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**Impaired alternative macrophage differentiation of peripheral blood mononuclear cells from
obese subjects**

G. Bories^{1,2,3,4}, **R. Caiazzo**^{1,5,6}, **B. Derudas**^{1,2,3,4}, **C. Copin**^{1,2,3,4}, **V. Raverdy**^{1,5,6}, **M. Pigeyre**^{1,5,7},
F. Pattou^{1,5,6}, **B. Staels**^{1,2,3,4} and **G. Chinetti-Gbaguidi**^{1,2,3,4}

¹ Univ Lille Nord de France, F-59000, Lille, France

² Inserm, U1011, F-59000, Lille, France

³ UDSL, F-59000, Lille, France

⁴ Institut Pasteur de Lille, F-59019, Lille, France

⁵ Inserm, UMR 859 Biotherapies for diabetes, F-59000, Lille, France

⁶ Department of Endocrine Surgery, University Hospital, F-59000, Lille, France

⁷ Department of Nutrition, University Hospital, F-59000, Lille, France

Running title: Macrophage alternative differentiation and obesity

Address correspondence to:

Bart Staels

Inserm UR 1011, Institut Pasteur de Lille

1, rue du Professeur Calmette, BP 245, Lille 59019, France

Tel: +33-3-20-87-73-88; Fax: +33-3-20-87-73-60

E-mail : bart.staels@pasteur-lille.fr

ABSTRACT

Visceral obesity, a chronic, low-grade inflammatory disease, predisposes to the metabolic syndrome, type 2 diabetes and its cardiovascular complications. Adipose tissue is not a passive storehouse for fat, but an endocrine organ synthesizing and releasing a variety of bioactive molecules, some of which are produced by infiltrated immune-inflammatory cells including macrophages. Two different sub-populations of macrophages have been identified in adipose tissue: pro-inflammatory “classical” M1 and anti-inflammatory “alternative” M2 macrophages and their ratio is suggested to influence the metabolic complications of obesity. These macrophages derive primarily from peripheral blood mononuclear cells (PBMC). We hypothesized that obesity and the metabolic syndrome modulate PBMC functions. Therefore, alteration of the monocyte response, and more specifically their ability to differentiate toward alternative anti-inflammatory macrophages was assessed in PBMC isolated from lean and obese subjects with or without alterations in glucose homeostasis. Our results indicate that PBMC from obese subjects have an altered expression of M2 markers and that their monocytes are less susceptible to differentiate toward an alternative phenotype. Thus PBMC in obesity are programmed, which may contribute to the inflammatory dysregulation and increased susceptibility to inflammatory diseases in these patients.

Keywords: obesity, macrophages, polarization, inflammation, gene expression

INTRODUCTION

Obesity is characterized by an excess of adipose tissue which predisposes to co-morbidities such as diabetes, atherosclerosis, liver disease and cancer. Obesity is associated with an inflammatory state: plasma concentrations of inflammatory markers including interleukin (IL)-6, tumor necrosis factor (TNF) α , soluble TNF α -receptor and C-reactive protein (CRP) are significantly elevated in obese subjects^{1, 2}. Therapies targeting obesity-associated immune and inflammatory defects are thus of potential interest. Most prior research on the cellular mediators of inflammation in obesity has focused on intrahepatic and adipose tissue immune cell function³. However, these inflammatory cells derive mainly from circulating precursors, such as peripheral blood mononuclear cells (PBMC), whose function in obesity-associated immune and inflammatory defects has been less studied. Circulating PBMC, are exposed to the systemic environment, including metabolic factors, such as dyslipidemia, and inflammatory molecules produced by other organs and tissues, and may thus be programmed and directly involved in the low-grade chronic inflammation related to obesity. PBMC are composed of lymphocytes and monocytes, the latter being the precursors of macrophages. Macrophages play a key role in inflammatory responses, by infiltrating adipose tissue of obese patients, as well as the vascular wall during development of atherosclerosis^{4,5}.

Macrophages are a heterogeneous cell population, which adapt and respond to a large variety of micro-environmental signals⁶. Th1 cytokines, such as interferon γ (IFN γ), interleukin 1- β (IL-1 β) or lipopolysaccharide (LPS), induce a “classical” activation profile (M1). M1 macrophages produce primarily pro-inflammatory cytokines, such as TNF α , IL-6 and IL-12⁷. On the other hand, Th2 cytokines, such as IL-4 and IL-13, induce an “alternative” activation program (M2) in macrophages. M2 macrophages dampen inflammatory and adaptive Th1 responses by producing anti-inflammatory factors (IL-10, transforming growth factor- β (TGF- β) and IL-1 receptor antagonist (IL-1Ra)), by scavenging debris and by promoting angiogenesis, tissue remodeling and repair^{7, 8}. Moreover, macrophages are plastic cells because they can switch from an activated M1 state back to M2, and vice versa, upon specific signals⁹. Human adipose tissue-infiltrated macrophages (ATM) are characterized by a mixed M1-M2 phenotype, since ATM express at their surface specific M2 markers but

secrete/release M1 pro-inflammatory factors^{10, 11}. No data are currently available regarding the consequences of obesity and its co-morbidities on the capacity of monocytes to differentiate into alternative macrophages. We thus tested whether obesity and associated alterations in glucose homeostasis affects monocyte differentiation into alternative anti-inflammatory M2 macrophages.

METHODS

Research subjects

Subjects were patients operated at the Department of Endocrine Surgery, Lille University Hospital, France, and enrolled in the ABOS study. Morbidly obese female patients (BMI>35) undergoing bariatric surgery were divided in different groups based on their metabolic characteristics (normoglycemic (n=10): fasting blood glucose ≤ 6 mmol/l and/or ≤ 7.8 mmol/l 120 minutes after oral glucose loading; glucose intolerant (IFG/IGT) (n=10): fasting blood glucose ≥ 6 and ≤ 7 mmol/l and/or ≥ 7.8 and ≤ 11.1 mmol/l 120 minutes after glucose loading; and diabetic (n=13): fasting blood glucose ≥ 7 mmol/l fasting and/or ≥ 11.1 mmol/l 120 minutes after glucose loading). A group of lean normoglycemic individuals operated for benign and non inflammatory diseases (n=10, BMI ≤ 27 , glycemia ≤ 6 mmol/l fasting and/or ≤ 7.8 mmol/l 120 minutes after glucose loading) was used as control (table 1). None of the patients had any clinical symptoms of systemic inflammation. This study was approved by the institutional review board and all patients gave written informed consent.

Cell culture

Peripheral blood was drawn from all patients during the pre-operative clinic visit and from lean control subjects. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation¹². The isolated monocytes were suspended in RPMI 1640 medium supplemented with 10% human pooled serum (Abcys, France), containing gentamicin (40 μ g/ml), glutamine (2 mM) (Sigma) and seeded at density of 1×10^6 cells/well in Primaria 24 well plastic culture dishes (Polylabo, Strasbourg, France) for 2 hours at 37°C. After this period non-adherent lymphocytes were removed by PBS washes. Differentiation of monocytes into macrophages occurs spontaneously by adhesion of cells to the culture dishes in the presence of RPMI medium containing gentamicin, L-glutamine and 10% pooled human serum (Abcys, France) for 7 days¹³. Monocyte differentiation into alternative macrophages (M2) was assessed by stimulating monocytes with recombinant human IL-4 (15 ng/mL) (Promocell, Germany) added at the beginning of differentiation and for 7 days¹³.

RNA extraction and analysis

Total cellular RNA was isolated from PBMC or differentiated macrophages using Trizol (Invitrogen), reverse transcribed and cDNAs were quantified by quantitative polymerase chain reaction (Q-PCR) on a MX4000 apparatus (Stratagene) using specific primers (table 2). The relative expression of each gene was calculated by the ΔCt method, where ΔCt is the value obtained by subtracting the Ct (threshold cycle) value of cyclophilin mRNA from the Ct value of the target gene. The amount of target relative to the cyclophilin mRNA was expressed as $2^{-\Delta\text{Ct}}$.

Statistical analysis

Statistically differences between groups were analyzed by Kruskal Wallis and Mann Whitney test and were considered significant when $p < 0.05$.

RESULTS

PBMC from obese subjects display a pro-inflammatory phenotype

In order to characterize the phenotype of circulating peripheral blood mononuclear cells (PBMC), a cell population including circulating monocytes and lymphocytes, blood was drawn from lean or obese patients with or without alterations in glucose homeostasis and PBMC were isolated by gradient centrifugation. Q-PCR analysis of inflammatory genes revealed that PBMC derived from obese subjects present a higher gene expression of TNF α , IFN γ and IL-1 β , indicative of a pro-inflammatory profile. Interestingly, while the expression of TNF α and IL-1 β are most pronouncedly increased in obese diabetic subjects, obesity *per se* confers high expression of IFN γ (figure 1).

PBMC from obese subjects display altered expression of alternative differentiation markers

No major difference was observed in the expression of the lymphocyte markers (CD3, CD4 and CD8) with the exception of CD4 which is slightly decreased in PBMC from obese subjects (figure 2).

To determine whether markers of alternative macrophage differentiation are altered in PBMC from these subject groups, MR, CD163 and CD200R mRNA levels were measured. The expression of other alternative macrophage markers (F13A1, AMAC1) was not detectable in circulating PBMC (data not shown). Expression of general monocyte markers, such as CD14 was slightly higher in obese diabetics, with no difference for CD16 (figure 1), whereas MR, CD163 and CD200R was lower in PBMC from obese compared to lean individuals, while no significant differences were observed between obese individuals with different metabolic complications (figure 3). These results suggest that precursor mononuclear cells from obese already display an altered expression of markers of alternative macrophage differentiation.

Monocytes from obese patients display a reduced capacity to differentiate toward an alternative phenotype

To determine the influence of donor phenotype on the ability of monocytes to differentiate toward alternative anti-inflammatory macrophages, monocytes from lean and obese donors (normoglycemic,

glucose intolerant and diabetic) were differentiated for 7 days in the presence of IL-4, a commonly used potent inducer of the macrophage alternative phenotype¹³. Our results demonstrate that the gene expression of CD200R, F13A1, TGF β , AMAC1, CD163 and MR, all established markers for the alternative differentiation, was drastically lower in the alternative macrophages derived from monocytes of obese subjects compared to those isolated from lean individuals (figure 4). Interestingly, no significant differences were observed between the three groups of obese patients, indicating that obesity alone is sufficient to cause altered monocyte responses (figure 4).

DISCUSSION

Obesity is a major underlying risk factor for cardiovascular disease, due to atherosclerosis, through its association/promotion of other major disease risk factors, namely dyslipidemia, hypertension and hyperglycemia. Recent studies have provided substantial evidence that obesity is a chronic low-grade inflammatory disease. Adipose tissue is not a passive storehouse for fat, but an endocrine organ synthesizing and releasing a variety of bioactive molecules that may modulate PBMC phenotype and monocyte functions.

PBMC are biosensors whose gene expression is influenced by circulating molecules produced by all organs as well as environmental factors, such as diet and exercise. As such, blood cell gene expression patterns might reflect the pathological processes developing in the organism. Available studies indicate that the gene expression profile of circulating PBMC can be useful as biomarkers in the identification and diagnosis of the disease, prediction of outcome and monitoring of the response to treatment in patient follow-up¹⁴.

During the last years, several studies have reported increased inflammatory mediators during obesity. Plasma concentrations of inflammatory markers such as IL-6, TNF α and C-reactive protein (CRP), are significantly elevated in obese subjects^{1, 2}. In addition, the expression of alternative macrophage differentiation markers IL-10 and CD163 was significantly decreased in monocytes from obese diabetic patients relative to obese non-diabetic patients¹⁵. In our study, we did not confirm a difference in the expression of the alternative markers MR, CD200R and CD163 between the three groups of obese patients with different metabolic disorders. However, a strong reduction in their expression was observed when obese were compared to lean individuals. Our results indicate that obesity itself is sufficient to alter the expression of alternative macrophage differentiation markers in human circulating precursor cells.

The influence of obesity and associated alterations in glucose homeostasis on the ability of monocytes to differentiate toward an alternative macrophage phenotype has not yet been evaluated in humans. Interestingly, the altered gene expression profile observed in PBMC from obese individuals was conserved in monocyte-derived alternative macrophages differentiated *in vitro* for 7 days in the presence of IL-4⁷ indicating that these cells are refractory to acquire an M2 phenotype. We did not

observe any difference in the expression of alternative differentiation markers in patients with glucose intolerance or diabetes, indicating that obesity *per se* is the major cause of the observed phenotype. These results indicate that the precursor cells are already programmed and maintain their phenotype once cultured *in vitro*, suggesting a potential involvement of epigenetic mechanisms.

The fact that monocytes from obese individuals are impaired to a stimulus promoting alternative macrophages can have more general consequences. Alternative macrophages are resident in adipose tissue as well as in atherosclerotic plaques, where they can exert local effects ¹⁶. In adipose tissue, macrophages produce the anti-inflammatory cytokines IL-10 and IL-1Ra, but can also secrete pro-inflammatory cytokines such as TNF, IL-6 and IL-1 β supporting a role of macrophages in obesity-induced adipocyte dysfunction and metabolic disorders ¹⁰. Thus, obesity may be associated not only by a sustained pro-inflammatory reaction, but also by a failure of anti-inflammatory control mechanisms. Understanding the consequences of obesity-associated pathological conditions on PBMC and monocyte functions can represent a promising novel strategy to elucidate the complexity of the obesity-related inflammation.

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FIGURE LEGENDS

Figure 1. Peripheral blood mononuclear cells (PBMC) from obese subjects display a pro-inflammatory profile.

PBMC were isolated from lean and obese subjects. mRNA levels of TNF α , IFN γ , IL-1 β , CD14 and CD16 were measured by Q-PCR. The relative expression of each gene was calculated as described above and represented in Boxplots indicating the median and the lower and the upper quartiles. Statistically significant differences are indicated (* $p < 0.05$, ** $p < 0.01$).

Figure 2. Expression of lymphocyte markers is similar in PBMC from lean and obese subjects.

PBMC were isolated from lean and obese subjects. mRNA levels of CD3, CD4 and CD8 were measured by Q-PCR. The relative expression of each gene was calculated as described above and represented in Boxplots indicating the median and the lower and the upper quartiles. Statistically significant differences are indicated (* $p < 0.05$).

Figure 3. Expression of markers of alternative differentiation is altered in PBMC from obese subjects.

PBMC were isolated from lean and obese subjects. mRNA levels of MR, CD163 and CD200R were measured by Q-PCR. The relative expression of each gene was calculated as described above and represented in Boxplots indicating the median and the lower and the upper quartiles. Statistically significant differences are indicated (** $p < 0.01$, *** $p < 0.001$).

Figure 4. Monocytes from obese subjects display an altered capacity to differentiate into IL4-driven alternative macrophages.

Monocytes from lean or obese subjects were differentiated into M2 macrophages in the presence of IL-4 (15 ng/ml) for 7 days. At the end of differentiation, mRNA was extracted and expression of markers of alternative differentiation (MR, CD200R, F13A1, TGF β , AMAC1 and CD163) analyzed by Q-PCR. The relative expression of each gene was calculated as described above and represented in

Boxplots indicating the median and the lower and the upper quartiles. Statistically significant differences are indicated (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Table 1. Clinical and biochemical characteristics of subjects

	Lean (n=10)	Obese (n=10)	IFG/IGT obese (n=10)	Diabetic obese (n=13)
BMI	23.58 ± 1.92	52.25 ± 8.15 ^{***}	52.10 ± 6.77 ^{***}	51.83 ± 11.23 ^{**}
Age (years)	45.80 ± 9.95	45.10 ± 5.47	45.3 ± 6.33	45.33 ± 9.65
Fasting Glucose (mmol/L)	4.15 ± 0.63	5.32 ± 0.49 [*]	6.12 ± 0.59 [*]	7.98 ± 1.92 [*]
Glycemia 2H (mmol/L)	-	7.20 ± 0.36	9.56 ± 0.94	13.81 ± 3.53
Treatment				
Insulin	No	No	No	4/13
Oral anti-diabetic (OAD)	No	No	No	1/13

Values are mean ± SD

(* p < 0.05, *** p < 0.001) compared to lean subjects

Table 2. Sequences of primers used

AMAC1	<i>forward</i>	5'-AGC TCT GCT GCC TCG TCT AT-3'
	<i>reverse</i>	5'-CCC ACT TCT TAT TGG GGT CA-3'
MR	<i>forward</i>	5'-CGA GGA AGA GGT TCG GTT CAC C-3'
	<i>reverse</i>	5'-GCA ATC CCG GTT CTC ATG GC-3'
IL-1β	<i>forward</i>	5'-AGC TCG CCA GTG AAA TGA TGG-3'
	<i>reverse</i>	5'-CAG GTC CTG GAA GGA GCA CTT C-3'
TNFα	<i>forward</i>	5'-CAG AGG GCC TGT ACC TCA TC-3'
	<i>reverse</i>	5'-GGA AGA CCC CTC CCA GAT AG-3'
CD200R	<i>forward</i>	5'-CAT CGT GGA TAT CAC CTC CAA G-3'
	<i>reverse</i>	5'-CTT GCT TAG TGG CAC AAT CGC-3'
F13A1	<i>forward</i>	5'-CTG ACC TTC CTG TTG GAT TTG G-3'
	<i>reverse</i>	5'-CTT GAT GGC TTG AAC CGA GG-3'
IFNγ	<i>forward</i>	5'-CTG ACT TGA ATG TCC AAC GC-3'
	<i>reverse</i>	5'-TCG AAA CAG CAT CTG ACT CC-3'
CD163	<i>forward</i>	5'-TTG CCA GCA GT TAA ATG TG-3'
	<i>reverse</i>	5'-AGG ACA GTG TTT GGG ACT GG-3'
CD14	<i>forward</i>	5'-CTG CAA CTT CTC CGA ACC TC-3'
	<i>reverse</i>	5'-CCA GTA GCT GAG CAG GAA CC-3'
TGFβ	<i>forward</i>	5'-CTC CGA GAA GCG GTA CCT GAA C-3'
	<i>reverse</i>	5'-CAC TTG CAG TGT GTT ATC CCT-3'
CD3	<i>forward</i>	5'-CTT ATC AGT TGG CGT TTG GG-3'
	<i>reverse</i>	5'-GCA TCT TCT GGT TTG CTT CC-3'
CD4	<i>forward</i>	5'-TTG CTT CTG GTG CTG CAA C-3'
	<i>reverse</i>	5'-TGG CAG TCA ATC CGA ACA C-3'
CD8	<i>forward</i>	5'-CCG CTG GCC TTG CTG CTC C-3'
	<i>reverse</i>	5'-GGT GGG CTT CGC TGG CAG G-3'
CD16	<i>forward</i>	5'-AAG TCC ATA TCG GCT GGC TG-3'
	<i>reverse</i>	5'-TGT GAC CTT ATG CAG AGC AGT G-3'
cyclophilin	<i>forward</i>	5'-GCA TAC GGG TCC TGG CAT CTT GTC C-3'
	<i>reverse</i>	5'-ATG GTG ATC TTC TTG CTG GTC TTG C-3'

