitaires

Élise Dalmas, Joan Tordjman, Michèle Guerre-Millo, Karine Clément

> L'existence d'un état inflammatoire chronique de bas niveau dans l'obésité pouvant intervenir dans la physiopathologie de la maladie et de ses nombreuses complications est bien établie. Le tissu adipeux lui-même est un site d'inflammation où s'accumulent des macrophages. Dans cette synthèse, nous décrivons les données expérimentales et cliniques qui ont permis d'élucider certains des mécanismes cellulaires et moléculaires impliqués dans la colonisation du tissu adipeux par les macrophages obtenus à partir de précurseurs monocytaires. Les macrophages sont des cellules dont le phénotype varie suivant l'état du microenvironnement. Dans l'obésité, ils peuvent exercer des effets délétères *via* la production de molécules pro-inflammatoires, mais contribuent également à l'homéostasie du tissu adipeux face aux changements de la masse grasse. Une autre conséquence de l'inflammation du tissu adipeux est la présence d'une fibrose dont la genèse et les conséquences sont encore mal connues. L'identification de mécanismes potentiellement protecteurs, tels que la neutralisation immunologique de certains types de lymphocytes ou encore le contrôle transcriptionnel des gènes de l'inflammation, pourrait suggérer de nouvelles perspectives thérapeutiques pour limiter l'inflammation dans le tissu adipeux. <



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En 2003, deux équipes américaines décrivent une accumulation de macrophages dans le tissu adipeux de souris obèses et chez l'homme, en relation avec l'augmentation de l'indice de masse corporelle [1, 2]. Les macrophages du tissu adipeux sont considérés comme une source majeure de facteurs pro-inflammatoires, ce qui leur confère un rôle potentiellement délétère au niveau local et systémique. Cependant, ces cellules sont particulièrement versatiles et peuvent adopter un profil anti-inflammatoire, ce qui ne permet pas d'exclure leur contribution physiologique à l'homéostasie du tissu adipeux. Depuis quelques années, la présence d'autres cellules immunitaires dans le tissu adipeux a été rapportée par plusieurs équipes, principalement dans les modèles murins d'obésité, mais également chez l'homme. Dans cette synthèse, nous montrons que ces invités inattendus, acteurs de l'immunité innée et adaptative, participent à la biologie du tissu adipeux et à ses altérations au cours du développement de l'obésité.

Pourquoi le tissu adipeux est-il enflammé ?

L'intervention de nombreux facteurs est évoquée, mais il reste difficile d'établir clairement le ou les événements à l'origine de l'inflammation dans le tissu adipeux. Les adipocytes eux-mêmes sont capables de produire de nombreuses biomolécules pro- ou anti-inflammatoires. L'accroissement de la taille des adipocytes, qui caractérise l'état obèse, altère leur profil de sécrétion et induit un statut pro-inflammatoire, alors que les signaux impliqués ne sont pas clairement définis. La répartition caractéristique des macrophages en couronne autour d'adipocytes présentant des signes de mort cellulaire [3] *(Figure 1)* suggère l'intervention de facteurs attractants spécifiques. Dans le tissu adipeux, des signaux exogènes comme les acides gras ou les lipopolysaccharides (LPS) d'origine bactérienne induisent une réponse inflammatoire en activant le récepteur TLR4 (*toll like receptor* 4) et les voies de signalisation intracellulaires associées, principalement la voie NFκB [4]. Localement, les adipocytes inflammatoires, dont l'activité lipolytique est élevée, libèrent des acides gras qui stimulent la voie TLR4/NFκB dans les macrophages, contribuant ainsi à

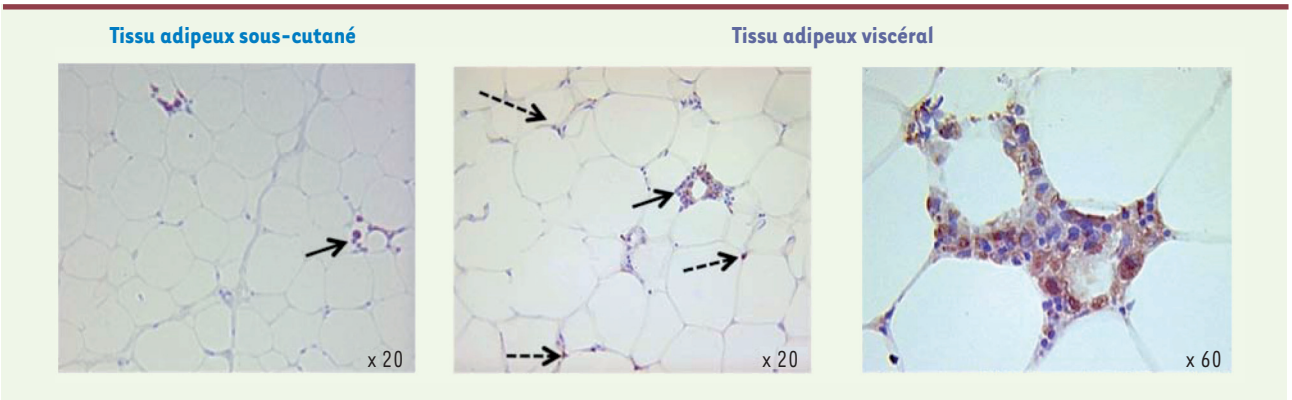

Figure 1. ***Aspect microscopique des macrophages dans le tissu d'un sujet obèse.*** Des biopsies chirurgicales de tissu adipeux sous-cutané et viscéral ont été traitées par un anticorps spécifique des macrophages. Les flèches pleines indiquent les macrophages disposés en couronne autour d'un adipocyte ; les flèches pointillées indiquent les macrophages disposés à l'intersection des adipocytes dans le parenchyme.

une boucle paracrine délétère entre les deux types cellulaires [5]. Dans ce contexte, l'inhibition de TLR4 par des facteurs tels que ATF3, facteur de transcription de la famille ATF/CREB, ou CTRP-3 (C1q/TNF*[tumor necrosis factor]-related protein-3*), membre de la famille C1q/TNF, pourrait représenter une cible thérapeutique pour contrôler l'inflammation du tissu adipeux [5, 6]. Récemment, un rôle anti-inflammatoire des acides gras omega 3 agissant *via* le récepteur GPR120 des macrophages a été identifié ; cette nouvelle approche reste à explorer [7]. Parmi d'autres facteurs, l'hypoxie tissulaire, cause classique d'attraction des macrophages dans certaines tumeurs et dans la plaque d'athérome, pourrait aussi intervenir dans le déclenchement de l'inflammation du tissu adipeux chez les sujets obèses [8]. Le stress du réticulum endoplasmique [44] et le stress oxydant [45] seraient aussi susceptibles d'intervenir dans l'inflammation locale. Les interactions entre ces différents types de stress cellulaire et les biomolécules de l'inflammation ouvrent tout un champ d'explorations au cours de l'évolution de l'obésité.

Lutte d'influence entre les cellules immunitaires dans le tissu adipeux

L'inflammation intervient dans le tissu adipeux alors qu'aucun microorganisme pathogène n'a été clairement identifié à ce jour, ce qui permet de la qualifier d'inflammation « stérile ». Plusieurs observations chez la souris rendue obèse par un régime hyperlipidique (DIO, *diet-induced obesity*) sont en faveur de l'intervention d'antigènes non microbiens. Ainsi, l'infiltration précoce et transitoire de neutrophiles suggère qu'une inflammation « aiguë » précède l'apparition des macrophages dans ce modèle [9]. Des cellules NKT (*natural killer T*), capables de reconnaître des antigènes glycolipidiques, colonisent également le tissu adipeux dans les premières phases de l'obésité, étape qui semble nécessaire à l'infiltration macrophagique [10]. Dans un modèle murin (souris $Kit^{W\text{-}sh/W\text{-}sh}$)[1], l'absence de mastocytes réduit l'accumulation de macrophages dans le tissu adipeux. Comment ces cellules surtout impliquées dans l'allergie contribuent au processus inflammatoire du tissu adipeux reste à clarifier [11]. Des lymphocytes, particulièrement des lymphocytes T, ont également été détectés dans le tissu adipeux chez la souris et chez l'homme *(Tableau I)*. Des études cinétiques dans le modèle DIO révèlent que les lymphocytes T précèdent les macrophages dans le tissu adipeux [12, 13]. De plus, les lymphocytes T $CD4^+$ isolés du tissu adipeux de souris DIO expriment un répertoire TCR restreint, suggérant qu'ils puissent reconnaître un - ou des - antigènes d'origine adipeuse [14].

On distingue plusieurs profils de mobilisation des lymphocytes T dans le tissu adipeux dans l'obésité : le nombre de lymphocytes T cytotoxiques ($CD8^+$) augmente alors que celui des lymphocytes T anti-inflammatoires Th2 ($CD4^+GATA3^+$) et Treg ($Foxp3^+$) diminue [13-15]. L'importance physiopathologique de ces changements est illustrée par le fait que l'inactivation des T $CD8^+$ [13] ou l'induction des lymphocytes Th2 ou des Treg [14, 15] diminue l'inflammation dans le tissu adipeux des souris DIO. Chez l'homme, une étude montre l'augmentation des lymphocytes T $CD8^+$ et lymphocytes $CD4^+$ avec le degré d'adiposité dans le tissu adipeux [16].

L'ensemble de ces observations suggèrent l'existence d'un jeu complexe d'influence entre les différents acteurs cellulaires de l'immunité innée (neutrophiles, mastocytes), adaptative (lymphocytes) ou à l'interface des deux systèmes (NKT). Ces différents protagonistes semblent agir séquentiellement ou de concert pour favoriser *in fine* l'accumulation de macrophages dans le tissu adipeux par des mécanismes encore mal connus. Un enjeu majeur de ces études reste l'identification

[1] Ces souris sont dépourvues du récepteur c-Kit, dont le ligand physiologique est le SCF (*stem cell factor*), cytokine essentielle à la différenciation des mastocytes.

Système immunitaire	Cellules de l'immunité		Marqueurs[1]	Effet de l'obésité	Références
	Type cellulaire	Phénotype			
Inné	Macrophages		CD68, CD14, F4/80	↑	[1-3, 23, 38, 40]
		M1[2]	CD40, CD11c, CD86, Mincle	↑	[17, 27, 32, 33, 35, 36]
		M2[2]	CD206, CD163, CD209	↑↔	[17, 27, 32]
		Mixte M1 M2		↑	[29-31]
	Neutrophiles		NIMP-R-14	↑	[9]
	Mastocytes		Tryptase, chymase	↑	[11]
Interface	NKT		CD3 et CD56, NK1.1	↑	[10]
Adaptatif	Lymphocytes T		CD3	↑	[13-16]
		Cytotoxiques	CD3 et CD8	↑	[13, 14, 16]
		Auxiliaires	CD3 et CD4	↓↑	[13, 14, 16]
		T_H1	CD3 et CD4 et IFNγ	↑	[14]
		T_H2	CD3 et CD4 et Gata3	↔	[14]
		T_{reg}	CD3 et CD4 et Foxp3, CD25	↓	[13-15]

Tableau I. ***Les cellules de l'immunité dans le tissu adipeux.*** [1]Sélection de marqueurs couramment utilisés. [2]Marqueurs d'activation M1 ou M2 associés aux marqueurs de macrophages, CD68 ou CD14 chez l'homme et F4/80 chez les rongeurs.

du ou des facteurs capables de déclencher une réponse immunitaire dans le tissu adipeux au cours des phases d'évolution de l'obésité humaine.

D'où viennent les macrophages du tissu adipeux ?

Les études de transplantation de moelle osseuse chez la souris ont clairement montré que les monocytes circulants sont les précurseurs de la majorité des macrophages présents dans le tissu adipeux [2], ce qui a été confirmé par des expériences de marquage des monocytes *in vivo* [17]. Plusieurs sous-populations de monocytes ont été décrites chez la souris et chez l'homme. Leur devenir dépend de leur phénotype, d'une part, et du microenvironnement dans lequel elles vont se différencier en macrophages, d'autre part. Chez la souris, les monocytes $Gr1^+Ly\text{-}6C^{high}$, équivalents des monocytes classiques $CD14^+CD16^-$ chez l'homme, seraient les précurseurs des macrophages pro-inflammatoires du tissu adipeux [18, 19]. Chez l'homme, des premiers travaux ont révélé que les cellules mononucléées circulantes expriment un statut pro-inflammatoire chez les sujets obèses [20]. Plus récemment, une étude clinique indique que la fréquence d'une sous-population de monocytes $CD14^{dim}CD16^+$ récemment identifiée est augmentée chez des sujets en surpoids [21]. Cette sous-population monocytaire pourrait contribuer préférentiellement à l'accumulation des macrophages dans le tissu adipeux.

Les mécanismes cellulaires et moléculaires qui favorisent le recrutement des monocytes *via* leur diapédèse à travers l'endothélium vasculaire sont peu explorés dans le tissu adipeux. Plusieurs molécules chémoattractantes peuvent intervenir, et un rôle important du système MCP-1/CCR2 (*monocyte chemotactic protein 1* et son récepteur CCR2) dans l'accumulation des macrophages du tissu adipeux a été suggéré par l'étude de modèles murins [22]. Les adipocytes eux-mêmes produisent des facteurs solubles qui augmentent la diapédèse monocytaire et stimulent la production de protéines d'adhésion, dont PECAM-1 (*platelet endothelial cell adhesion molecule*) et ICAM-1 (*intercellular adhesion molecule*) exprimées sur les cellules endothéliales [23]. Nos travaux montrent que la cytokine CCL5 (*alias* RAN-

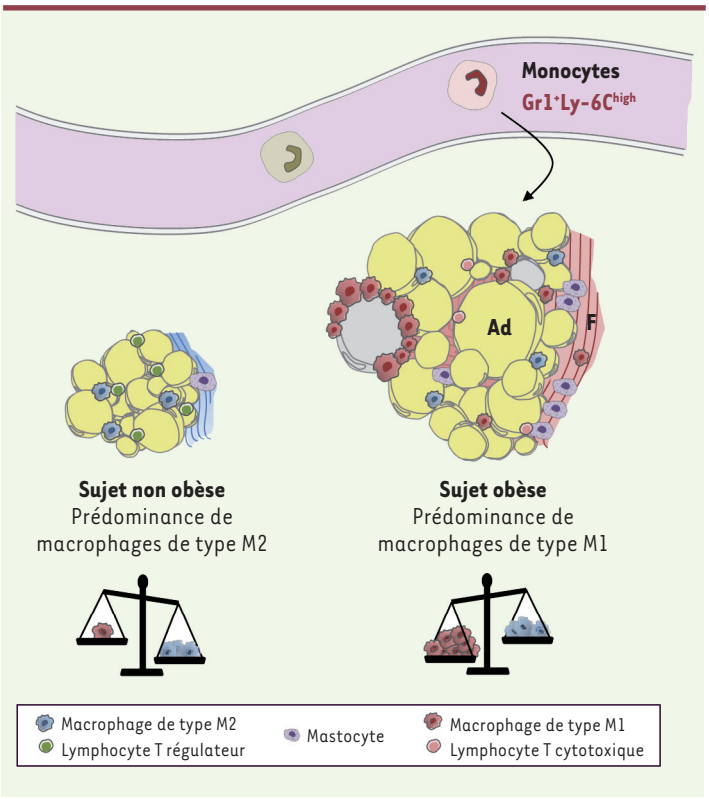

Figure 2. ***Altérations cellulaires et structurales du tissu adipeux induites par l'obésité.*** Chez les souris non obèses, les macrophages résidents ont un phénotype de type M2. Des lymphocytes Treg sont présents, particulièrement dans les tissus profonds. Dans le modèle DIO, l'obésité induit le recrutement de monocytes inflammatoires de phénotype $Gr1^{+}Ly\text{-}6C^{high}$, qui seraient les précurseurs de macrophages de type M1. Ces macrophages recrutés sont disposés en couronne autour d'adipocytes présentant des caractéristiques de mort cellulaire. Les mécanismes impliqués sont divers et complexes et pourraient mettre en jeu la contribution d'autres cellules de l'immunité, dont les lymphocytes cytotoxiques. La présence d'une fibrose a été décrite principalement dans le tissu adipeux humain *(voir Figure 3)*. Plusieurs types cellulaires sont associés aux zones fibrotiques, dont des macrophages et des mastocytes. Ad : adipocytes ; F : fibrose.

TES [*regulated upon activation, normal T cell expressed and secreted*]) pourrait contribuer à ce processus [24]. Il s'avère que les cellules endothéliales sont plus inflammatoires dans le tissu adipeux viscéral que dans le tissu adipeux superficiel, ce qui pourrait favoriser le recrutement des monocytes dans les tissus profonds [25]. Clairement, d'autres études sont nécessaires pour définir le spectre complet des facteurs contribuant à la diapédèse des monocytes et à l'activation endothéliale dans le tissu adipeux au cours de l'obésité.

Les macrophages du tissu adipeux : un phénotype variable et complexe

Il existe un large spectre phénotypique de macrophages, mais on distingue surtout deux catégories suivant une classification qui s'apparente à celle des lymphocytes Th1/Th2 : les macrophages M1 activés par la voie classique et les macrophages M2 qui utilisent une voie alterne d'activation [46]. Comme pour d'autres types cellulaires, cette classification repose en partie sur l'expression de marqueurs dont la spécificité reste souvent imprécise *(Tableau I)*. Les macrophages M1 sont stimulés par le LPS, l'interféron-γ et le TNF-α et produisent des cytokines pro-inflammatoires comme l'IL(interleukine)-6 et l'IL-1. Les macrophages M2 sont induits par l'IL-4 et IL-13 et, *via* la production de facteurs tels que l'IL-10 et le TGF-β (*transforming growth factor*), ont un effet anti-inflammatoire. À ces phénotypes sont associées des fonctions distinctes : élimination des pathogènes et induction d'inflammation pour les macrophages M1, angiogenèse et réparation tissulaire pour les macrophages M2. Qu'en est-il dans le tissu adipeux ? L'injection d'un marqueur spécifique des monocytes chez la souris a permis de montrer que l'obésité (modèle DIO) provoque le recrutement de macrophages M1, qui sont ceux qui s'organisent en couronne autour des adipocytes [26]. Ces macrophages sont chargés en gouttelettes lipidiques, qui témoignent de leur activité de phagocytose des lipides [1, 3]. En l'absence d'obésité, des macrophages résidents sont également détectés dans le tissu adipeux. Ils présentent un phénotype de type M2 et leur nombre n'est pas ou peu modifié dans l'obésité [26, 27]. Une étude cinétique dans le modèle DIO suggère une situation plus complexe : l'accumulation de macrophages de type M1 se poursuit jusqu'à 16 semaines de régime puis diminue au profit de macrophages de type M2, sans que l'on sache s'il s'agit d'un recrutement ou d'une repolarisation de ces macrophages *in situ* [28]. De plus, une troisième population de phénotype intermédiaire M1/M2 a été identifiée dans le tissu adipeux de souris DIO ; elle pourrait constituer un groupe de cellules en cours de repolarisation [29].

Dans le tissu adipeux humain, les macrophages sont essentiellement de phénotype mixte intermédiaire entre les états de polarisation M1 et M2 [30, 31]. Dans une population de sujets massivement obèses, nous avons observé une augmentation du nombre des cellules positives pour le marqueur CD40 et un nombre constant de cellules exprimant des marqueurs de type M2 (CD206 [*mannose receptor*] et CD163 [membre de la famille des *scavenger receptors*]) par rapport à des sujets témoins, suggérant que l'obésité favorise l'accumulation de macrophages de type M1 [32]. Une caractérisation plus

précise montre que, comme chez la souris, les macrophages disposés en couronne autour des adipocytes présentent un phénotype de type M1, alors que les macrophages interstitiels ont un phénotype orienté M2 [33, 34]. Chez l'homme, cependant, il est impossible de distinguer les macrophages recrutés des macrophages résidents.

Les lipides activateurs de la voie TLR4/NFκB [35] ou l'accumulation de lipides dans les macrophages eux-mêmes [36] favorisent la polarisation M1 dans le tissu adipeux des souris DIO. De manière surprenante, l'augmentation de la capacité de stockage des triglycérides dans les macrophages *via* la surexpression de la diacylglycérol acyltransférase 1 (DGAT1) les rend résistants à une activation M1 [37]. Ce phénotype s'explique par le fait que les triglycérides seraient moins pro-inflammatoires que certaines autres espèces lipidiques dans les macrophages. L'ensemble de ces travaux chez la souris et chez l'homme révèlent la complexité et l'aspect évolutif du phénotype des macrophages du tissu adipeux dans l'obésité *(Figure 2)*, une situation certainement associée à des aspects fonctionnels qui restent à clarifier.

Les macrophages du tissu adipeux : amis ou ennemis ?

La question du rôle des macrophages dans les complications métaboliques de l'obésité s'est rapidement posée. Dans les modèles murins, l'abondance des macrophages peut être modifiée dans le tissu adipeux par des moyens pharmacologiques ou *via* des manipulations génétiques ciblées, ce qui a permis d'établir l'importance de ces cellules dans l'induction d'une insulinorésistance. Chez l'homme, en revanche, cette relation reste plutôt controversée. Certaines études rapportent une relation inverse entre la sensibilité à l'insuline et l'expression de marqueurs de macrophages dans le tissu adipeux, particulièrement dans le tissu adipeux viscéral [38]. La présence accrue de macrophages en couronne a également été associée à l'altération de la sensibilité à l'insuline [39]. À l'inverse, nos travaux ne permettent pas d'établir de relation entre l'abondance des macrophages et les paramètres sanguins de l'homéostasie glucidique chez des sujets très obèses [40]. L'association entre l'abondance d'une fraction minoritaire de macrophages de phénotype mixte et l'insulinorésistance a été rapportée, suggérant qu'une analyse précise des phénotypes pourrait permettre de distinguer des types de macrophages plus délétères que d'autres [33].

Bien que fortement soupçonné, un lien entre l'accumulation macrophagique et l'augmentation du risque cardiovasculaire n'a pu être établi chez l'homme. En revanche, nos études cliniques dans une large population de sujets massivement obèses révèlent que la gravité de l'histopathologie hépatique (stéatose et fibrose inflammatoire) est liée à l'ampleur de l'accumulation macrophagique dans le tissu adipeux viscéral, indépendamment du statut glycémique [40]. Les facteurs qui relaient les signaux pro-inflammatoires du tissu adipeux viscéral vers le foie sont en cours d'identification.

Les macrophages peuvent-ils contribuer à maintenir l'homéostasie du tissu adipeux face aux changements du bilan énergétique ? Une

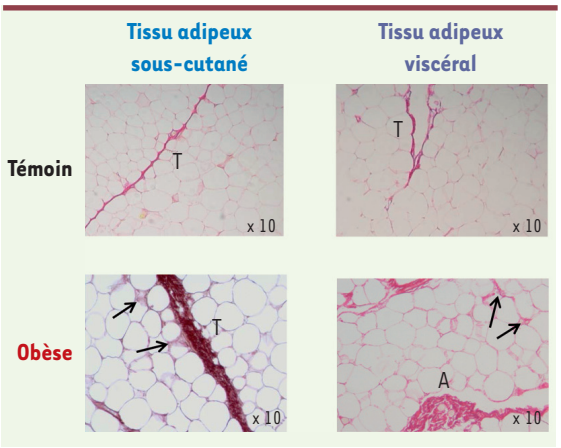

Figure 3. ***Aspect microscopique des dépôts de fibrose dans le tissu adipeux humain.*** Des biopsies chirurgicales de tissu adipeux sous-cutané et viscéral ont été marquées au rouge picrosirius (marqueur des collagènes) pour révéler les zones fibrotiques. A : amas ; T : travée. Les flèches localisent la fibrose péricellulaire présente uniquement chez les sujets obèses.

étude cinétique au cours de la perte de poids chez la souris DIO permet de répondre en partie à cette question. En effet, contrairement à l'hypothèse initiale, une accumulation importante de macrophages a été observée dans le tissu adipeux lors des phases précoces d'une restriction calorique, au moment où la lipolyse adipocytaire est stimulée. Ces macrophages exercent une activité de phagocytose des lipides sans déclencher d'inflammation, et contribuent ainsi à restaurer localement l'homéostasie lipidique [41]. D'autres observations chez la souris suggèrent que la présence de macrophages pourrait contribuer à contenir l'hypertrophie adipocytaire. Cette possibilité est évoquée pour rendre compte du phénotype des souris déficientes pour le gène *CCR2,* qui code pour le récepteur de MCP1 : elles présentent une diminution du nombre des macrophages associée à une augmentation du diamètre adipocytaire [26]. Il faut noter que les adipocytes étant les seules cellules de l'organisme capables de stocker efficacement et sans dommage l'excès d'apport calorique sous forme de triglycérides, la limitation de leur expansion peut se révéler plus néfaste que bénéfique en favorisant les dépôts ectopiques dans d'autres organes non spécialisés tels que le foie.

La fibrose, une nouvelle conséquence de l'inflammation du tissu adipeux

Associé à une inflammation chronique, le remodelage de la matrice extracellulaire peut aboutir à une accumulation anormale d'éléments matriciels qui carac-

térise la fibrose. C'est effectivement ce qui se produit dans le tissu adipeux humain, où nous avons détecté récemment la présence de dépôts fibrotiques en amas denses ou en travées [42]. Chez le sujet obèse, il existe également de la fibrose péricellulaire entourant les adipocytes *(Figure 3)*. Les macrophages sont considérés comme des régulateurs majeurs de la fibrose dans différents tissus où ils exercent des rôles pro- ou antifibrotiques suivant leur phénotype. *In vitro*, le milieu conditionné de macrophages M1 activés par le LPS ou isolés du tissu adipeux de sujets obèses confère un phénotype profibrotique aux préadipocytes humains [43]. Ces observations suggèrent un rôle plutôt profibrotique des macrophages du tissu adipeux chez les sujets obèses.

Quelles sont les conséquences de la présence de fibrose dans le tissu adipeux ? Dans notre étude chez des sujets obèses, nous observons que plus la quantité de fibrose est faible, plus les sujets perdent de la masse grasse en réponse à la chirurgie gastrique. La présence de fibrose en excès pourrait altérer le remodelage du tissu adipeux et potentiellement les adaptations métaboliques qui interviennent au cours de la perte de poids rapide et drastique induite dans ce modèle [42]. Élucider les acteurs cellulaires et moléculaires de la fibrose et ses conséquences locales ou systémiques est un nouveau défi dans le domaine de l'obésité.

Conclusion

Dans l'obésité, le tissu adipeux blanc est la cible d'un remaniement cellulaire et structural majeur dont les composants ne sont probablement pas encore tous identifiés. Les types cellulaires et les signaux impliqués sont multiples et complexes, créant localement un microenvironnement pro-inflammatoire qui favorise le recrutement de macrophages et la formation de fibrose. La cinétique de ces altérations est également mal connue au cours des différentes phases du développement de l'obésité. Bien que partiellement réversibles, certaines de ces altérations persistent après une perte de poids. L'identification des mécanismes potentiellement protecteurs, tels que la neutralisation immunologique de certains types de lymphocytes ou encore le contrôle transcriptionnel de l'inflammation, pourrait ouvrir de nouvelles perspectives thérapeutiques visant à contenir l'inflammation dans le tissu adipeux. ◊

SUMMARY

Adipose tissue, a new playground for immune cells

Adipose tissue has been under focus in the last decade and pivotal concepts have emerged from the studies of its complex biology. Low-grade inflammation both at the systemic level and in adipose tissue itself characterizes obesity. Among the different cell types contributing to inflammation, this review focuses on the mechanisms and consequences of macrophage accumulation in obese adipose tissue. Mechanisms for monocyte recruitment to adipose tissue, and how macrophages' phenotypes are modified in this environment in response to increasing fat mass, are considered. We review recent studies addressing the complex and versatile phenotype of adipose tissue macrophages that contribute to inflammatory and metabolic alterations, but could also help to maintain adipose tissue homeostasis in the setting of obesity both in mouse and human situations. A newly discovered consequence of adipose tissue inflammation is fibrosis. Whether macrophages and/or other immune cells exert a pro-fibrotic effect in adipose tissue is still unclear. This wealth of new information will hopefully help to design new ways to control adipose tissue inflammation and its deleterious sequels. ◊

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TIRÉS À PART

K. Clément

ANNEX 2

Krüppel-like factor 4 regulates macrophage polarization

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Current paradigms suggest that two macrophage subsets, termed M1 and M2, are involved in inflammation and host defense. While the distinct functions of M1 and M2 macrophages have been intensively studied — the former are considered proinflammatory and the latter antiinflammatory — the determinants of their speciation are incompletely understood. Here we report our studies that identify Krüppel-like factor 4 (KLF4) as a critical regulator of macrophage polarization. Macrophage KLF4 expression was robustly induced in M2 macrophages and strongly reduced in M1 macrophages, observations that were recapitulated in human inflammatory paradigms in vivo. Mechanistically, KLF4 was found to cooperate with Stat6 to induce an M2 genetic program and inhibit M1 targets via sequestration of coactivators required for NF-κB activation. KLF4-deficient macrophages demonstrated increased proinflammatory gene expression, enhanced bactericidal activity, and altered metabolism. Furthermore, mice bearing myeloid-specific deletion of KLF4 exhibited delayed wound healing and were predisposed to developing diet-induced obesity, glucose intolerance, and insulin resistance. Collectively, these data identify KLF4 as what we believe to be a novel regulator of macrophage polarization.

Introduction

Clinical and experimental studies support an important role for the macrophage in a broad spectrum of acute (e.g., pathogen infection, sepsis) and chronic inflammatory conditions (e.g., insulin resistance, atherosclerosis, chronic wounds, and tumorigenesis) (1–6). Macrophages exhibit remarkable plasticity that allows them to modulate their phenotype and efficiently respond to environmental signals (7). For purposes of simplicity, researchers have established a model system that classifies macrophages on a continuum in which M1 macrophages represent one extreme — the proinflammatory state — and M2 macrophages represent the contradistinct antiinflammatory state. Thus M1 and M2 macrophages can serve distinct functions in the regulation of the inflammatory response (7, 8). While the classification of the functions of M1 and M2 macrophages has provided an important tool for understanding the regulation of the inflammatory process, at present the molecular mechanisms that govern M1/M2 polarization remain incompletely understood.

Macrophages stimulated with LPS or INF-γ assume an M1 proinflammatory phenotype characterized by a high expression level of iNOS, a high capacity to present antigen, and production of proinflammatory cytokines such as TNF-α, IL-1β, and monocyte chemoattractant protein–1 (MCP-1) (9). Mechanistically, stimuli such as LPS can activate TLR, resulting in the activation of key transcriptional mediators including NF-κB that, in turn, regulate the production of proinflammatory factors. M1 macrophages are critical effector cells that kill microorganisms and thus benefit the host. However, this proinflammatory activity must be carefully titrated, as sustained activation can predispose to chronic inflammatory states such as obesity and insulin resistance. Indeed, evidence derived from murine experimental models suggests that infiltration of adipose tissue by M1 macrophages contributes to the development of obesity and insulin resistance (1, 4). Corroborative evidence has also been found in studies of human obesity. For example, clinical data indicate that macrophages residing in adipose tissue of obese human subjects can produce robust amounts of inflammatory cytokines and thus contribute to the development of insulin resistance (10). In contrast, M2 macrophages are involved in the resolution of inflammation. This cell type is more heterogeneous and is further classified into at least 3 subcategories — namely M2a, M2b, and M2c — that express different subsets of M2 marker genes and have specialized functions (8). Macrophages are differentiated to M2a by IL-4 and IL-13; M2b by immunoglobulin complexes in combination with TLR agonists; and M2c by IL-10, TGF-β, or glucocorticoids (8, 11). Accumulating evidence suggests that M2 macrophages can protect against insulin resistance, eliminate parasites, and promote tissue remodeling and repair. Interestingly, weight loss, known to improve insulin sensitivity and to induce remodeling of adipose tissue, is associated with an increase in macrophages labeled with M2 surface markers (e.g., CD206) (12). Mechanistically, IL-4 stimulation leads to phosphorylation of Stat6, which then translocates to the nucleus and activates transcription of target genes (13). More recently, studies from several laboratories support a critical role for nuclear receptors and specific

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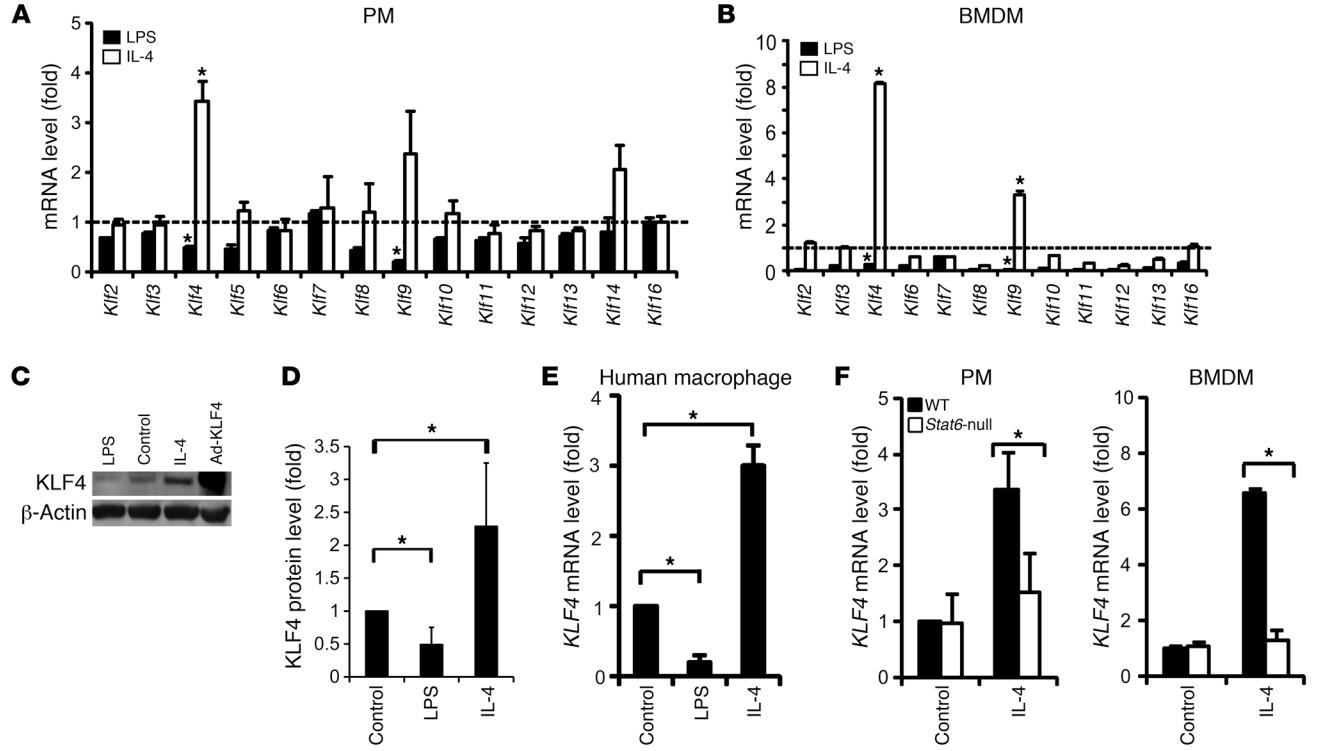

Figure 1

Klf4 expression is augmented by M2 and inhibited by M1 stimuli in macrophages. (**A** and **B**) KLF mRNA levels in (**A**) mouse PMs and (**B**) BMDMs after 16 hours of stimulation with LPS (50 ng/ml) or mouse IL-4 (5 ng/ml). Gene expression levels were assessed by qPCR and normalized to those in untreated cells (dashed line). *Klf1*, *Klf15*, and *Klf17* were not detectable in PMs or BMDMs. *Klf5* and *Klf14* were not detectable in BMDMs. $n = 5$. (**C** and **D**) KLF4 protein levels after LPS and IL-4 treatment. $n = 3$. (**E**) *KLF4* mRNA level in M-CSF–differentiated primary human macrophages (from 3 donors) stimulated with LPS (50 ng/ml) or human IL-4 (10 ng/ml) for 16 hours. (**F**) IL-4–mediated induction of the *Klf4* gene was diminished in *Stat6*-null PMs and BMDMs. $n = 3$. C57BL/6J mice were used as control (WT). $^*P < 0.05$, Student's *t* test.

coactivators (e.g., PPARγ, PPARδ, and PPARγ coactivator 1α [PGC-1α]) and the transcriptional factor HIF-2α in regulating the M2a phenotype (4, 14–17). However, the precise molecular mechanisms governing M2a polarization remain incompletely understood. Krüppel-like factors (KLFs) are a subfamily of the zinc finger class of DNA-binding transcriptional regulators. Members of this gene family have been shown to play important roles in a diverse array of cellular processes including hematopoiesis (18–20). Our group and others have reported that KLF4 is expressed in a stage-specific pattern during myelopoiesis and functions to promote monocyte differentiation (21, 22). While several studies have evaluated the role of KLF4 in macrophage cell lines (23–28), the physiologic role of myeloid KLF4 in vivo has not been elucidated. Given the important roles of the KLF family members in cellular differentiation, we hypothesized that KLF4 might be involved in the transcriptional control of macrophage polarization. Here we present data identifying KLF4 as an essential regulator of macrophage M1/M2 polarization and attendant functions. These results may have implications for the physiology and pathophysiology associated with a broad spectrum of inflammatory states.

Results

KLF4 expression is differentially regulated by M1/M2 stimuli. To determine whether members of the KLF gene family are critical in macrophage polarization, we first performed quantitative RT-PCR (qPCR) analysis for KLF1-17 in thioglycollate-elicited mouse peritoneal macrophages (PMs) (purified by differential adhesion, Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI45444DS1) and M-CSF–differentiated mouse bone marrow–derived macrophages (BMDMs) after stimulation with well-established M1 (LPS) or M2 (IL-4) polarizing agents. In both PMs and BMDMs, treatment with these two stimuli differentially regulated the expression of several KLF family members (Figure 1, A and B). The expression of *Klf4* was particularly noteworthy, as it was greatly increased by IL-4 and strongly attenuated following LPS treatment. Time course experiments revealed that a significant increase in *Klf4* expression was maintained for at least 16 hours after treatment with M2 stimuli such as IL-4 or IL-13 but not IL-10 (Supplemental Figure 2). In contrast, treatment with M1 stimuli (LPS or INF-γ) caused a mild, transient increase in *Klf4* expression between 1 and 4 hours after stimulation, followed by a significant reduction in expression at 16 hours after stimulation (Supplemental Figure 2). These differential effects on *Klf4* mRNA expression were confirmed at the protein level by Western blot analyses (Figure 1, C and D). Similar effects on *KLF4* mRNA expression were also obtained in primary human macrophages (Figure 1E). Next, we sought to determine how M2 stimuli induce KLF4 expression. A key mechanism by which the effects of IL-4 and IL-13 are mediated is the activation of the Stat6 pathway (13, 29). As shown in Figure 1F, the IL-4–mediated induc-

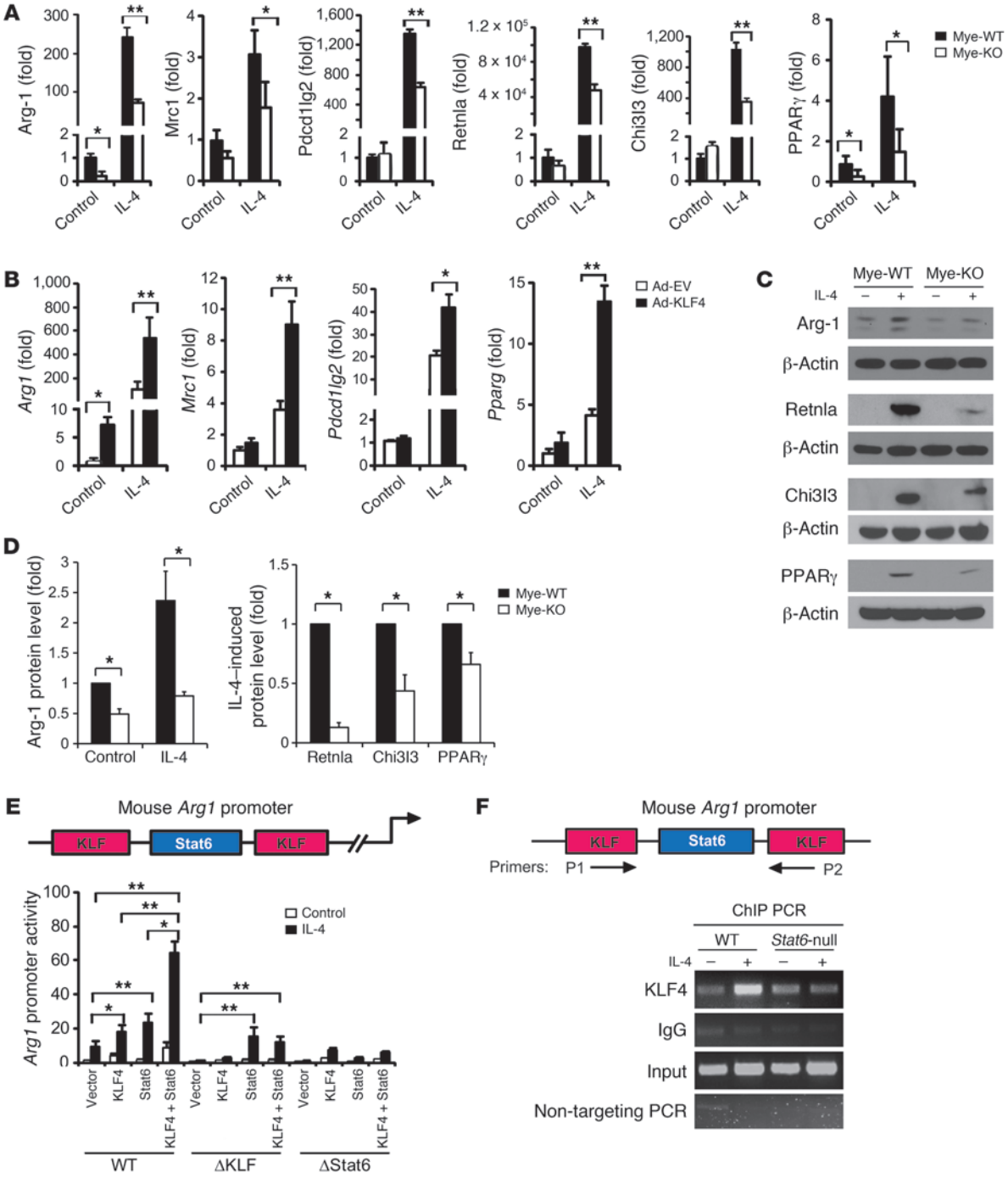

Figure 2

KLF4 is essential for IL-4–mediated macrophage M2 polarization. (**A**) Impairment of M2 marker gene expression in KLF4-deficient macrophages. PMs isolated from Mye-WT and Mye-KO mice were treated with IL-4 for 16 hours. $n = 3$ in each group. (**B**) KLF4 overexpression enhances M2 gene expression. RAW264.7 cells were infected with either Ad-GFP (Ad-EV) or Ad-KLF4 for 24 hours prior to treatment with IL-4 for 16 hours. $n = 3$ in each group. (**C**) Representative Western blot showing IL-4–mediated protein induction of Arg-1, Retnla, Chi3l3, and PPARγ in Mye-WT and Mye-KO macrophages. (**D**) Quantification of Western blot data by densitometry. For Arg-1, protein levels were normalized to Mye-WT control group. For Retnla, Chi3l3 and PPARγ, only IL-4–induced protein levels were calculated and normalized to the IL-4–treated Mye-WT group, due to extremely low levels of expression at baseline. Data were calculated from 3–5 independent blots. (**E**) Synergistic activation of the mouse *Arg1* promoter by KLF4 and Stat6 as assayed by transient transfection. WT, ~4 kb WT mouse *Arg1* promoter; ΔKLF, *Arg1* promoter with both KLF-binding sites mutated; ΔStat6: *Arg1* promoter with Stat6-binding site mutated. Transient transfection experiments were performed in RAW264.7 cells. $n = 3$. (**F**) KLF4 binding to *Arg1* promoter detected by ChIP assay in WT and *Stat6*-null BMDMs with or without IL-4 treatment (4 hours). $^*P < 0.05$, $^{**}P < 0.01$, Student's *t* test with Bonferroni correction.

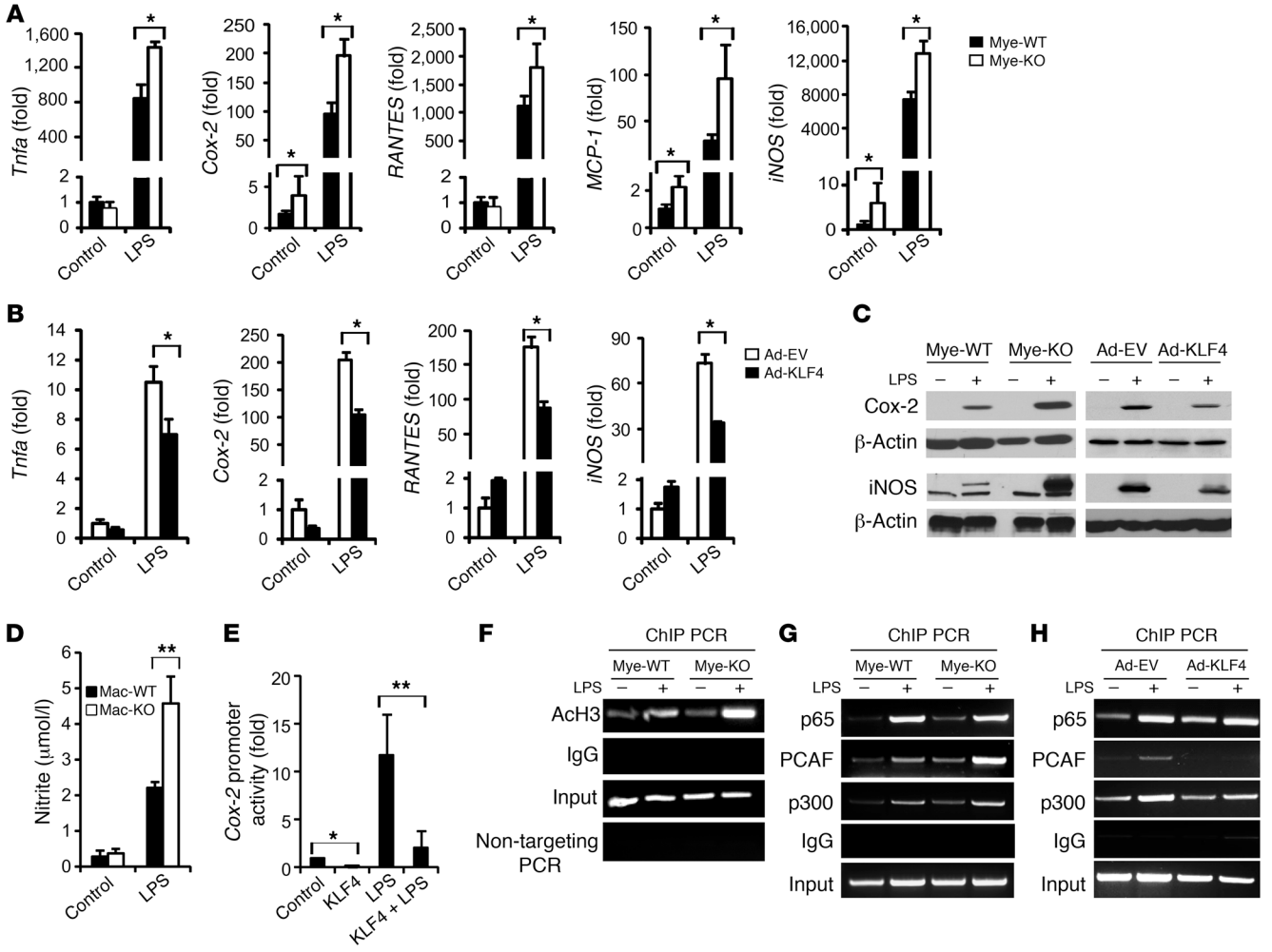

Figure 3

KLF4 deficiency enhances macrophage M1 polarization. (**A**) Enhanced expression of M1 marker genes in KLF4-deficient macrophages stimulated by LPS for 16 hours. $n = 3$ in each group. (**B**) Attenuated expression of M1 marker genes in RAW264.7 cells with overexpression of KLF4. $n = 3$ in each group. (**C**) Protein levels of Cox-2 and iNOS in PMs (left) and RAW264.7 cells (right). (**D**) KLF4-deficient macrophages generate more NO after LPS stimulation. $n = 3$ in each group. (**E**) Inhibition of *Cox-2* promoter by KLF4. Transient transfection experiments were performed in RAW264.7 cells. $n = 3$. (**F**) Enhanced acetylation of histone H3 (AcH3) in LPS-treated KLF4-deficient macrophages demonstrated by ChIP. (**G** and **H**) KLF4 regulates LPS-induced recruitment of p300/PCAF as shown by deficiency (**G**) and overexpression (**H**) experiments. $^*P < 0.05$, $^{**}P < 0.01$, Student's *t* test with Bonferroni correction.

tion of *Klf4* was abolished in *Stat6*-null PMs and BMDMs, demonstrating that *Klf4* induction by M2 agonists is Stat6 dependent.

KLF4 promotes macrophage M2 polarization. To study the function of KLF4 in the process of macrophage polarization, we performed both gain- and loss-of-function studies. Adenoviral overexpression of KLF4 in RAW264.7 cells was employed as a gain-of-function model. For the loss-of-function model, we developed myeloid-specific KLF4-deficient mice ($LysM^{Cre/Cre}Klf4^{fl/fl}$, designated Mye-KO). Mye-KO mice demonstrated greater than 90% deletion of KLF4 in PMs and BMDMs compared with the $LysM^{Cre/Cre}$ controls (designated Mye-WT), but maintained normal complete blood count (CBC) parameters and distribution of $Ly6C^{hi}Ly6C^{lo}$ monocyte populations (Supplemental Figure 3). IL-4 induces expression of prototypical target genes that characterize the M2 phenotype, including arginase-1 (*Arg1*), the mannose receptor (*Mrc1*), resistin-like α (*Retnla*, *Fizz1*), chitinase 3–like 3 (*Chi3l3*, *Ym1*), and other molecules that mediate several critical functions, including deposition of ECM, macrophage adhesion, and clearance of cell debris (30–32). In addition, PPARγ has been shown to be induced by M2 stimuli in macrophages and thereby promote the M2 phenotype (4, 17, 30). As shown in Figure 2A, the IL-4–mediated induction of characteristic M2 marker genes was significantly attenuated by approximately 42.2%–69.4% in KLF4-deficient macrophages, indicating that this factor is required for optimal M2 activation. Concordant effects were seen at the protein level (Figure 2, C and D). Consistent with these observations, overexpression of KLF4 in RAW264.7 cells enhanced basal and/or IL-4–mediated induction of *Arg1*, *Mrc1*, programmed cell death 1 ligand 2 (*Pdcd1lg2*), and *Pparg* by 2.0- to 4.7-fold (Figure 2B). KLF4 overexpression also augmented Arg-1 protein levels (Supplemental Figure 4A), and co-transfection of KLF4 induced *Pparg* promoter–driven luciferase activity (Supplemental Figure 4B). *Arg1* encodes an

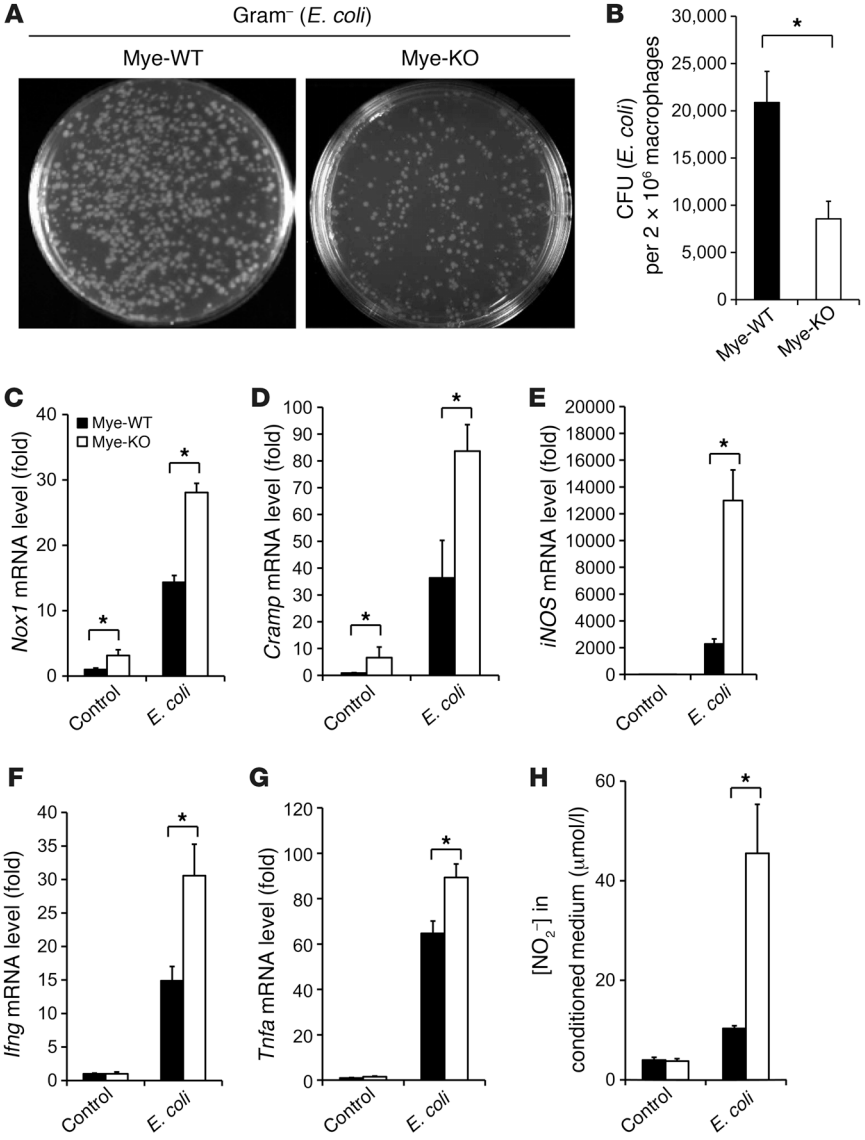

Figure 4
KLF4-deficient macrophages exhibit enhanced bactericidal activity. (**A** and **B**) KLF4-deficient macrophages exhibit enhanced bactericidal activity against *E. coli* ex vivo. $n = 3$ in each group. (**C**–**G**) Expression of genes involved in bactericidal activity is augmented in KLF4-deficient macrophages after incubation with *E. coli*. $n = 3$ in each group. (**H**) NO production in macrophage was determined by nitrite (NO_2^-) levels in conditioned medium. $n = 3$ in each group. *$P < 0.05$, Student's t test with Bonferroni correction.

L-arginine–degrading enzyme that limits NO production and promotes polyamine synthesis (13, 33). The *Arg1* promoter has been well characterized (13), and therefore we focused our mechanistic studies on understanding the molecular basis for its regulation by KLF4. Sequence analysis revealed two consensus KLF-binding sites (CACCC) within a previously identified IL-4–responsive enhancer region of the *Arg1* promoter (13, 30) (Figure 2E and Supplemental Figure 4C). This region contains a Stat6-binding site that is essential for IL-4–mediated induction of the *Arg1* gene (13). Thus, gene reporter assays were undertaken using luciferase constructs under control of a 4-kb mouse *Arg1* promoter with either the normal (WT) or mutated enhancer region (ΔKLF, ΔStat6, as described in Figure 2E). As expected, IL-4 strongly induced the *Arg1* promoter (Figure 2E); this effect was strongly attenuated with mutation of either the KLF4- or Stat-binding site. The IL-4–mediated induction of promoter activity was enhanced further by transfection of either KLF4 or Stat6 and maximally induced following co-transfection of both plasmids (Figure 2E). While mutation of the individual KLF sites conferred a mild reduction in KLF4-mediated transactivation, a more significant loss of activity was observed when both KLF sites were mutated (ΔKLF) (Figure 2E and Supplemental Figure 4C). Further, activation of Arg-1 by Stat6 was maintained with mutation of the KLF sites, but the synergistic activation of Arg-1 by KLF4 and Stat6 required both of the KLF4 sites and Stat6 to be intact (Figure 2E). Finally, to determine whether KLF4 is recruited to the *Arg1* enhancer region, we performed ChIP assays. As shown in Figure 2F, recruitment of KLF4 to the *Arg1* enhancer region was enhanced following IL-4 treatment, and this increase was abrogated in *Stat6*-null cells. In contrast, the binding of Stat6 to the *Arg1* promoter was independent of KLF4 as revealed by ChIP assay with KLF4-deficient PMs and KLF4-overexpressing RAW264.7 cells (Supplemental Figure 4D). Additionally, deficiency of KLF4 did not affect IL-4–mediated Stat6 phosphorylation, a key upstream event during Stat6 activation (Supplemental Figure 4E). Thus, we conclude that KLF4 is induced by IL-4 in a Stat6-dependent manner and that these two factors cooperate to induce M2 genes such as *Arg1*. This cooperative interaction between KLF4 and Stat6 was also found on the *Pparg* promoter (Supplemental Figure 4B).

KLF4 inhibits macrophage M1 polarization. M1 macrophages produce characteristic molecules such as prostaglandins (through induction of prostaglandin-endoperoxide synthase 2 [Cox-2]), nitric oxide (through induction of iNOS), and proinflammatory cytokines (e.g., TNF-α and IL-1β), that are critical for the immune response (8). Because KLF4 expression was reduced by M1 stimuli, we posited that this reduction in expression may be important for the M1 phenotype. As shown in Figure 3A, in response to LPS stimulation, KLF4-deficient macrophages demonstrated a marked enhancement (~1.63- to 3.24-fold) in the expression of several M1 genes such as *Cox-2*, *Tnfa*, *MCP-1* (*Ccl2*), *RANTES* (*Ccl5*), and *iNOS* (*Nos2*). Conversely, overexpression of KLF4 in RAW264.7 cells attenuated the LPS-mediated induction of M1 genes (~33.6%–53.4%; Figure 3B). These changes in mRNA levels were recapitulated by parallel changes in protein levels, as determined by Western blot and ELISA assays (Figure 3C and Supplemental Figure 5). Further, KLF4-deficient macrophages exhibit enhanced NO pro-

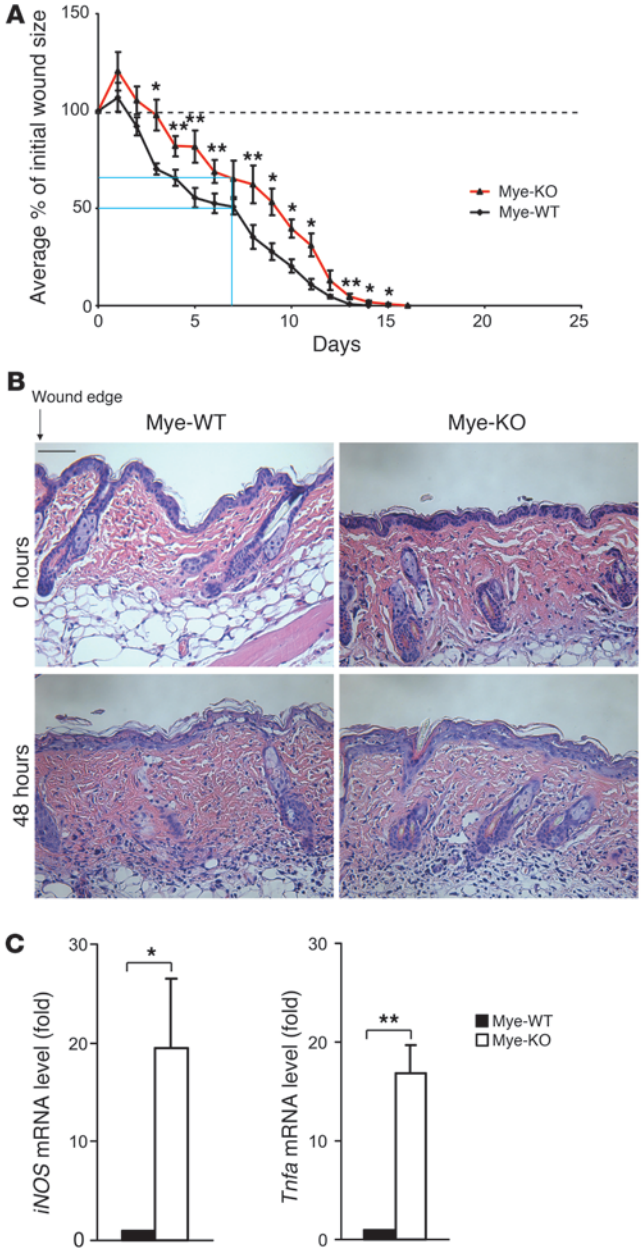

Figure 5
Myeloid KLF4 deficiency results in delayed wound healing. (**A**) Wound healing graphs of the mean percentage of initial wound area demonstrate a delay in wound healing in Mye-KO mice. At 1 week after wounding, Mye-WT mice had a 49.3% total reduction in average wound size, in contrast to a 34.9% reduction in Mye-KO mice (blue lines). Furthermore, complete wound closure in Mye-KO mice occurred on day 16, compared with day 14 for WT mice. Dashed line at 100% indicates "baseline" percentage of wound area. $n = 9$ in each group. (**B**) H&E staining of skin surround wound edge at 48 hours demonstrates similar levels of myeloid cells in the perilesional wound tissue. Scale bar: 150 μm. (**C**) Expression of *iNOS* and *Tnfa* from skin surrounding the wound edge as assessed by qPCR at 48 hours after wounding. $n = 3$ per genotype. $^*P < 0.05$, $^{**}P < 0.01$, Student's *t* test.

duction following LPS stimulation as assessed by the Griess assay (Figure 3D). Of note, we did not observe any enhancement of LPS-induced M1 gene expression or NO production in *Stat6*-null macrophages (Supplemental Figure 6), suggesting that the enhanced expression of some M1 targets seen in KLF4-deficient macrophages is not a consequence of global impairment in M2 activation. To elucidate the molecular mechanism by which KLF4 inhibits M1 gene expression, we focused our attention on Cox-2, a classic pro-inflammatory target. Transient transfection assays in RAW264.7 cells showed that KLF4 inhibited basal and LPS-induced *Cox-2* promoter activity (Figure 3E). However, sequence analysis of the *Cox-2* promoter did not reveal a canonical KLF-binding site. Thus, we reasoned that KLF4 may function indirectly by altering pathways that mediate LPS induction of the *Cox-2* promoter. Indeed, the increased levels of acetylated histone H3 associated with the *Cox-2* promoter in LPS-treated KLF4-deficient macrophages would correspond to greater transcriptional activation of the *Cox-2* gene (Figure 3F). Because activation of the NF-κB pathway is critical for LPS-mediated Cox-2 induction (34), we assessed whether KLF4 affects this pathway. KLF4 overexpression or deficiency did not significantly affect IκB phosphorylation or degradation (data not shown) and resulted in a modest increase in p65 recruitment to the *Cox-2* promoter in LPS-treated cells (Figure 3, G and H). However, KLF4 deficiency enhanced and overexpression attenuated the recruitment of the coactivators p300 and PCAF to the *Cox-2* promoter following LPS stimulation (Figure 3, G and H). The recruitment of p300 and PCAF is specific for the NF-κB site on the *Cox-2* promoter (35), as no enrichment was observed to a different region of the *Cox-2* promoter (Supplemental Figure 7). These data suggest that KLF4 deficiency allows for increased recruitment of cofactors known to augment NF-κB transcriptional activity. Finally, because KLF4 induces the M2 and inhibits the M1 phenotype, we sought to determine whether KLF4 may be required for suppression of inflammatory gene expression in M2 macrophages. For these studies, PMs from Mye-WT and Mye-KO mice were treated with IL-4 for 16 hours, and LPS was added for an additional 6 hours. As expected, pretreatment with IL-4 significantly inhibited LPS-mediated M1 gene induction (e.g., *iNOS*, *Tnfa*, *MCP-1*, and *RANTES*) by approximately 42.8%–51.3% in WT macrophages. However, this inhibitory effect was strongly attenuated in KLF4-deficient macrophages (Supplemental Figure 8). These data suggest that KLF4 may also be important in suppressing inflammatory gene expression in M2 macrophages.

KLF4-deficient macrophages exhibit enhanced bactericidal activity. The bactericidal activity of macrophages is generally viewed as within the purview of M1-polarized macrophages (9). Because KLF4-deficient macrophages exhibit enhanced M1 polarization, we hypothesized that these cells may exhibit enhanced bactericidal activity. Thus, we performed phagocytosis and bacterial killing assays in control and KLF4-deficient primary macrophages. Indeed, KLF4-deficient macrophages exhibited significantly greater bactericidal activity toward both Gram-negative (*E. coli*) (Figure 4, A and B) and Gram-positive (*Staphylococcus aureus*; Supplemental Figure 9, A and B) bacteria. This effect was not due to alterations in phagocytosis, as assessed by fluorescence microscopy and FACS analysis (Supplemental Figure 10). To determine the basis for the enhanced bactericidal activity, we assessed for expression of effector molecules known to be involved in pathogen killing. Exposure of KLF4-deficient macrophages to *E. coli* (Figure 4C) or *S. aureus* (Supplemental Figure 9C) increased expression of the ROS-generating enzyme

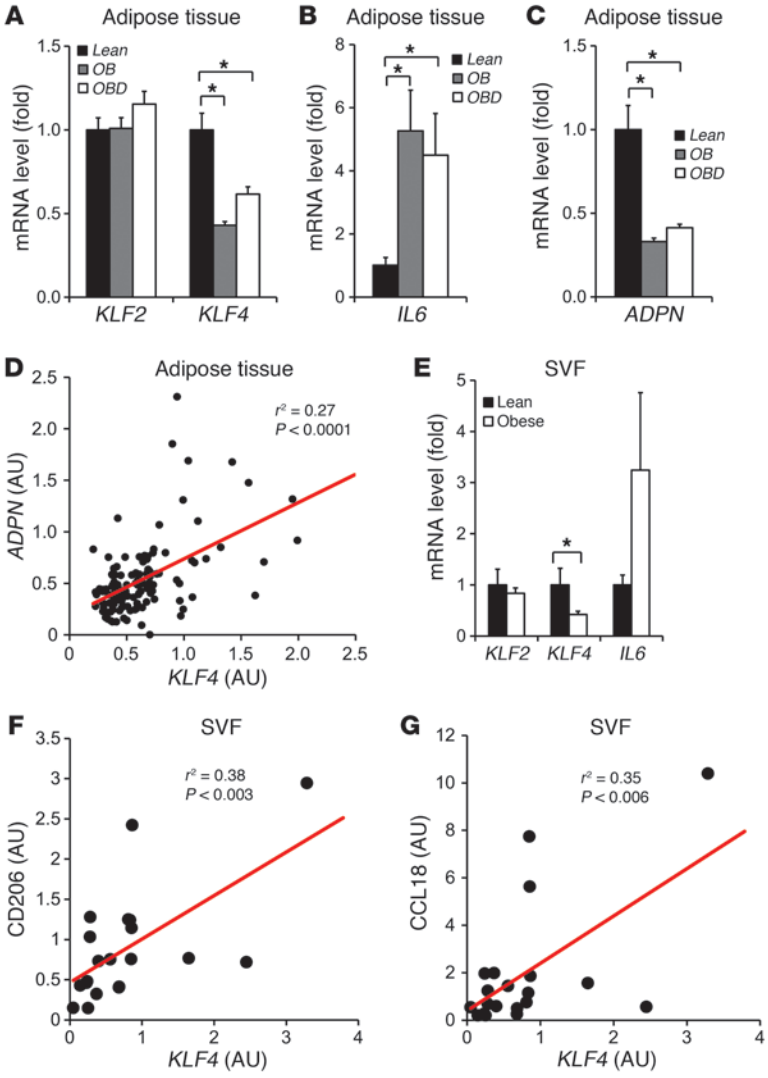

Figure 6
Relationship of KLF4 to obesity in human subjects. (**A**–**C**) Gene expression of *KLF2*, *KLF4*, *IL6*, and *ADPN* in subcutaneous adipose tissue (needle biopsies) from 48 nondiabetic obese (OB), 37 obese diabetic (OBD), and 20 control (Lean) subjects. (**D**) Significant correlation between gene expression of *KLF4* and *ADPN* in human adipose tissue. r^2 and *P* values by Pearson correlation test. (**E**) *KLF2*, *KLF4*, and *IL6* gene expression in SVF from 9 non-obese and 12 obese patients. (**F** and **G**) Significant correlations between gene expression of *KLF4* and CD206 and CCL18 in SVF from 9 non-obese and 12 obese patients. r^2 and *P* values by Pearson correlation test. **P* < 0.05, Kruskal-Wallis test (vs. lean group). Gene expression in SVF samples was normalized to *CD68*.

NADPH oxidase 1 (*Nox1*) by approximately 2.0-fold. In addition, exposure to *E. coli* also increased expression of several additional bactericidal targets such as cathelicidin-related antimicrobial peptide (*Cramp*; ~2.3-fold), *iNOS* (~5.7-fold), *Ifng* (~2.1-fold), and *Tnfa* (~1.4-fold) (Figure 4, D–G). Consistent with the effect on iNOS, nitric oxide production was approximately 3.4-fold higher in KLF4-deficient macrophages infected by *E. coli* (Figure 4H).

Myeloid KLF4 deficiency results in delayed wound healing. Macrophages are rapidly recruited to sites of tissue injury and become key regulators of inflammation, tissue repair, and cellular debris clearance (8, 36). The initial inflammatory response is regulated by M1 macrophages, while the healing/repair response is thought to lie in the purview of M2 macrophages (37). Importantly, excess or dysregulated inflammation in the skin can lead to delayed wound closure and further tissue destruction. Because KLF4-deficient macrophages exhibit enhanced M1 polarization, we hypothesized that these cells may increase proinflammatory cytokine expression in the surrounding tissue and lead to delayed wound closure due to tissue destruction. Thus, we assessed the time for wound closure in Mye-WT and Mye-KO mice following cutaneous wounding. Data from wound healing curves showed that Mye-KO mice have delayed wound healing (Figure 5A). At 1 week after wounding, Mye-WT mice demonstrated a 49.3% total reduction in average wound size, in contrast to a 34.9% reduction in Mye-KO mice. Ultimately, complete closure of wounds was delayed by 2 days in Mye-KO mice compared with Mye-WT mice (Figure 5A). Histologic examination revealed that the skin adjacent to wounds in both genotypes had equivalent monocyte/macrophage infiltration in the dermal and subcutaneous layers (Figure 5B and Supplemental Figure 11A). Furthermore, ex vivo studies confirmed that migration of KLF4-deficient macrophages in response to the prototypical monocyte/macrophage chemokine MCP-1 was similar to that of control cells (Supplemental Figure 11B). To determine whether delayed wound healing is mediated by enhanced M1-polarized macrophages in Mye-KO mice, we analyzed the skin surrounding the wound for proinflammatory factors. Data from qPCR analysis of RNA harvested from skin at 48 hours after wounding show that *iNOS* and *Tnfa* expression was significantly increased (>17-fold) in Mye-KO mice compared with Mye-WT mice (Figure 5C). These data demonstrate that Mye-KO mice have increased proinflammatory factors in the skin associated with a concomitant delay in wound healing.

KLF4 expression is regulated in human obesity. Macrophages are abundantly present in obese adipose tissue, and these adipose tissue macrophages (ATMs) contribute to many of the circulating inflammatory molecules postulated to be involved in insulin resistance and type 2 diabetes progression (1, 14). Increased presence of M1 macrophages has been described in adipose tissue from obese human subjects (12). Thus, we assessed *KLF4* gene expression in subcutaneous adipose tissue (needle biopsies) from nondiabetic obese (n = 48), diabetic obese (n = 37), and lean patients (n = 20) (Supplemental Table 1). Data from qPCR analysis showed that *KLF4* expression was significantly reduced by 50% in obese patients compared with lean subjects ($P < 0.05$), regardless of their diabetic status (Figure 6A). Age adjustments did not change this relationship. The expression of *KLF2*, a closely related family member (38, 39), was unchanged (Figure 6A). Consistent with previous studies (40), *IL6* expression was increased, while adiponectin (*ADPN*) levels were reduced in obese adipose tissue (Figure 6, B and C). Further, a significant positive correlation ($r^2 = 0.27$, $P < 0.0001$, Pearson correlation test) was observed between mRNA levels of *KLF4* and

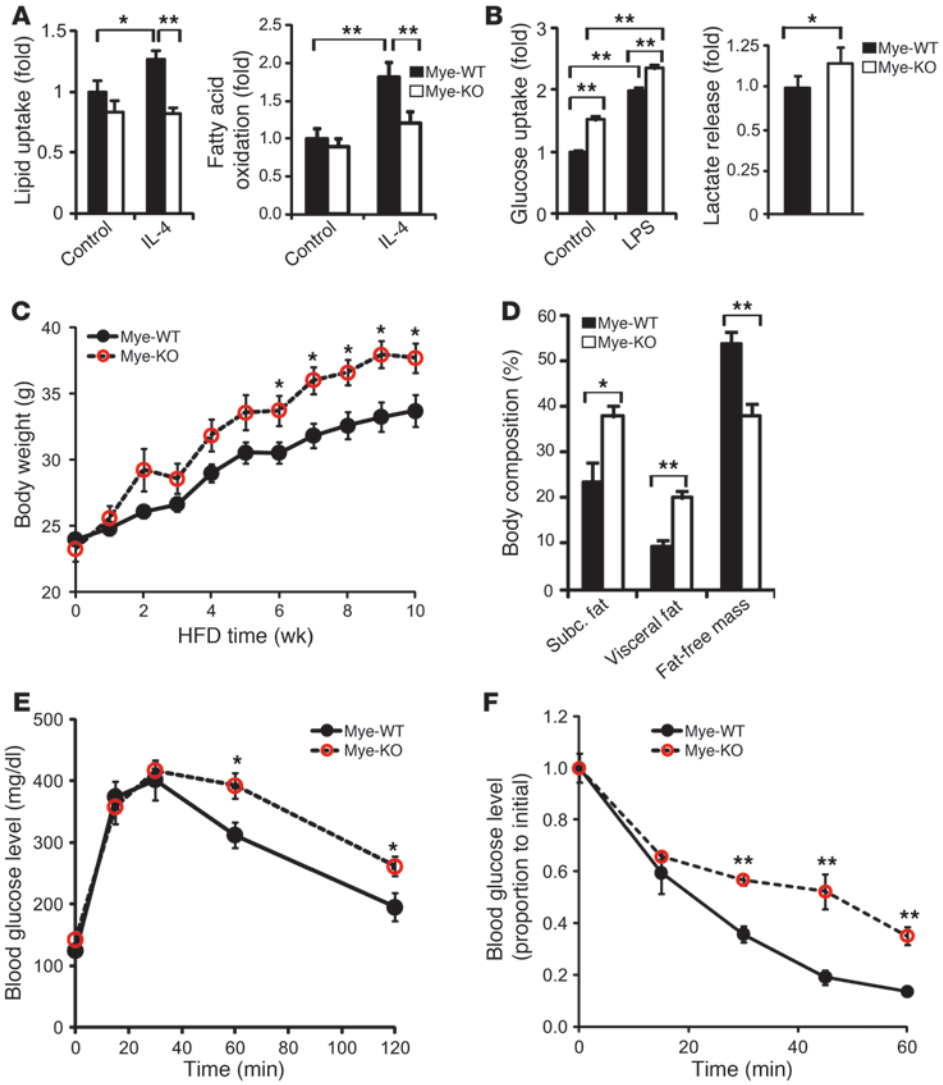

Figure 7
Myeloid-specific deficiency of KLF4 exaggerates HFD-induced obesity and insulin resistance state in mice. (**A**) Impairment of IL-4–mediated lipid uptake (left panel) and β-oxidation (right panel) in KLF4-deficient macrophages. [^{3}H] counts were normalized to protein content and expressed as fold increase relative to untreated controls. n = 8 in each group. (**B**) KLF4-deficient macrophages exhibit enhanced glucose uptake before and after LPS stimulation (left panel) and release more L(+)-lactate (right panel), a product of glycolysis. [^{3}H] counts from 2-deoxy-[^{3}H]-D-glucose in the cell lysate and L(+)-lactate concentration in the conditioned medium were normalized to protein content and expressed as fold increase over untreated controls. n = 8 in each group. (**C** and **D**) Mye-KO mice on HFD gained more weight (**C**) and stored more fat (**D**) than Mye-WT controls. n = 6 in each group. Subc., subcutaneous. (**E** and **F**) After 10–12 weeks of HFD, Mye-KO mice show impaired glucose metabolism as revealed by glucose intolerance (**E**) and resistance to exogenous insulin (**F**). n = 6 per genotype. *P < 0.05, **P < 0.01, Student's t test with Bonferroni correction.

ADPN in human adipose tissue (Figure 6D). Finally, since visceral fat is more metabolically active and closely linked to insulin resistance, we compared *KLF4* expression in this compartment versus subcutaneous fat. *KLF4* (but not *KLF2*) expression in visceral fat was significantly lower (~50%) than that in subcutaneous fat samples from obese subjects (Supplemental Figure 12A).

Macrophages are typically present in the stromal vascular fraction (SVF), and thus we also assessed KLF4 expression in the SVF of lean and obese subjects. Cellular fractionation of human SVF revealed that *KLF4* expression in macrophages was higher than in other cellular constituents (Supplemental Figure 12B). Consistent with the adipose tissue data (Figure 6A), a 50% reduction of *KLF4* expression was observed in SVF from obese subjects (Figure 6E). Finally, in SVF samples, the expression of *KLF4* was positively correlated with that of prototypical human M2 macrophage makers such as CD206 (mannose receptor) (r^2 = 0.38, P < 0.003, Pearson correlation test) and CCL18 (r^2 = 0.35, P < 0.006, Pearson correlation test) (Figure 6, F and G). Collectively, these data indicate that *KLF4* expression is regulated in human obesity.

Myeloid KLF4 deficiency promotes insulin resistance. We next asked whether myeloid-specific deficiency of KLF4 could affect macrophage metabolism and systemic metabolic homeostasis. M2 stimuli, such as IL-4, induce a metabolic switch to fatty acid oxidation, while M1 stimuli promote glycolysis in macrophages (15, 30). As shown in Figure 7A, in the absence of KLF4, the IL-4–mediated uptake and β-oxidation of oleic acid by macrophages was significantly attenuated. However, in response to an M1 stimulus such as LPS, KLF4-deficient macrophages exhibited higher glucose uptake both in untreated and LPS-treated conditions and released more L(+)-lactate, indicating an enhanced rate of glycolysis (Figure 7B). To determine the role of myeloid KLF4 in the development of obesity and insulin resistance, we subjected Mye-WT and Mye-KO mice to diet-induced obesity (DIO) via a high-fat diet (HFD). After 10 weeks of HFD (started at age of 8 weeks), the body weight of Mye-KO mice (37.7 ± 1.1 g) exceeded that of Mye-WT controls (33.7 ± 1.2 g) by approximately 12% (Figure 7C). Mye-KO mice accumulated more adipose tissue than the Mye-WT controls (60% more subcutaneous fat and 110% more visceral fat) as revealed by MRI (Figure 7D and Supplemental Figure 13A). This higher fat mass was associated with a moderate increase in circulating triglyceride levels (Mye-KO: 91.7 ± 8.6 mg/dl vs. Mye-WT: 75.2 ± 5.2 mg/dl, P = 0.067) (Supplemental Figure 13B).

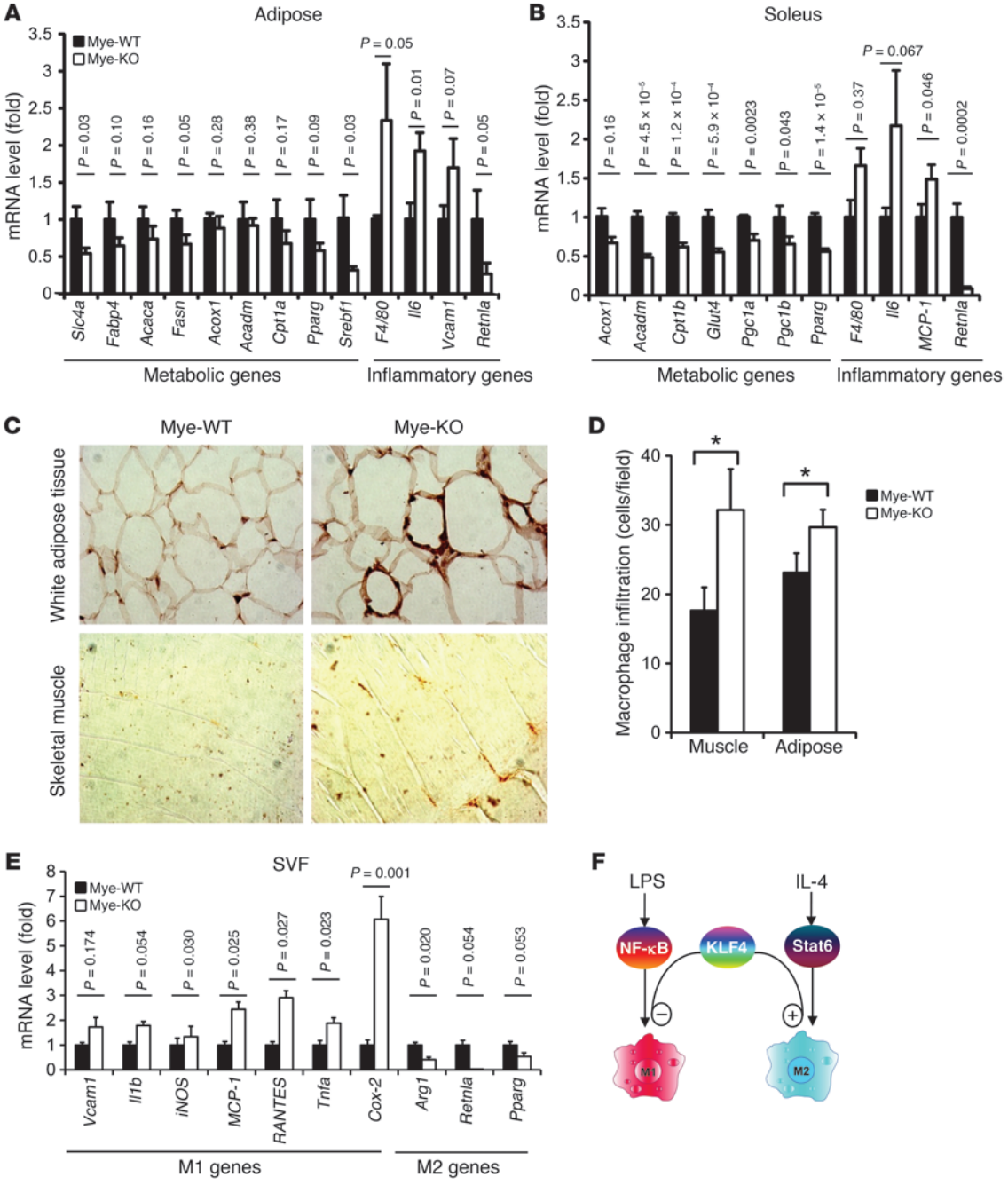

Figure 8

Mye-KO mice exhibit altered inflammatory and metabolic gene expression in tissues following HFD. (**A** and **B**) Myeloid deficiency of KLF4 in HFD-mice affects transcription of many genes involved in nutrition uptake, β-oxidation, oxidative phosphorylation and inflammation in white adipose tissue (**A**) and skeletal muscle (**B**). $n = 6$ in each group. (**C**) Increased macrophage infiltration of white fat tissues and muscle tissues as determined by Mac-3 antibody staining. Original magnification, ×400. (**D**) Quantification of Mac-3 staining. $n = 6$ in each group. (**E**) Myeloid deficiency of KLF4 in HFD-fed mice affects expression of macrophage M1 and M2 genes in SVF. $n = 6$ in each group. $^*P < 0.05$, Student's t test. (**F**) Schematic of proposed mechanism. See text for details.

HDL and LDL levels were similar in the two groups (Supplemental Figure 13, C and D). These data indicated that Mye-KO mice developed more pronounced DIO. This phenotype was not due to differences in food consumption (Supplemental Figure 14A), indicating that total body energy expenditure may be reduced in the Mye-KO mice. Indeed, indirect calorimetric assays confirmed that Mye-KO mice exhibited reduced oxygen consumption, heat production, and respiratory quotient (Supplemental Figure 14B). To determine whether myeloid KLF4 deficiency resulted in insulin resistance, we performed glucose and insulin tolerance tests

in HFD-fed Mye-WT and Mye-KO mice. Serum insulin levels in HFD-fed Mye-KO mice were significantly increased, indicating that these mice may have a propensity toward insulin resistance (Supplemental Figure 15A). Glucose tolerance tests revealed that Mye-KO mice had increased glucose levels 30 minutes after challenge (Figure 7E). As expected with decreased insulin sensitivity, Mye-KO mice were more resistant to the glucose-lowering effects of exogenous insulin (Figure 7F). Lean mice on a normal chow diet showed a similar trend, in that Mye-KO mice were also less glucose tolerant than Mye-WT controls, though the difference did not reach statistical significance (Supplemental Figure 15B). Finally, in keeping with an insulin-resistant state, phosphorylation of Akt was reduced in liver and skeletal muscle of Mye-KO mice after HFD (Supplemental Figure 15C).

To better define the HFD-induced alterations in adipose tissue and muscle, we quantified transcript levels of genes important in fatty acid metabolism and inflammation. As shown in Figure 8A, mRNA levels of a large number of genes encoding key enzymes in nutrient uptake (*Slc4a*, sodium-coupled bicarbonate transporter 4A), fatty acid binding (*Fabp4*), fatty acid synthesis (*Fasn*, fatty acid synthase; *Acaca*, acetyl-coenzyme A carboxylase α), and β-oxidation (*Cpt1a*, carnitine palmitoyltransferase 1a), as well as transcriptional factors known to regulate fatty acid metabolism (*Pparg*; *Srebf1*, sterol regulatory element–binding protein 1), were reduced by 33.3%–68.0% in white adipose tissue from HFD-fed Mye-KO mice. To determine whether macrophage infiltration and the polarization status of infiltrated macrophages contributed to adiposity, we analyzed macrophage-specific gene expression in the white adipose tissue of HFD-fed Mye-WT and Mye-KO mice. Notably, expression of the macrophage-specific gene *F4/80* increased more than 2.3-fold in adipose tissue of HFD-fed Mye-KO mice, indicating enhanced infiltration of macrophages into adipose tissue of these mice (Figure 8A). The inflammatory genes *Il6* and *Vcam1* were increased 1.7- and 1.9-fold, respectively, while *Rentla*, a marker of M2 macrophages, was reduced by 73.4% in adipose tissue (Figure 8A). In skeletal muscle, genes encoding fatty acid β-oxidation enzymes (*Acox1*, peroxisomal acyl-coenzyme A oxidase 1; *Acadm*, acyl-coenzyme A dehydrogenase for medium-chain fatty acids; *Cpt1b*, carnitine palmitoyltransferase 1b) and the insulin-sensitive glucose transporter gene *Glut4* were reduced by 29.4% to 51.3% in HFD-fed Mye-KO mice (Figure 8B and Supplemental Figure 16A). *F4/80* expression was increased by 66.3% in skeletal muscle, indicating enhanced macrophage infiltration. Moreover, the inflammatory genes (*MCP-1*, *Il6*) were increased, and the M2 macrophage marker gene *Rentla* was reduced significantly in skeletal muscle (Figure 8B and Supplemental Figure 16A). These gene expression data demonstrate that, after HFD, Mye-KO mice have enhanced macrophage infiltration in adipose tissue and skeletal muscle and that the infiltrating macrophages exhibit an inflammatory M1 phenotype. The enhanced infiltration of macrophages in adipose tissue and skeletal muscle of obese Mye-KO mice was further confirmed by immunohistochemistry for Mac-3 (Figure 8, C and D). By comparison, livers from Mye-KO mice did not show significant changes in metabolic genes, but did show a trend toward increased expression of inflammatory genes (e.g., *MCP-1*, *Vcam1*) (Supplemental Figure 16B). Finally, we assessed for M1/M2 targets in the SVF of Mye-WT and Mye-KO mice following HFD feeding. As shown in Figure 8E, many M1 genes (e.g., *Vcam1*, *Il1b*, *iNOS*, *RANTES*, *Tnfa*, *Cox-2*) were expressed at significantly higher levels in Mye-KO SVF, while M2 genes (e.g., *Arg1*, *Rentla*, *Pparg*) were significantly reduced. Collectively, these data suggest that KLF4 deficiency may affect the polarization status of ATMs.

Discussion

Transcriptional control of macrophage differentiation is currently the subject of intense investigation. To date, control of macrophage polarization has largely been attributed to the function of a small group of factors including NF-κB, AP-1, HIFs, STATs, and PPARs. Our observations introduce the KLF family as contributors to this process and, more specifically, identify KLF4 as critical in regulating M1/M2 polarization (Figure 8F). Since the first identification of mammalian KLF family members in 1993 (41), a total of 17 KLF factors have been identified. Several recent reports have implicated KLFs in myeloid cell biology. With respect to KLF4, Feinberg et al. showed that KLF4 promotes monocyte differentiation (21). These observations were extended by elegant studies from the Civin laboratory that determined, by transplantation of hematopoietic *Klf4*-KO fetal liver cells into lethally irradiated WT mice, that KLF4 has an essential role in the differentiation of mouse monocytes (22). Specifically, these studies identified an essential role for KLF4 in Ly6C^{hi} and Ly6C^{lo} monocyte development. In light of these observations, and in order to minimize developmental effects, we employed the LysM-Cre mouse line, in which the Cre gene is under control of the endogenous LysM promoter. Owing to the temporal and spatial nature of LysM promoter activation in myeloid cells, the Cre gene is expressed at low levels during myeloid development but is highly induced upon transformation of mature monocytes into macrophages (42, 43). Consistent with our expectation, KLF4 deficiency, as achieved in the LysM$^{Cre/Cre}$*Klf4*$^{fl/fl}$ mouse, did not significantly affect the peripheral blood cell differential or monocyte subsets (Supplemental Figure 3). Thus, our approach enabled a detailed evaluation of KLF4 in macrophage biology and led to our identification of KLF4 as an important regulator of M1/M2 polarization. Collectively, the results of the current study coupled with previous work implicate KLF4 in multiple stages of monocyte/macrophage biology, from cellular differentiation to activation.

A key observation provided by our studies is that KLF4 can differentially affect a large repertoire of genes that characterize the M1 and M2 phenotype. With respect to M2 polarization, our studies indicate that KLF4 is both induced by and cooperates with Stat6 to induce quintessential M2 targets such as Arg-1 following IL-4 stimulation. Our studies of the *Arg1* promoter reveal that maximal transcriptional activity of Stat6 requires intact KLF4 sites (and vice versa). This type of inductive and cooperative relationship has also been observed between Stat6 and PPARγ in macrophages during M2 activation (30). The fact that PPARγ levels were reduced in *Klf4*-null cells and tissues raises the possibility that KLF4 and Stat6 may also cooperate to augment PPARγ expression — a possibility that is supported by the inductive effect of KLF4 and Stat6 on the *Pparg* promoter (Supplemental Figure 4B). The importance of this relationship between PPARγ and KLF4 is also supported by the fact that myeloid deletion of PPARγ leads to a phenotype that is similar to that of KLF4 Mye-KO mice observed in this study (30, 44). While additional studies are clearly warranted, current observations raise the possibility that a cascade of inductive and cooperative interactions in the Stat6/KLF4/PPARγ axis may allow for optimal and sustained M2 activation. Finally, while much of our work here has focused on M2 polarization in the context of IL-4 stimulation, a recent study suggested that alternative IL-4–independent mechanisms may also regulate the M2 phenotype (45).

Whether KLF4 participates in this pathway is an important issue that will require further investigation.

The induction of KLF4 provides a molecular mechanism to not only induce the M2 phenotype but also inhibit the M1 pathway. The importance of KLF4's inhibition of proinflammatory targets is highlighted by gain- and loss-of-function studies in vitro (Figures 3 and 4) and by corroborative evidence in disease models (Figures 5, 7, and 8). Furthermore, the observation that KLF4 deficiency attenuates the ability of IL-4 to inhibit M1 targets (Supplemental Figure 8) suggests that KLF4 may also be important in suppression of proinflammatory genes in M2 macrophages. Our mechanistic studies demonstrate that this antiinflammatory effect occurs at least in part through the ability of KLF4 to inhibit NF-κB transcriptional activity. Importantly, altering KLF4 levels did not significantly affect activation of IKK/IκBα. However, optimal NF-κB transcriptional activity requires key coactivators such as p300/CBP and associated factors such as PCAF. Because KLF4 interacts with p300/CBP (46, 47), we reasoned that it may compete with NF-κB for these rate-limiting amounts of this coactivator, a notion supported by our ChIP studies (Figure 3, G and H). Finally, in addition to competing for coactivators, the KLF4-mediated induction of PPARγ likely contributes to the potent inhibition of M1 polarization by KLF4. Efforts to understand whether these two factors cooperate to inhibit classical macrophage activation is an important issue that is currently being investigated. Our studies also provide insights regarding discrepant observations in the literature regarding the kinetics of KLF4 expression and its targets in response to proinflammatory stimuli. It is likely that (a) idiosyncrasies of various cell lines employed (e.g., J774a, THP-1, RAW, and BV-2) and (b) differences in the concentration (ranging from 10 ng/ml to 1,000 ng/ml) and source of LPS employed (e.g., *E. coli*, *Porphyromonas gingivalis*, and *Salmonella enterica*) may account for many of the disparate results seen among various studies (23–28). For example, several studies in cell lines and/or primary cells have reported an increase in KLF4 expression. Most of these studies assessed expression at early time points following stimulation. In the current study, the most dramatic effect on KLF4 expression in primary murine macrophage was a reduction that occurred at late time points. While this appears to be contradictory, we note that a modest but reproducible increase (statistical significance achieved in PMs [10 ng/ml at 4 hours] and BMDMs [50 ng/ml at 4 hours]; Supplemental Figure 2) was also seen in primary cells. The magnitude is clearly modest but is consistent with observations in cell lines. Importantly, our studies suggest that the reduction in KLF4 expression, observed in both murine and human macrophages as well as human tissue samples (SVF fraction; Figure 6E), is critical, as evidenced by the in vitro and in vivo gene expression and functional studies. A second issue relates to the regulation of iNOS expression by KLF4. The increase in this M1 marker in the setting of KLF4 deficiency was surprising given the previous finding by our group and others that KLF4 induces iNOS expression (23). An important difference between these two studies is that the previous work was performed in the mouse monocyte/macrophage tumor cell line J774a. Indeed, repeat studies have confirmed that overexpression of KLF4 in this cell line robustly induces iNOS. Interestingly, in contrast to the induction of iNOS, KLF4 overexpression reduced the expression of MCP-1 in J774a cells (Kapadia and Jain, unpublished observation). Thus, while gain-of-function studies (in cell lines) and loss-of-function studies (in primary cells) revealed the anticipated anti-parallel effect on some M1 markers such as MCP-1, the basis for the induction of iNOS in both contexts remains incompletely understood. It is noteworthy that differential effects of KLF4 on target genes and cellular functions have been reported in other systems. In the cancer literature, a number of studies support both a pro- and anti-tumorigenic role for KLF4. A potential explanation based on the p21 status of cancer cells was put forth by Rowland and colleagues. This study and others suggest that the cellular context likely affects KLF4 action (48, 49). While additional studies are required to elucidate the molecular basis for the differential effect of KLF4 on iNOS expression in J774a cells versus primary cells, the concordant effects on an entire cassette of M1 genes in primary macrophages observed in the current study suggest that KLF4 inhibits the M1 phenotype. Furthermore, the fact that iNOS was robustly induced in the skin wounds of myeloid-specific KLF4-deficient mice (Figure 5C) strongly supports this in vitro finding. Finally, our observations reinforce the notion that the use of primary cells and corroboration of findings in vivo will be critical in future studies aimed at understanding the biology of myeloid KLF4.

Converging studies reveal that phenotypic polarization of macrophages by the inflammatory microenvironment is critical for both induction and resolution of the inflammatory response. For example, in lean adipose tissue, production of IL-13 can induce the M2 phenotype in ATMs (14). However, obesity can induce a proinflammatory state that promotes the M1 phenotype and initiates a feed-forward circuit that amplifies the inflammatory response and contributes to insulin resistance (1). Similarly, Th1 cytokines predominate early in the course of an infection and thereby enhance macrophage bactericidal effects, while Th2 cytokines confer an M2 phenotype to help in the resolution and repair following an inflammatory insult. In this regard, our observations that KLF4 expression is differentially regulated by Th1 and Th2 cytokines is of particular interest. Furthermore, these studies predict that in the absence of KLF4, the balance would be tipped in favor of an M1 phenotype. Our functional studies are aligned with this hypothesis, as we observed that KLF4-deficient macrophages exhibit robust bactericidal activity. However, proinflammatory activation can be deleterious in the setting of chronic inflammatory conditions, resulting in delayed wound healing and, if sustained, insulin resistance. Indeed, the observation that KLF4 MyeKO mice exhibited delayed wound healing and susceptibility to DIO/insulin resistance is consistent with this idea.

An important aspect of this work is the association of KLF4 with human obesity. Consistent with the view that ATMs in obese subjects assume an M1 phenotype, a significant reduction in KLF4 expression was seen in adipose tissue from obese human subjects. Furthermore, the observation that KLF4 expression is reduced in visceral versus subcutaneous fat in obese subjects is particularly intriguing given the epidemiologic association between visceral fat and insulin resistance. Our studies also extended our assessment of KLF4 in obesity to the SVF fraction where the macrophage population resides. We note that although murine and human M1 macrophages express similar M1 markers, there are distinct differences in M2 markers (33). For example, some studies indicate that human monocytes and monocyte-derived macrophages do not induce Arg-1, YM1, or FIZZ1 in response to IL-4/IL-13 (31, 33, 50). However, like murine macrophages, alternatively activated human macrophages do express CD206 (C-type receptor 1; MRC1) and CCL18. This receptor belongs to the group of pattern recognition receptors involved in host cell immune responses through

phagocytosis and endocytosis. Previous studies demonstrate that the ratio of $CD40^+$ to $CD206^+$ macrophages was 2-fold lower after weight loss in the subcutaneous adipose tissue of obese subjects due to a concomitant decrease in $CD40^+$ and increase in $CD206^+$ macrophages (12). Furthermore, we also assessed CCL18, which was identified in the late 1990s by several groups (5–9, 51) and given different names owing to the manner of discovery, including alternative macrophage activation–associated CC chemokine–1 (AMAC-1). Although a rodent counterpart has not been identified, CCL18 expression by macrophages has been implicated in lymphocyte and immature dendritic cell recruitment. CCL18 has also been identified as being differentially expressed in adipose tissue of patients with nonalcoholic steatohepatitis with fibrosis (52). Interestingly, we found a significant correlation between both human M2 markers and KLF4 in SVF samples. In addition, and consistent with the observation in adipose tissue, KLF4 expression was reduced in the SVF of obese subjects (Figure 6E). Collectively, these observations suggest that the reduction in KLF4 expression may have important implications for human obesity.

The ability for KLF4 to regulate both M1 and M2 polarization has potentially broad implications for numerous inflammatory disease states beyond insulin resistance and wound healing. The fact that KLF4-deficient macrophages exhibited enhanced bactericidal activity (Figure 4) supports a role for this factor in disease processes such as sepsis. Importantly, since KLF4 is also expressed in granulocytes, and LysM-Cre deletes floxed genes in both cell types (53, 54), such studies will need to account for effects of KLF4 deficiency in both macrophages and granulocytes in order to fully elucidate the integrated host response to bacterial infection. In addition, since macrophages are critical in combating chronic infections (e.g., *Mycobacterium tuberculosis*) and parasites (e.g., *Listeria monocytogenes*), investigation of these pathologic states is also likely to be fruitful. Furthermore, given the importance of macrophages in atherosclerosis and tumorigenesis, assessment of these processes in the myeloid KLF4-deficient model is clearly warranted. Finally, as KLF4 levels can be manipulated by diverse agonists such as statins, resveratrol, proteosome inhibitors (e.g., bortezomib), and dietary compounds (e.g., broccoli), KLF4 is a potentially promising therapeutic target.

Methods

Human subjects. The study enrolled 114 morbidly obese subjects involved in a gastric surgery program and 20 lean controls recruited at the Department of Nutrition, Hôtel-Dieu Hospital, Paris, France. Obese subjects met the criteria for obesity surgery and were categorized into diabetic and nondiabetic groups (Supplemental Table 1). Fat specimens were obtained by needle aspiration in the periumbilical area under local anesthesia (12). The Ethics Committee, Hôtel-Dieu Hospital, approved all clinical investigations, and informed written consent was obtained from all subjects.

Animals. To generate macrophage-specific *Klf4*-knockout mice, we crossed $Klf4^{fl/fl}$ mice with $LysM^{Cre/Cre}$ mice (53, 54). $LysM^{Cre/Cre}$ mice (designated Mye-WT) were used as control animals, and $LysM^{Cre/Cre}Klf4^{fl/fl}$ mice (designated Mye-KO) were used as experimental animals. $Klf4^{fl/fl}$ mice were generated as described previously (55, 56). $LysM^{Cre/Cre}$ mice, C57BL/6J wide-type mice, and *Stat6*-null mice were obtained from The Jackson Laboratory. All experimental procedures were approved by the IACUC of Case Western Reserve University.

Cell culture and transfection assays. Mouse peritonitis was induced by i.p. injection of 3% thioglycolate broth in 8- to 20-week-old mice as described previously (57). Peritoneal cells were harvested at 72 hours after i.p. injection, and macrophages were enriched by quick adhesion. BMDMs were differentiated with M-CSF as described previously (58). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. Macrophage culture purity was assessed by CD115-based flow cytometry. All transient transfection and adenoviral overexpression procedures were performed in the RAW264.7 cell line (ATCC). For overexpression, RAW264.7 cells were infected with the empty control virus (Ad-EV) or the adenovirus carrying the human *KLF4* gene (Ad-KLF4). Transient transfection assays were done with FuGENE 6 transfection reagent (Roche Applied Science) in RAW264.7 cells. The *Arg1* promoter luciferase construct was a gift from Peter Murray (St. Jude Children's Research Hospital, Memphis, Tennessee, USA). KLF- and Stat6-binding sites (13, 30) were mutated using a site-directed mutagenesis kit (Agilent Technologies). The Stat6 expression plasmid was a gift from Thomas A. Hamilton (Cleveland Clinic). KLF4 and p65 expression plasmids and Cox-2 luciferase plasmid were described previously (21, 38).

Phagocytosis and bacteria killing. To assess the phagocytic capability of macrophages, opsonized Alexa Fluor 488–conjugated *E. coli* (strain K-12) bioparticles (Invitrogen) were incubated with macrophages and intracellular uptake was visualized by fluorescence microscopy and quantified by flow cytometry. To determine the bactericidal function of macrophages, 2×10^6 adherent PMs were incubated with 5×10^7 *E. coli* (strain ATCC-29522) (59) or 1×10^6 *S. aureus* (strain Newman) (57) for 2 hours, and then cells were thoroughly washed with PBS and incubated for 24 hours in DMEM medium containing antibiotics before harvesting of intracellular bacteria. Cell lysate from macrophages containing intracellular bacteria was serially diluted with PBS and spread onto agar plates to determine bacterial viability (expressed as CFU).

Wound preparation, treatment, and measurement. Each mouse was subjected to three 6-mm excisional wounds, 1 midline and 2 on each side of the midline, followed by a single dose of 72 mJ/cm^2 UVB. This dose of UVB has been previously shown to recruit monocytes and macrophages into the skin (60, 61). Wounds were measured daily (length × width), and total wound area was calculated, until they healed (loss of serum crust and reepithelialization).

Mouse HFD model and assessment of metabolic status. Male mice were fed either standard rodent chow or Clinton/Cybulsky High Fat Rodent Diet (HFD: D12108C, Research Diet) (62) starting at 8 weeks of age for 8–12 weeks. These mice were subjected to a glucose tolerance test or an insulin tolerance test at 10 weeks after initiation of HFD (44) (Case Mouse Metabolic Phenotyping Center, Case Western Reserve University). After 12 weeks of HFD, animals were assessed for body composition on a 7T small animal MRI scanner in the Case Center for Imaging Research (Case Western Reserve University) and sacrificed for harvesting of serum, fat tissue (perigonadal, visceral, and subcutaneous), skeletal muscle (quadriceps and soleus), and liver. To investigate the potential sites of insulin resistance, HFD-fed Mye-WT and Mye-KO mice were injected with insulin (5 mU/g) through the inferior vena cava, and liver and skeletal muscle were quickly harvested for biochemical analysis. Samples were stored at –80°C or fixed in 10% formalin for subsequent analysis. Food intake and energy expenditure were determined after 10 weeks of HFD at the Mouse Metabolic Phenotyping Center, University of Cincinnati, Cincinnati, OH.

RNA isolation and qPCR. Total RNA from cultured cells, human tissue samples or mouse tissue samples was isolated using either TRIzol reagent (Invitrogen) or RNeasy kit (QIAGEN). First-strand cDNA was synthesized and subjected to qPCR with either SYBR green or Roche universal probe reagents (Universal ProbeLibrary, Roche Applied Science) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression was normalized to GAPDH, 18SRNA, or β-actin using the ΔΔCt method.

Western blot analysis and immunohistochemistry. Western blots were performed following standard protocols. Chicken anti–arginase-1 antibody was a gift of Sidney M. Morris Jr. (University of Pittsburgh, Pittsburgh, Pennsylvania,

USA). Other antibodies were iNOS (SC-8310), Cox-2 (SC-7951), PPARγ (SC-7273), and HRP-conjugated anti-goat IgG (SC-2020) from Santa Cruz Biotechnology Inc.; Stat6 (catalog 9362) and phospho–Tyr641-Stat6 (catalog 9361) from Cell Signaling Technology; goat anti-KLF4 (AF3158) from R&D Systems; mouse anti–β-actin (A1978) from Sigma-Aldrich; HRP-conjugated anti-rabbit IgG (NA934V) and anti-mouse-IgG (NA931V) from GE Healthcare; and HRP-conjugated anti-chicken IgY (SA1-300) from Affinity BioReagents. Mac-3 antibody (ab22506) was from Abcam. Immunohistochemical studies on tissue samples were performed as described previously (44), and images were collected with a ×40 objective.

ChIP. ChIP assays were performed as previously described (38, 47). In brief, 2×10^7 cells were stimulated with LPS (50 ng/ml) for 30 minutes or IL-4 (5 ng/ml) for 1–4 hours prior to crosslinking for 10 minutes with 1% formaldehyde. Antibodies recognizing p65 (SC-372), p300 (SC-585), PCAF (SC-8999), and Stat6 (SC-981) were from Santa Cruz Biotechnology Inc. Acetyl-histone 3 antibody (06-599B) and Magna ChIP Protein A Magnetic Beads (catalog 16-661) are products of Millipore. Normal rabbit IgG (PP64B, Upstate) was used as negative control. Mouse KLF4 ChIP was performed with an ExactaChIP Mouse KLF4 Chromatin Immunoprecipitation Kit (ECP3158, R&D Systems). *Arg1* promoter PCR was performed with the specific primers flanking the Stat6 enhancer region (sense: TCACGCGTGGTAGCCGACGAGAG; antisense: CGCACGCGTAAAGTGGCACAACTCACGTA) and *Cox-2* promoter PCR was performed with specific primers flanking the NF-κB–binding site (sense: ATGTGGACCCTGACAGAGGA; antisense: TCTCCGGTTTCCTCCCAGTC) (13, 35). Nontargeting primers for *Arg1* promoter (sense: CGCCTCGAGGCTGCATGTGCTCGG; antisense: CGCACGCGTAGAACTGCTTTGGGTTGTCA), and *Cox-2* promoter (sense: ATTCAAGCAGCAGAAGAGGGCAG; antisense: CTGGGATGCCAGAGCACACTG) were used to perform nonspecific control PCR.

In vitro metabolic assays. Macrophage polarization was induced by treating cells with either LPS (50 ng/ml) or IL-4 (5 ng/ml) for 16 hours prior to performing metabolic assays. For glucose uptake, 2-deoxy-[^{3}H]-D-glucose (0.4 μCi/ml) was added to the cells for 10 minutes at 37°C, and the reaction was terminated by washing cells twice in ice-cold PBS, followed by cell lysis for radioactivity determination (30). To determine fatty acid uptake, cells were incubated at 25°C for 5 minutes with 1-[^{3}H]-oleic acid (0.2 μCi/ml), followed by washing with cold PBS and lysis with RIPA buffer. Fatty acid oxidation was determined by radioassay for [^{3}H]-H_2O in the aqueous phase of cell lysate chloroform extracts (63). Nitrite derived from NO was determined with the Griess Reagent System (Promega) in macrophage-conditioned medium per the manufacturer's instructions. Glycolysis was determined by an L(+)-lactate release assay (K627-100, BioVision) following the manufacturer's instructions. All sample values were normalized for protein content and expressed as fold change relative to control samples.

Statistics. Results are presented as mean ± SEM. To analyze the difference between 2 groups, 2-tailed Student's *t* test was used. Bonferroni correction was used when more than 2 groups were present. A P value less than 0.05 was considered significant. Human gene expression data were analyzed with Kruskal-Wallis test and Pearson correlation test.

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ABSTRACT

Adipose tissue inflammation in obesity and surgery-induced weight loss: emerging paracrine dialogue between macrophages and lymphocytes

Obesity is now considered a chronic low-grade inflammatory condition with increased levels of inflammatory mediators both in the circulation and adipose tissue. During this project, we followed a group of obese patients before and at three consecutive time points after gastric bypass surgery. In a first study, we observed unexpected bi-phasic regulation of serum pro-inflammatory factors, characterized by drastic decreases at month 3 post-surgery, followed by a rebound and eventual stabilization after 1 year, in close relation with patient's nutritional status. Besides, we showed that obesity and obesity-induced type 2 diabetes were associated with enrichment in a non-classical circulating $CD14^{dim}CD16^{+}$ monocyte subset that decreased after bariatric surgery. In a second study, we focused on adipose tissue that is now recognized as a site of infiltration of numerous immune cells in obesity. Both macrophages and T lymphocytes contribute to set up an inflammatory microenvironment that alters adipose tissue biology. However, little is known about cross-talk between adipose tissue immune cell types. We showed that adipose tissue macrophages secreted significant amounts of IL-1β through activation of NLRP3 inflammasome. This process was overactivated in type 2 diabetic patients and down-regulated by weight loss. Strikingly, the production of IL-17 by adipose tissue $CD4^{+}$ lymphocytes was altered the same way as IL-1β in these clinical conditions. In primary cell co-culture experiments, adipose tissue macrophage-derived IL-1β markedly enhanced IL-17 production by adipose tissue lymphocytes, while IL-17 reciprocally induced IL-1β secretion by macrophages. This identified IL-1β and IL-17 as key cytokines mediating a paracrine inflammatory cross-talk between adipose tissue macrophages and lymphocytes. Type-2 diabetes and weight reduction are associated, respectively, with deterioration and amelioration of glycemic parameters. Since they provide opposite responses with respect to IL-1β and IL-17 production, this strengthens the implication of these cytokines and their deleterious interplay in obesity-induced alteration of glycemic status.

RESUME

Inflammation du tissu adipeux chez le sujet obèse et au cours de la perte de poids : nouveau dialogue paracrine entre macrophages et lymphocytes

L'obésité est considérée comme un état inflammatoire chronique dit de « bas grade », associé à une augmentation de facteurs pro-inflammatoires circulants qui diminuent lors de la perte de poids. Pour ce projet, nous avons suivi des patients obèses morbides candidats à la chirurgie du by-pass gastrique. Dans une première étude, nous avons montré que les facteurs inflammatoires sériques évoluaient selon une régulation multiphasique au cours de la perte de poids, alternant diminution puis re-augmentation, en lien étroit avec le statut nutritionnel des patients. En plus des facteurs inflammatoires, l'obésité et le diabète de type 2 sont associés à un enrichissement en monocytes $CD14^{dim}CD16^{+}$ circulants qui diminuent après chirurgie. Dans une deuxième étude, nous nous sommes intéressés au tissu adipeux qui apparaît être également un site d'inflammation avec l'accumulation de nombreuses cellules immunitaires. Lors de l'obésité, l'accumulation de macrophages et de lymphocytes T contribue au développement d'un microenvironnement pro-inflammatoire du tissu adipeux, capable d'interférer avec la biologie adipocytaire. Il reste à définir comment ces cellules immunitaires communiquent au sein du tissu adipeux et leur lien avec le statut diabétique des patients. Nous avons donc montré que les macrophages du tissu adipeux sécrètent de l'IL-1β nécessitant l'activation de l'inflammasome NLRP3, chez le patient obèse diabétique. D'autre part, IL-17 produit par des lymphocytes $CD4^{+}$ du tissu adipeux est également augmenté chez ces mêmes patients et l'IL-1β des macrophages stimulent de manière significative la production d'IL-17 par les lymphocytes. En contradiction avec d'autres cytokines pro-inflammatoires, les niveaux de l'IL1β et de l'IL17 sécrétés par des explants de tissu adipeux diminuent au cours de la perte de poids. Ainsi, ce projet a permis de montrer que, d'une part, la perte de poids est un phénomène complexe qui n'induit pas une amélioration systématique de l'inflammation systémique et locale associées à l'obésité. D'autre part, nous avons identifié l'IL-1β et l'IL-17 comme deux cytokines clés à la base d'un

dialogue paracrine et pro-inflammatoire entre macrophages et lymphocytes du tissu adipeux, spécifiquement chez les patients obèses diabétiques.