Tuberculosis 114 (2019) 30–41

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: www.elsevier.com/locate/tube

Immunological Aspects

Non-classical circulating monocytes in severe obesity and obesity with uncontrolled diabetes: A comparison with tuberculosis and healthy individuals

Danilo Pires Resende[a], Adeliane Castro da Costa[a], Lorena Pereira de Souza Rosa[b,c], Ana Paula Rodrigues[b,c], Annelise Silva e Alvez Santos[b,c], Camila Kellen Cardoso[b,c], Jaqueline Danesio Sousa[d], André Kipnis[e], Erika Aparecida Silveira[b,c], Ana Paula Junqueira-Kipnis[a,*]

[a] Laboratório de Imunopatologia das Doenças Infecciosas, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235, Setor Universitário, Goiânia, Goiás, CEP 74605050 Brazil
[b] Hospital das Clínicas- Programa de Pós-Graduação em Ciências da Saúde, Faculdade de Medicina- UFG, Goiânia, GO, Brazil
[c] Primeira avenida, Setor Universitário, Goiânia, Goiás, CEP 75605020 Brazil
[d] Universidade Norte do Paraná, Avenida Paris, 675, Jardim Piza, CEP 86041-120, Londrina, Paraná, Brazil
[e] Laboratório de Bacteriologia Molecular, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235, Setor Universitário, Goiânia, Goiás, CEP 74605050 Brazil

ARTICLE INFO

Keywords:
Metabolism
Co-infection
Risk groups
Severe obesity
$CD14^+CD16^+$

ABSTRACT

Severe obesity and diabetes lead to a significant decrease in quality of life. Although controversial, population-wide studies have implicated obesity in the development of tuberculosis (TB). Non-classical monocytes have been described in obesity and TB, whereas in diabetes they have been associated with poorer clinical outcomes. The present study focuses on the functional significance of several monocyte populations of obese, obesity-related diabetic (OBDM), non-obese/diabetic tuberculosis and non-obese healthy control patients. Monocytes were evaluated by measuring expression of CD86, CD206, TLR-2 and TLR-4 as well as production of IL-6, IL-12, and by using a mycobacterial growth inhibition assay for both *Mycobacterium tuberculosis* and *M. abscessus* subsp. *massiliense*. Non-classical monocytes from OBDM and non-obese TB patients exhibited similar activation profiles (CD86/CD206/TLR-2 and TLR-4 expressions). Only monocytes from TB patients had a higher positivity for IL-12 and IL-6, whereas adiponectin serum levels increased similarly between TB and OBDM patients. Monocytes from active TB patients and OBDM were more permissive to Mtb growth than obese individuals, but this susceptibility was not observed for *M. abscessus* subsp. *massiliense*. From these findings, we conclude that diabetes and tuberculosis had similarities in the population of circulating non-classical monocytes, improving our understanding of the association of these diseases.

1. Introduction

Chronic conditions that are associated with inflammation, such as obesity (OB), diabetes mellitus (DM) and tuberculosis (TB) are serious public health problems. The obesity epidemic is increasing around the world; between 1980 and 2014, the prevalence of obesity among adults, aged 18 and older, nearly doubled. In 2014, 39% of adults were overweight and 11% were classified as obese, totalizing more than 500 million people with obesity [1]. As diabetes is associated with obesity, the prevalence of diabetes also increased in the same period, rising to 9% of the adult population worldwide, totalizing 422 million people with diabetes [2].

Obesity is characterized by secretion by the adipose tissue of inflammatory mediators like IL-1, IL-6, leptin, TNF-α and monocyte chemoattractant protein-1 (MCP-1) among others [3,4]. Diabetes patients also present elevated circulating levels of acute phase proteins and inflammatory cytokines such as IL-1 and IL-6 [5,6], as well as NLRP3 activation [7]. Thus, obesity and diabetes are classified as chronic inflammatory diseases [3].

Monocytes are mononuclear phagocytes generated in the bone

* Corresponding author. Rua 235 esquina com Primeira Avenida, S/N. Laboratório de Imunopatologia das Doenças Infecciosas, sala 325, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Setor Universitário, Goiânia, Goiás, CEP 74605-050, Brazil.
E-mail address: ana_kipnis@ufg.br (A.P. Junqueira-Kipnis).

https://doi.org/10.1016/j.tube.2018.11.003
Received 25 June 2018; Received in revised form 1 November 2018; Accepted 2 November 2018

marrow [8] and are constantly liberated into the circulation, forming a heterogeneous population of cells. In humans, these cells can be divided into three subpopulations accordingly to the expression of the surface markers CD14 and CD16 into, classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{+}$) monocytes [9]. The proportions of these subpopulations have been shown to shift during several inflammatory illnesses like diabetes, obesity and tuberculosis. In obesity, for instance, it has been shown that the circulating non-classic monocytes are increased and could give rise to the inflammatory macrophages resident in the adipose tissue [10], while circulating classical monocyte population appears to correlate with CD11c + macrophages in the adipose tissue [11]. In diabetes, the variation in the populations of circulating monocytes is also present and some studies show that this variation is correlated with disease severity [12–14]. In tuberculosis, the non-classical and intermediate monocyte populations are expanded [13] and were related to the severity of the infection and modulated by the treatment [14].

Within the monocyte subsets, the non-classical monocytes were described to increase in obesity [15], diabetes [10] and tuberculosis [16]. Obese patients were shown to present a more inflammatory population of non-classical monocytes than lean controls, with greater production of inflammatory mediators [9]. The non-classical monocyte population is also increased in diabetes [17], and is related with complications caused by the disease [12]. This monocyte subset is also increased in tuberculosis [13] and correlated with the severity of the infection [14]. Thus, non-classical monocyte population might be inversely associated with the capacity to deal with intracellular pathogens.

As monocytes directly participate in the inflammation generated during the abovementioned diseases, and non-classical monocytes might contribute to this chronic inflammation; in this work we hypothesized that monocytes from obese individuals with diabetes may be involved in the risk of developing TB, and wished to explore whether the effect of a high proportion of non-classical monocytes in severe obesity (BMI > 35 kg/m2) and uncontrolled obesity-related diabetes (OBDM) (glycosylated Hb level > 6,5%) differed from that found in patients with TB.

2. Material and methods

2.1. Study population

This study is part of a major project (DietBra Trial) that was approved by the Research Ethics Committee (CEP) of the Clinics Hospital of the Universidade Federal de Goiás (protocol number 747.792). Forty-five severely obese individuals (BMI > 35 kg/m^2) aged between 18 and 65 years old and living in Goiânia and/or the metropolitan area were recruited from a single blind randomized clinical trial. The study was performed at the Outpatient Clinic of Nutrition in Severe Obesity (ANOG) of the Clinics Hospital of the Universidade Federal de Goiás (HC/UFG). Patients were sent to ANOG through the Municipal Health Center (Secretaria Municipal de Saúde - SMS) or through other clinics from HC/UFG; therefore, every patient in this study was from the Unified Health System of Goiânia (Sistema Único de Saúde - SUS). The inclusion criteria were OB patients with or without DM, dyslipidemia, metabolic syndrome or arterial hypertension. A recruit was excluded from the study for the following: underwent bariatric surgery or lost 5% of body weight in the previous three months; had been previously treated at ANOG; or was using aspirin and antioxidants, was pregnant or lactating, or was an alcoholic. Additionally, patients were excluded if they were receiving nutritional or medical treatment for weight reduction, had received some type of nutritional treatment in the last 2 years, were using anti-obesity drugs like statins, or had food allergies or any disability. The study was conducted in accordance with the Declaration of Helsinki for experiments with human beings. The subjects participated in the study voluntarily and signed informed consent prior to participation.

Samples from active TB patients (All TB patients were selected on clinical symptoms of tuberculosis confirmed by thorax radiography suggestive of TB. Sputum from all 21 patients with suggestive tuberculosis was processed for acid fast staining and culture. However sample from five patients did not produce positive results in culture but responded positively to the anti-TB treatment) recruited within 15 days of treatment and asymptomatic healthy control individuals with normal blood parameters (controls) who were tuberculin skin test negative and SPOT-TB negative were obtained from the biological sample collection of the Immunopathology and Tropical Diseases Laboratory from the Tropical Pathology and Public Health Institute (IPTSP-UFG) under an approved protocol (CEP/HC/UFG number 055/2009).

2.2. Blood collection and peripheral blood mononuclear cell (PBMC) isolation

Blood samples were harvested for serum and PBMC separation in the morning after 12 h of fasting, in a reserved room next to the clinics where the patients were being interviewed. Another 10 mL of blood was collected into a clot accelerator tube to obtain serum, which was stored at −80 °C for analysis.

PBMCs were isolated by Ficoll density gradient separation (Ficoll-Paque Plus, GE Healthcare Bio-Sciences) of heparinized blood. After isolation, the cells were counted in a Neubauer chamber; the concentration was adjusted to 1×10^6 cells/mL, and the cells were frozen with dimethyl sulfoxide (DMSO) at −80 °C for analysis. For culture and flow cytometric analysis, the cells were gently thawed, washed three times with RPMI-1640 (GIBCO, Invitrogen Corporation Grand Island, NY, USA) and transferred to 24 (500 μL) or 96 (200 μL)-well plates at a concentration of 10^6 cells/well with RPMI (GIBCO, Invitrogen Corporation Grand Island, NY, USA) supplemented with 2 nM glutamine, 10 nM pyruvate, 2 nM non-essential amino acids, 50 μg/mL streptomycin, 50 μg/mL penicillin and 10% heat-inactivated fetal calf serum.

2.3. Circulating monocyte flow cytometry analysis

The following antibodies were used for labeling surface and intracellular molecules for flow cytometry analysis: FITC CD206 (BD Pharmingen, San Jose, CA, USA clone 87315)/FITC TLR-2 (BD Pharmingen, San Jose, CA, USA clone TL2.1)/FITC IL-6 (BD Pharmingen, San Jose, CA, USA clone MQ2-13A5), PE CD86 (BD Pharmingen, San Jose, CA, USA clone GL1)/PE TLR-4 (BD Pharmingen, San Jose, CA, USA clone HTA125) PE IL-12p40/23 (BD Pharmingen, San Jose, CA, USA clone eBioHP40), PercP CD14 (BD Pharmingen, San Jose, CA, USA clone 61D3) and APC CD16 (BD Pharmingen, San Jose, CA, USA clone B73.1), APC CD11b (BD Pharmingen, San Jose, CA, USA clone ICRF44). Cells were treated with phosphate-buffered saline (PBS) containing 0.05% sodium azide for 20 min before antibody labeling. After centrifugation ($3000 \times g$ for 10 min), the cells were labeled for 30 min at 4 °C with the following labeled surface-marker antibodies: FITC (CD206/TLR-2), PE (CD86/TLR-4), PercP (CD14) and APC (CD16/CD11b). The plates were then washed twice with PBS/0.05% sodium azide and fixed with Perm Fix (BD Pharmingen, San Jose, CA, EUA) for 20 min. For intracellular labeling, cells were permeabilized with Perm Wash (BD Bioscience Pharmingen) and incubated for 20 min at 4 °C with the following labeled antibodies: FITC (IL-6) (BD Pharmingen, San Jose, CA, EUA) and PE (IL-12p40/23) (BD Pharmingen, San Jose, CA, USA). After washing, the samples were immediately analyzed using a FACS Verse (BD Pharmingen, San Jose, CA, USA) in the Multiuser Laboratory at the Veterinary School of the Universidade Federal de Goiás (UFG). At least 50,000 events were acquired for each sample. Data analysis was performed with the software FlowJo Vs 7.0.

Table 1
Severe obese, Active TB and healthy patient demographic data.

	Obesity (BMI > 35 kg/m^2)		Control[a]	Active TB[a]	p[b]
	Non-diabetic	Diabetic			
	N = 22	N = 25	N = 20	N = 21	
Gender (M/F)	3/19	4/21	11/9	18/3	
Age (Years)	40.7 ± 8.8	45.5 ± 9.2	34 ± 14.3	41.5 ± 13.5	
Weight (kg)	113.3 ± 18.5	117.4 ± 18.2	–	–	
BMI (kg/m^2)	45 ± 6.4	47.1 ± 7.0	–	–	
Blood glucose[c] (mg/dL)	101.1 ± 25.2	154.4 ± 75.8	94 ± 17	117 ± 32	**p = 0.0003**
Insulin (mg/dL)	20 ± 10.9	20.8 ± 9.6	–	–	
HbA1c (%)	< 6,4%	> 6,5%	–	–	
BAAR	NA	NA	NA	21/21 positive	
Culture	NA	NA	NA	16/21 positive	

NA: Not-applicable.
[a] Samples were obtained from the biological sample collection of the Immunopathology and Tropical Diseases Laboratory IPTSP-UFG, protocol of CEP/HC/UFG number 055/2009.
[b] ANOVA comparison between the four groups.
[c] Tukey post-test for glucose serum levels showed significant differences between diabetic and non-diabetic obese groups $p < 0.01$ and between diabetic obese and healthy control groups $p < 0.01$.

2.4. In vitro infection of monocytes with M. tuberculosis (H37Rv) and Mycobacterium abscessus subsp. massiliense

PBMCs were plated in 24-well polystyrene plates with 3-mm glass slides at the bottom using RPMI-1640 culture medium (GIBCO, Invitrogen Corporation Grand Island, NY, USA) supplemented with 2 nM glutamine, 10 nM pyruvate, 2 nM non-essential amino acids, 50 μg/mL streptomycin, 50 μg/mL penicillin and 10% heat-inactivated fetal calf serum. After plating, the cells were incubated for 3 h with no stimulus to allow the monocytes to adhere to the glass slide, after which the cells were washed with RPMI (37 °C) to remove non-adherent cells. *In vitro* Mtb (H37Rv) infection was performed at a 1:1 multiplicity of infection (MOI) in incomplete RPMI without streptomycin, kanamycin or penicillin for 2 h. The cells were then washed with RPMI (37 °C) supplemented with 100 μg/mL kanamycin. One plate was incubated for 24 h at 37 °C to determine the number of phagocytosed mycobacteria, and the other plate was incubated for 24 and 48 h for quantification of cytokines and nitric oxide released into the medium. Cells were also harvested on the glass slides for Fite-Faraco staining.

2.5. Colony forming unit (CFU) determination

After 24 h of incubation, cells were washed twice with PBS (37 °C), lysed with 200 μL of PBS 0.05%/Triton X-100, and serially diluted 10^{-1}, 10^{-2}, and 10^{-3}. Next, 50 μL of each dilution was plated in duplicate on 7H11 Middlebrook agar supplemented with oleic acid-albumin-dextrose-catalase. The plates were incubated for 3 weeks at 37 °C in a 0.05% CO_2 atmosphere.

2.6. Cytokine analyses

The serum or supernatant of cell cultures was used for adiponectin (limit of detection: 1.64 pg/mL), TNF-α (limit of detection: 6 pg/mL) and TGF-β (limit of detection: 8 pg/mL) enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions; Human Adiponectin Platinum ELISA, TNF-α and TGF-β ELISA Ready-SET-Go (eBioscience, USA) kits were used. Optical density (OD) at 450 nm was obtained using an ELISA reader (Thermo LabSystems Multiskan RC/MS/EX Microplate Reader). For each cytokine, a standard curve was calculated from the readings of different concentrations of recombinant cytokines provided by the commercial kits. The obtained OD values were then converted to actual concentrations based on the respective cytokine standard curve.

2.7. Nitric oxide determination

Supernatants (100 μL) from monocyte cultures that had been infected with Mtb were stored in a 96-well plate at −20 °C until use. Fifty microliters of the supernatant was transferred to another 96-well plate, and 50 mL Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylene diamine dihydrochloride) was added. The samples were incubated for 15 min at room temperature, protected from light. A serial dilution of nitrite was included in separate wells to provide a standard curve for comparison. Absorbance at 595 nm was measured using a spectrophotometer (Thermo LabSystems Multiskan RC/MS/EX Microplate Reader).

2.8. Statistical analysis

All results were tabulated using Microsoft Excel software, and graphics and statistical analysis were performed using GraphPad Prism 5 software. Normality was defined using the Shapiro-Wilk test. Variances among parametric results were tested by one-way analysis of variance (ANOVA) followed by Student's *t*-test or Tukey test. Non-parametric results (median fluorescence intensity, MFI analysis) were analyzed by one-way ANOVA, and groups were compared using the Mann-Whitney test. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Study sample characteristics

Our cohort consisted of a severely obese population of 45 individuals, among whom 22 were OB patients and 25 OBDM patients. Most of the patients were female (n = 40), with an average age of 40.7 ± 8.8 years. The blood glucose and glycated hemoglobin levels of the diabetic patients were both higher than those of the OB patients (Glucose: 154.4 vs.101.1; *$p < 0.05$ and Glycated hemoglobin: 7.4 vs. 5.9; *$p < 0.05$). To investigate if either OB or OB in combination with DM is a risk factor for TB development, an active TB patient group and a respective control group were included. As shown in Table 1, 21 active TB individuals were selected. Most were male (n = 18), with an average age of 40 years old, and had a confirmed TB diagnosis. Ten control individuals with negative tuberculin skin test (TST) were selected (Table 1). The monocytes of all recruited patients were marked for $CD14^+CD11b^+$ and $CD14^+CD16^+$ but we show here only the $CD14^+CD16^+$ cells of 11 selected patients, as this phenotype is better described in the literature.

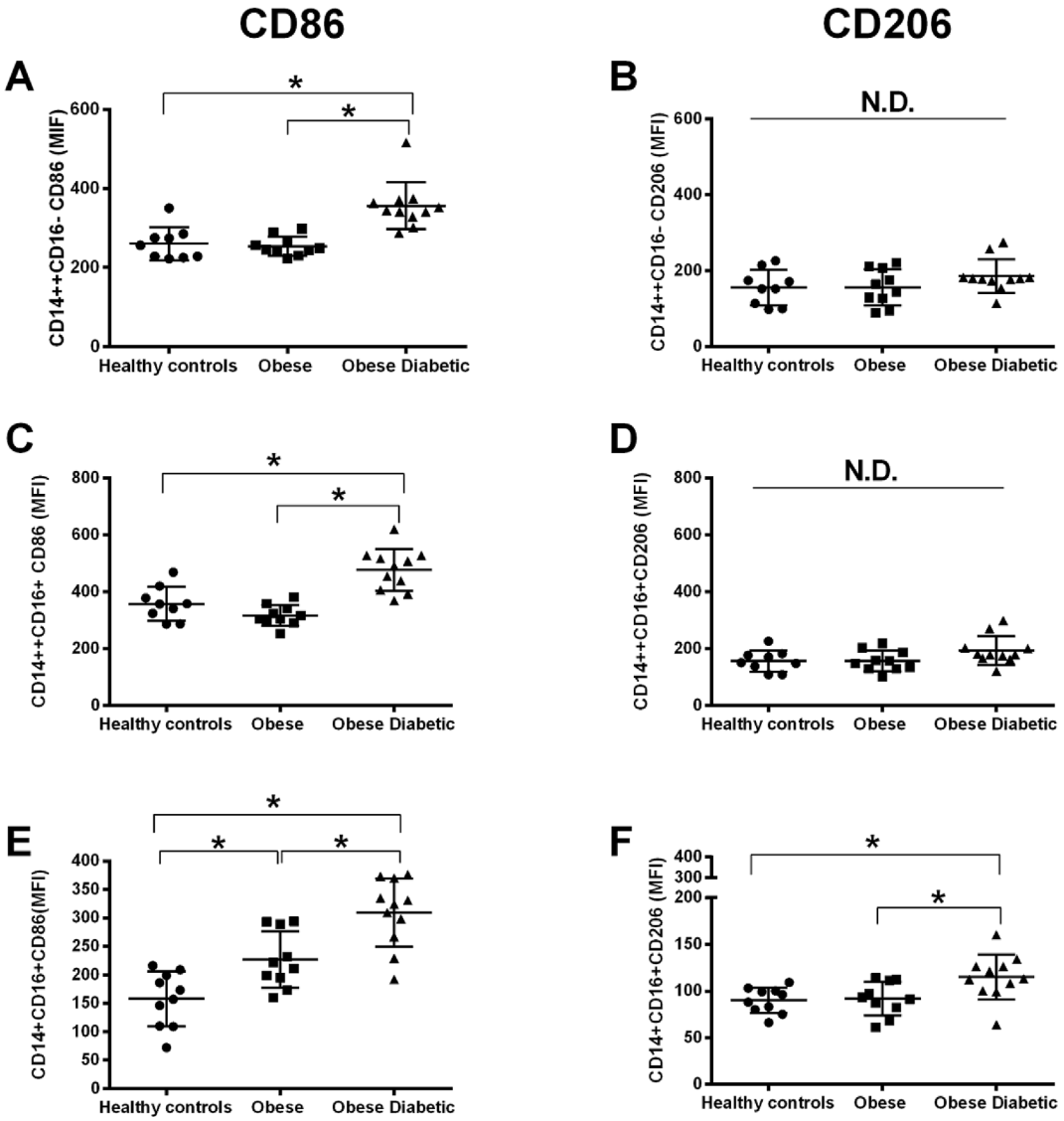

Fig. 1. Median fluorescence intensity (MFI) of CD86 and CD206 molecule expression by $CD14^{++}CD16$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes isolated from PBMCs of OB and OBDM patients. A and B: CD86 and CD206 MFI of classical monocytes from healthy control, OB and OBDM individuals. **C and D**: CD86 and CD206 MFI of intermediate monocytes from healthy control, OB and OBDM individuals. **E and F**: CD86 and CD206 MFI of non-classical monocytes from healthy control, OB and OBDM individuals. *$p < 0.05$.

3.2. OBDM individuals have a mixed population of activated non-classical monocytes

In vitro, macrophages differentiate toward a pro-inflammatory profile in the presence of glucose [18]. Therefore, we hypothesized that higher blood glucose levels in OB patients might influence the phenotype of circulating monocytes. Based on glucose level, glycated hemoglobin and insulin level (HOMA index, Homeostatic Model Assessment), individuals were classified into OBDM and OB groups, and the three major populations of circulating monocytes in the blood of control, OB and OBDM individuals were evaluated by flow cytometry. Monocyte populations were separated based on expression of CD14 and CD16 molecules into $CD14^{++}CD16^{-}$ (classical monocytes), $CD14^{++}CD16^{+}$ (intermediate monocytes) and $CD14^{+}CD16^{+}$ (non-classical monocytes) groups. Through analysis of these monocyte populations, we calculated the MFI of the molecules CD86 (the co-stimulatory protein, also known as B7-2, which binds to CD28 and CTLA-4, present on antigen presenting cells and working with CD80 to prime T cells) and CD206 (mannose receptors). According to the results, classical and intermediate monocytes from OBDM patients had higher expression of CD86 than did healthy individuals or OB patients (Fig. 1A and C; *$p < 0.05$). Conversely, no difference in expression of CD206 was observed among the populations (Fig. 1B and D; $p > 0.05$).

However, when analyzing non-classical monocyte populations, we found that monocytes from both OB and OBDM patients had higher expression of CD86 and CD206 than did monocytes from control individuals. Nevertheless, non-classical monocytes from OBDM individuals still showed increased expression of CD86 and CD206 compared to the OB group (Fig. 1E; *$p < 0.05$). Furthermore, non-classical monocytes were the only cell type that presented differential expression of CD86 between the OB and control groups. Maybe the metabolic changes in OBDM patients caused the increase of non-classical monocytes levels that express both CD86 and CD206 [19].

3.3. Non-classical monocytes from OBDM patients are similar to active TB non-classical monocytes

Considering the alterations in monocytes during DM and the historical relationship between DM and TB, we sought to determine whether non-classical monocytes population could be related in both

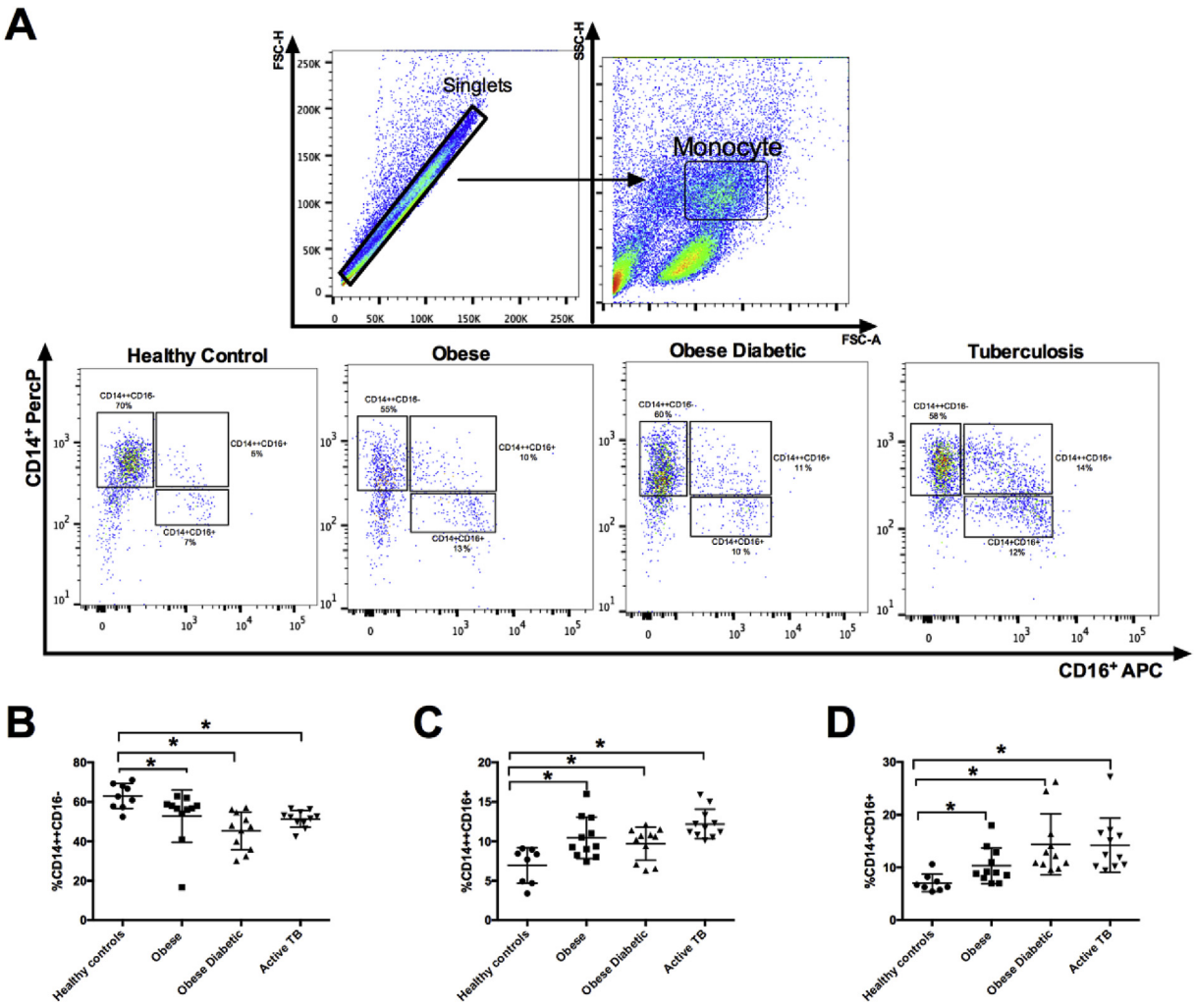

Fig. 2. Gating strategy for circulating monocyte analysis and monocyte sub-population percentage from healthy control, OB, OBDM and active TB patients. A: First, singlets were selected, and the monocyte population was gated based on cell size and granularity. Subsequently, the classical, intermediate and non-classical subpopulations of monocytes of control, OB, OBDM and TB individuals were analyzed. **B:** Percentage of classical monocyte population of control, OB, OBDM and TB individuals. **C:** Percentage of intermediate monocyte population of healthy control, OB, OBDM and TB individuals**. D:** Percentage of non-classical monocyte population of control, OB, OBDM and TB individuals, *$p < 0.05$.

diseases. Although we analyzed peripheral monocytes, in regard to TB, Mtb interacts with macrophages through complement receptors, TLRs and CD206, inducing activation of the cells and promoting an inflammatory response as well as increased expression of activation molecules [20–22]. As the response profile of TB infection is similar to that in other inflammatory diseases such as DM, we questioned whether similarities might exist in the circulating monocyte phenotype in both illnesses once this may increase the likelihood of an OBDM individual developing TB.

To address this question, the circulating monocyte populations of control, OB, OBDM and active TB patients were analyzed. As shown in Fig. 2A, classical, intermediate and non-classical monocyte populations were identified by flow cytometry. Compared to control individuals, cells isolated from all patient groups exhibited decreased numbers of classical monocytes (Fig. 2B, $p < 0.05$) and an increase in both intermediate and non-classical monocytes (Fig. 2C and D, $p < 0.05$). Furthermore, the percentage of each monocyte population was altered in a similar manner in the different disease states, which prompted us to consider that these cells may participate in the pathology of OB, OBDM and active TB patients.

Next, expression levels of CD86 and CD206 in monocytes from TB compared to OB and OBDM patients were evaluated. Expression levels of CD86 and CD206 in classical (Fig. 3A and B) and intermediate (Fig. 3C and D) monocytes were assessed. Classical and intermediate monocytes from OBDM individuals presented CD86 MFI higher than all the other groups. No differences were observed on the CD206 level of expression. Increased levels of CD86 MFI were observed on non-classical monocytes among all groups investigated (Fig. 3 E) when compared to healthy controls. In a similar trend, the expression of CD206 among non-classical monocytes was increased in OBDM and TB patients (Fig. 3F) compared to OB and healthy controls.

In light of these results, we conclude that active TB, OB, and OBDM patients have a similar phenotype of non-classical circulating monocytes, indicating a possible function of these cells in both illnesses and a possible connecting point in the historical association between tuberculosis and diabetes.

3.4. Activation of circulating monocytes in OBDM does not affect cytokine production

To further investigate a possible similarity in function of these cells, we measured the production of cytokines by the non-classical monocyte population using flow cytometry. We evaluated the production of two main inflammatory cytokines, IL-12 and IL-6, which are both important for the establishment of inflammation and are related to the immune responses of TB and OBDM. To our surprise, significant production of

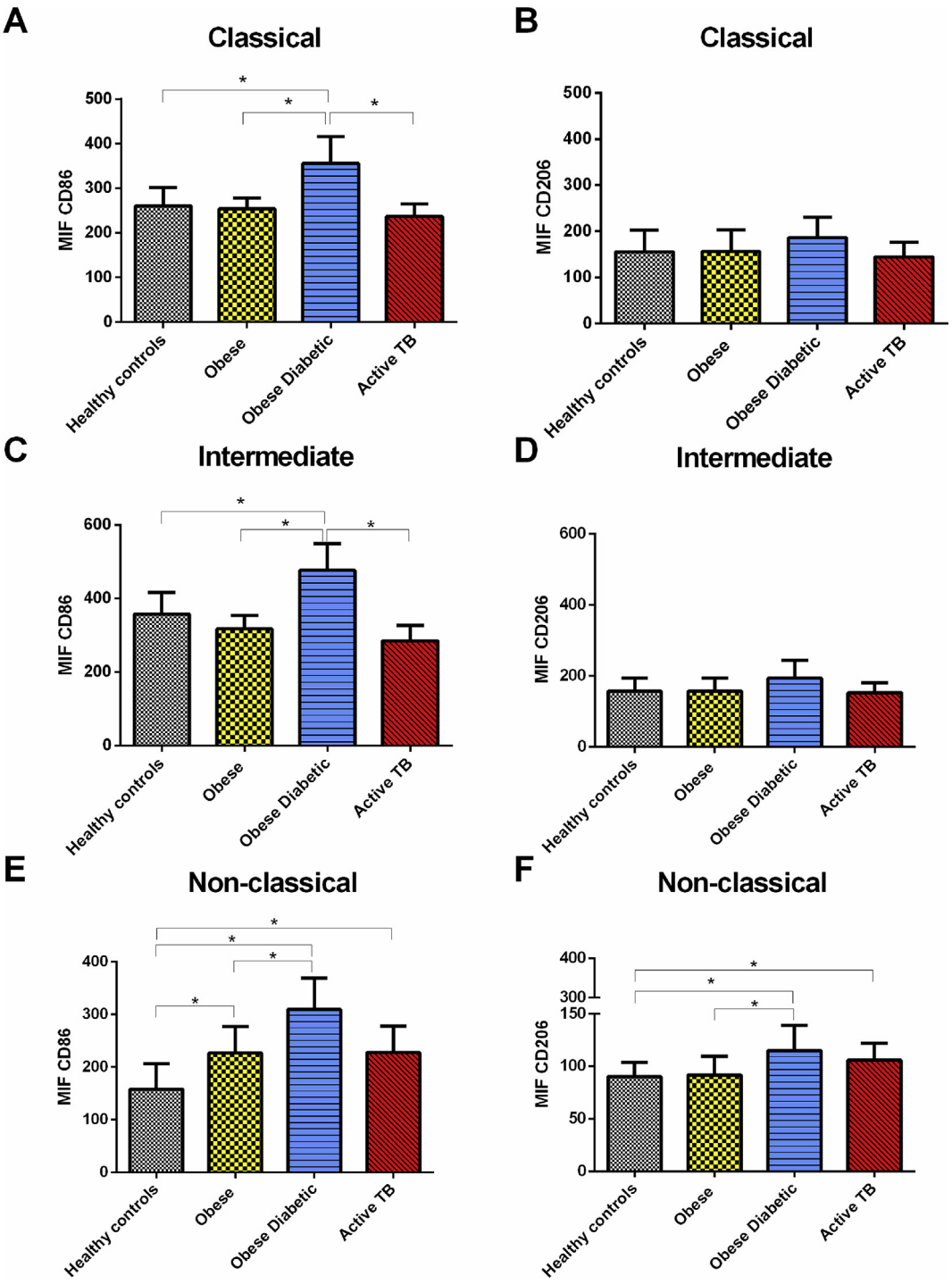

Fig. 3. CD86 and CD206 expression by classical, intermediate and non-classical monocytes from healthy control, OB, OBDM and active TB patients. A and **B**: CD86 and CD206 MFIs of classical monocytes from healthy control, OB, OBDM and active TB individuals. **C and D**: CD86 and CD206 MFIs of intermediate monocytes from healthy control, OB, OBDM and active TB individuals. **E and F:** CD86 and CD206 MFIs of non-classical monocytes from healthy control, OB, OBDM and active TB individuals. *p $<$ 0.05.

the cytokines was only found in active TB patients (Fig. 4A and B).

Different cell populations produce a variety of inflammatory cytokines that contribute to the inflammatory state of these diseases. For instance, TNF-α, produced mainly by activated macrophages, is implicated in insulin resistance in DM, and TGF-β, produced by many cell types, results in differentiation of Th17 cells, which is important in both diseases; adiponectin, produced exclusively by adipocytes, is also related to insulin resistance [23–25]. Adiponectin has also been considered a serum marker for severity of tuberculosis infection, and hence was found increased in the serum of active TB patients [26,27]. We investigated the serum levels of adiponectin and found that it was similarly increased in TB and OBDM patients (Fig. 4C). We also investigated the systemic production of inflammatory cytokines by testing serum from these patients. We found that TNF-α levels were not different from control individuals (Fig. 4E) and that TB patients showed decreased TGF-β production (Fig. 4D). As cytokine production was not similar between the diseases, we investigated the activation and microbicide capacity of monocytes in both illnesses.

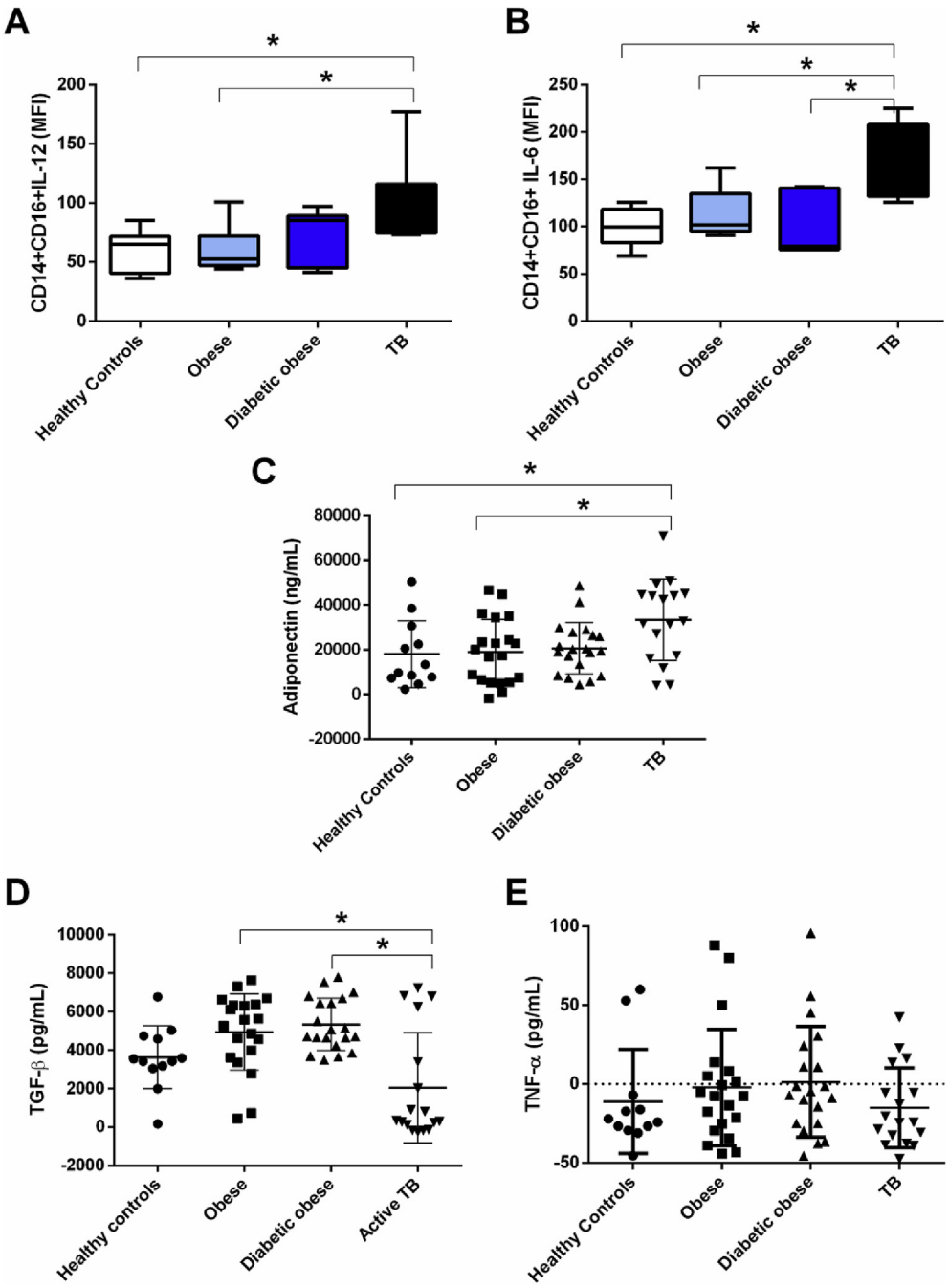

Fig. 4. Median fluorescence intensity (MFI) of IL-12p40/23 and IL-6 cytokines in $CD14^+CD16^+$ monocytes from PBMCs, and serum cytokines from OB and active TB patients. A and B: MFI of IL-12p40/23 and IL-6 of non-classical monocytes from healthy, OB, OBDM and active TB patients. **C, D and E**: Adiponectin, TGF-β and TNF-α levels measured in the serum from healthy controls, OB, OBDM and active TB patients *$p < 0.05$.

3.5. Expression of TLRs is similar between OBDM and TB patient monocytes

It is known that in OB and other metabolic diseases, the presence of free fatty acids and glucose can lead to activation of macrophages through interaction with TLR-2 and TLR-4 [28]. These same receptors are also crucial for the recognition of Mtb by several innate immunity cells. To determine whether the expression profiles of these molecules in circulating monocytes are similar for both diseases, the MFI of TLR-2 and TLR-4 in $CD14^+CD11b^+$ monocytes from the peripheral blood of control, OB, OBDM and active TB patients was evaluated (Fig. 5A); as CD11b is a global marker of monocytes, the $CD14^+CD11b^+$ population was chosen for analysis such that all three CD14/CD16 monocyte populations could be investigated. Based on our results, TLR-2 expressions were increased in OB and TB, but OBDM presented a tendency to increase as well (Fig. 5B); in contrast, TLR-4 expressions were reduced compared to the healthy control group (Fig. 5C). These data suggest that TLR-2 signaling may be involved in the inflammation process in both diseases, reinforcing the importance of monocytes in these conditions.

3.6. OB and OBDM contribute to M. tuberculosis growth in monocytes

It has been observed that monocytes from OB and OBDM individuals share similarities with monocytes from active TB patients. TLRs are important receptors for inflammation initiation in both diseases and are related to Mtb phagocytosis. The similarity between these

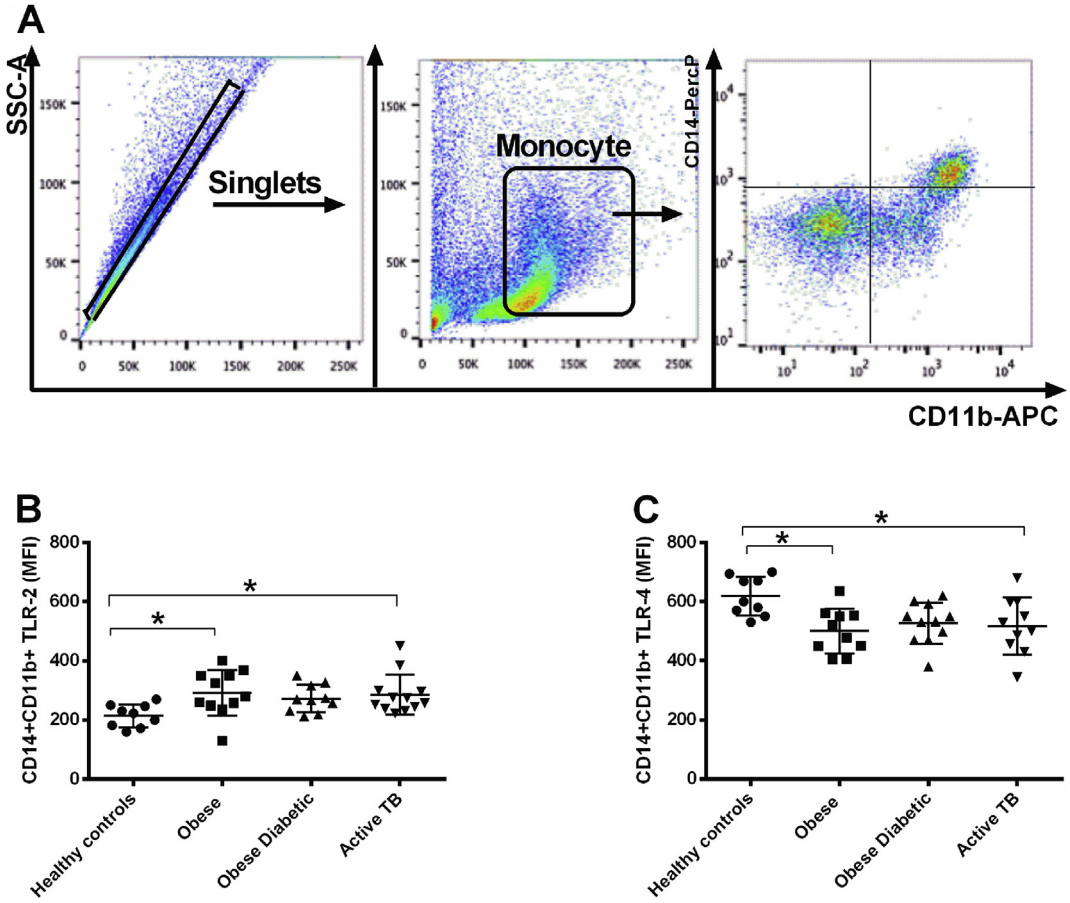

Fig. 5. Median fluorescence intensity (MFI) of TLR-2 and TLR-4 of $CD14^+CD11b^+$ monocyte populations. A: The gating strategy for circulating monocyte analyses started with singlet selection, and the monocyte population was then gated. Subsequently, analysis of the $CD14^+CD11b^+$ monocyte population was performed. **B:** MFI of TLR-2 of healthy control, OB, OBDM and active TB groups. **C:** MFI of TLR-4 in healthy control, OB, OBDM and active TB groups. *p < 0,05.

cells, together with the fact that TLR-2 levels were altered in both OBDM and TB patients, prompted us to assess if similar alterations in monocyte TLR expression, result in a change in cellular killing capacity that may be implicated in TB susceptibility in DM patients. Macrophages derived from the monocytes from control, OB, OBDM and active TB patients were infected with Mtb *in vitro* for 24 and 48 h. After *in vitro* infection, macrophages from OB patients were more permissive to Mtb growth, whereas OBDM and TB patient macrophages exhibited similar increases in Mtb growth, which was higher than that in macrophages from the OB and control individuals (Fig. 6A, D and E). Nitric oxide production by these cells during infection was also measured, with greater production by OBDM and TB monocytes observed after 24 (Figs. 6B) and 48 (Fig. 6C) hours.

To assess whether Mtb cell wall components or virulence factors exclusive to Mtb were responsible for this phenomenon, we repeated the *in vitro* infection using *M. abscessus* subsp. *massiliense,* a rapidly growing environmental mycobacterium. We observed that the growth of *M. abscessus* subsp. *massiliense* remained unaltered, independent of the monocyte with which it was cultured. After 48 h of infection, overall bacterial growth was reduced by almost one log, as expected for this bacterium (Fig. 7). This result suggests that components exclusive to Mtb may play a role in its survival in TB and OBDM monocytes.

4. Discussion

Our data confirm the previous literature where non-classical circulating monocytes were increased in obese individuals [10–15]. Additionally, we show for the first time that this cellular involvement occurs among OBDM and OB patients with body mass index (BMI) greater than 35 kg/m^2. Classical and intermediate monocytes from OBDM patients presented higher levels of CD86 expression, while non-classical monocytes from OBDM patients presented increased CD86 and CD206 levels when compared to OB individuals. Non-classical monocytes from OB patients had similar levels of CD86 expression as those from TB patients. However only the non-classical monocytes from TB patients had increased expression of IL-12 and IL-6 cytokines that did not correlate with the TLR-2 expression increase seen in all patient groups. Interestingly the expression of TLR-4 was reduced similarly in all groups. Despite the differences in pro-inflammatory cytokine expression observed on monocytes from OBDM and TB individuals, they were both more permissive to Mtb growth but not to other mycobacteria (*M. abscessus* subsp. *massiliense* for example), when compared to OB and control individuals, suggesting that maybe monocytes could be one of the common factors associated with increased TB susceptibility.

In this work, it was found that the subpopulations of monocytes were increased, as expected, in OBDM patients [9], whereas in OBDM, only non-classical monocytes exhibited mixed expression of the surface markers CD86 and CD206. Moreover, expression of CD86 by OBDM patients differed from OB patients only with regard to the non-classical population. CD86 is a costimulatory molecule present in antigen-presenting cells important for the generation of the specific immune response [29]. High expression of this molecule by monocytes has been associated with the development of inflammatory M1 macrophages [30]. CD206 is a *C*-type lectin, also known as mannose receptor, previously described as participating in the phagocytosis of pathogens such as Mtb [31] and is related to insulin resistance in human obesity [32]. The increased expression of these molecules could reflect the

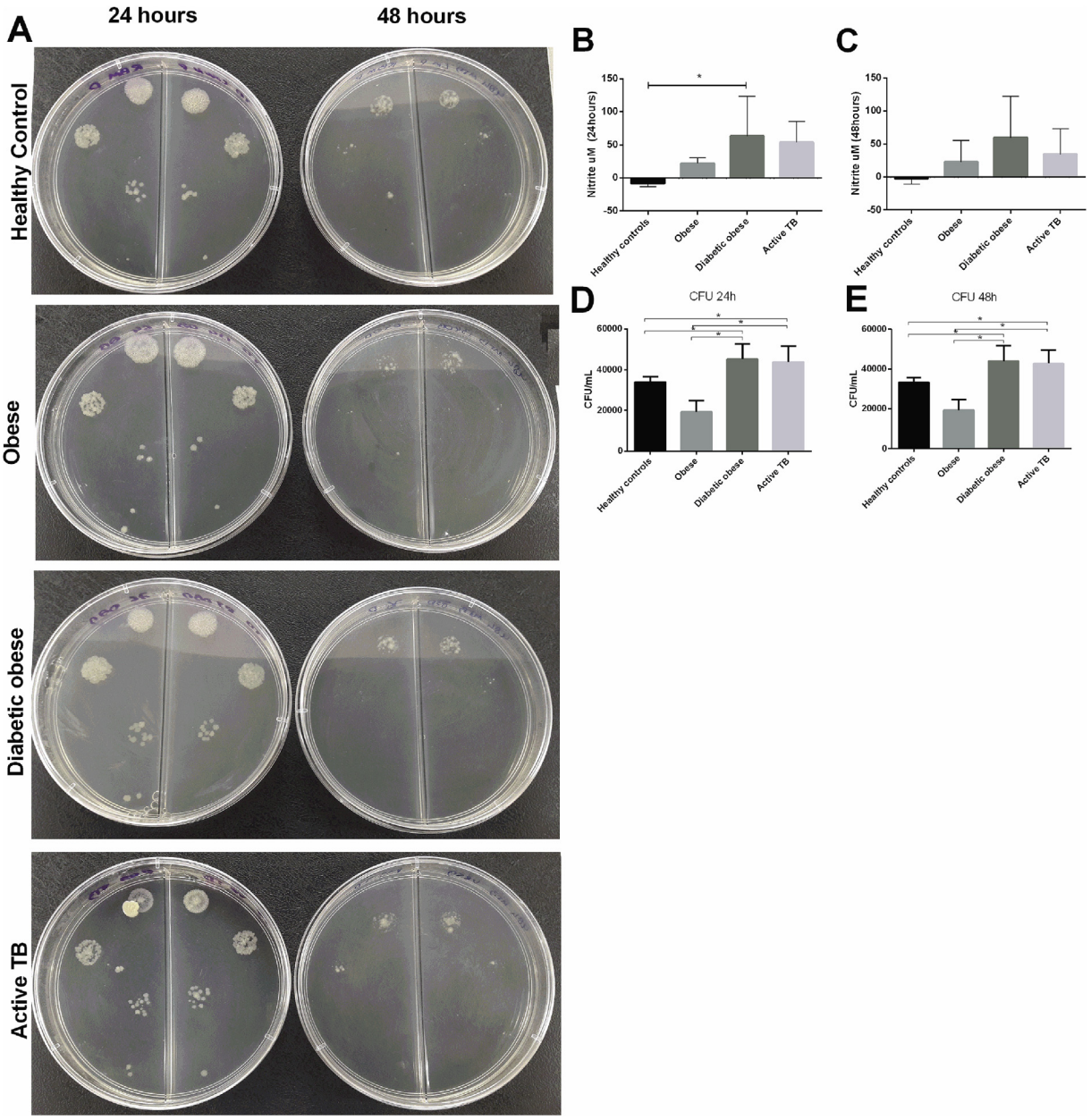

Fig. 6. Mtb (H37Rv) infection of monocytes from healthy controls, OB, OBDM and active TB patients. A: Monocytes obtained from healthy individuals, OB, OBDM and active TB patients were infected *in vitro* with Mtb (MOI 1:1). After 24 h of infection, the cells were lysed with 0.05% Triton. The lysates were diluted to 10^{-1}, 10^{-2} and 10^{-3}, plated (50 μL) on 7H11 agar medium and incubated for 21 days at 37 °C with a 5% CO_2 atmosphere. **B and C:** After 24 and 48 h of infection, respectively, the culture supernatants were collected for nitric oxide determination. *$p < 0.05$. **C and D:** CFU growth 24 and 48 h post-infection, respectively.

inflammatory state of these conditions, as CD206, for instance, could mediate inflammation through recognition of DAMPs resulting in release of pro-inflammatory cytokines like IL-1β and IL-6 [33]. As for CD86, the expression of this molecule has already been correlated with inflammation and production of TNF-α, IL-18, IL-8 and other inflammatory cytokines [34], corroborating with our findings.

As macrophages participate directly in the pathology of both diseases, we analyzed circulating monocyte profiles in OBDM and TB patients. Although the non-classical monocytes of these individuals expressed both CD86 and CD206, there was a predominance of CD86 expression. In the tissue, macrophages with a $CD206^+$ profile have also been defined as inflammatory and contribute to insulin resistance, metabolic syndrome and DM development [32,35]. An increase in inflammatory CD86 monocytes in TB patients, could contribute to tissue damage in the lungs, once this cells infiltrate and differentiate into inflammatory macrophages, worsening the disease [36].

In the liver and subcutaneous tissues of OBDM patients, there is a predominance of M1 pro-inflammatory macrophages (TREM-1, CD86, CCR-7, iNOS, IFN-γ, TNF-α, IL-6, MCP-1, CCR-2 and CCR-5) and a reduction in M2 macrophages (TREM-2, CD163, IL-4 and IL-10) compared to obese and lean individuals [37]. As observed in OBDM patients, macrophages from OB patients, also increased molecules that are mostly associated with M1 markers [37]. Circulating classical monocytes from healthy individuals preferentially differentiate into M1 macrophages, while non-classical monocytes into M2 macrophages [38]. Our data, however, demonstrate that for OBDM and TB patients, the non-classical monocytes have a mixed expression of molecules (CD86 and CD206). Moreover, non-classical monocytes from TB patients showed a tendency towards M1 polarization, once these cells were positive for IL-12 and IL-6 (Fig. 4). Furthermore, despite the belief

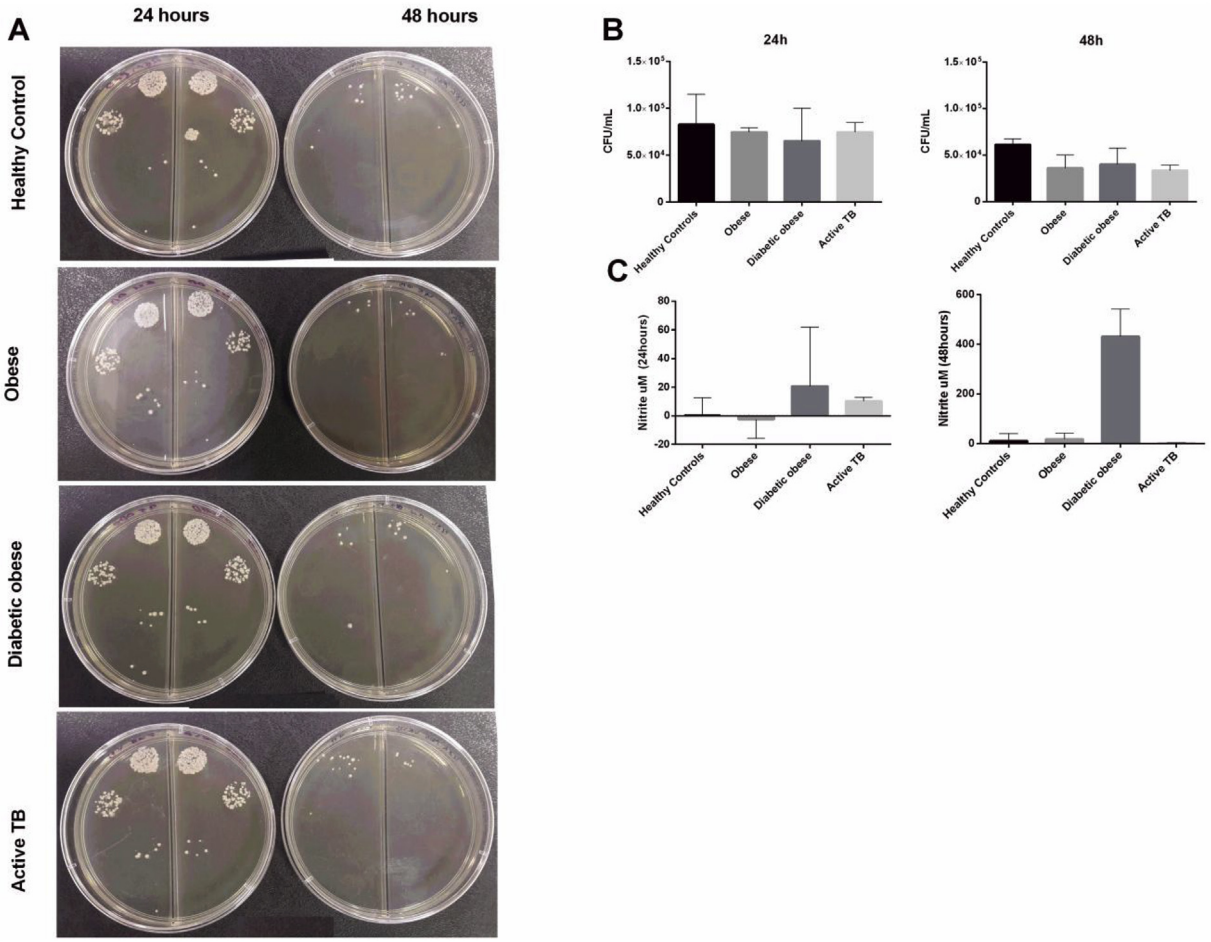

Fig. 7. *Mycobacterium abscessus* subsp. *massiliense* infection of monocytes from healthy controls, OB, OBDM and active TB patients. A: Monocytes obtained from healthy, OB, OBDM and active TB patients were infected *in vitro* with *M. abscessus* subsp. *massiliense* (MOI 1:1). After 24 or 48 h of infection, the cells were lysed with 0,05% Triton. The lysate was diluted to 10^{-1}, 10^{-2} and 10^{-3}, plated (50 μL) in MH agar medium and incubated for up to 7 days at 37 °C with a 5% CO_2 atmosphere. **B:** CFU growth 24 and 48 h post-infection. **C:** At 24 and 48 h after infection, the culture supernatants were collected for nitric oxide determination.

that non-classical monocytes are the source of M2 macrophages, it was characterized here and by others that in OB, as well as in TB, these cells produce mostly inflammatory cytokines like IL-1β, IL-6, IL-8 and IL-12 [15,16], which are cytokines classically produced by M1 macrophages.

The M1/M2 paradigm should be evaluated with caution, as monocyte phenotypes are very plastic and any variation in the microenvironment of the host, like obesity/diabetes, could influence the polarization of these cells. Due to the intrinsic high production of pro-inflammatory cytokines within the microenvironment of those tissues, circulating monocytes might be modulated toward an M1 profile, as observed in this study (Figs. 1 and 5), favoring induction of insulin resistance, although the authors are aware that more markers are needed to completely define an M1 population.

Although we observed similarity in the expression of activation molecules in monocytes from TB and OBDM patients, only those from active TB patients showed increased expression of IL-12 and IL-6 (Fig. 4), but the TGF-β blood levels were decreased. This fact might be explained because during Mtb infection, interaction of PAMPs from the bacterial cells results in direct activation of monocytes and these cells could be previously activated, while the monocytes from OB patients were not. The difference in cytokine production generates a paradox, because obesity and TB are chronic inflammatory diseases that could induce monocyte activation, but one possible explanation is that the cells from OB and OBDM patients could be in an immune exhaustion state [39] and to a lower degree of innate immune cells [40–42]. Thus, the absence of cytokine production by OB and OBDM cells could reflect an inability of these cells to respond to an infection. In addition, in the studied diseases no increase of TNF-α level was observed. It is very well known that IL-12 and TNF-α are important macrophage activators that significantly contribute to the control of Mtb infection [43,44]. Maybe the inability of non-classical monocytes from OB and OBDM patients to produce those cytokines could contribute to an increased risk of TB development.

Our results demonstrate that in relation to healthy individuals, OB and active TB patients have increased expression of TLR-2 in circulating monocytes (Fig. 5). In contrast, expression of TLR-4 is reduced in OB, OBDM and active TB patients. There are many questions about the factors that predispose OBDM individuals to develop TB. It was recently demonstrated that macrophages from overweight DM individuals (BMI = 28 kg/m^2) exhibit defects in phagocytosis of *M. bovis* BCG [45], prompting us to question whether the phagocytosis receptors of these monocytes could be defective in the internalization of Mtb, as it is the main etiologic agent of TB, and also *M. abscessus* subsp. *massiliense*, a rapidly growing mycobacterium.

We have shown a reduction in TLR-4 in circulating monocytes in OB and OBDM individuals (Fig. 5). One of the possible explanations for this reduction is that these receptors might be unavailable for identification on the monocyte surface due to internalization of bonded receptors. This interaction promotes activation of these cells resulting in production of pro-inflammatory cytokines such as IL-6, TNF-α and adhesion molecules that promote inappropriate recruitment of circulating monocytes to adipose tissue [46]. However, our results show that in

circulation, those monocytes do not possess the ability to produce some of these cytokines (Fig. 4), even after activation of TLR-4 due to a possible immune exhaustion as already discussed above.

When monocytes are infected with Mtb, increased bacterial growth can be observed in the cells of OBDM individuals (BMI > 40) in relation to monocytes from healthy individuals. This phenomenon is similar to that found for the macrophages of active TB individuals (Fig. 6). The increase in TLR-2 in monocytes from OBDM and active TB patients might contribute to enhanced Mtb growth, as the TDM present in the Mtb cell wall interacts with TLR-2, which leads to infection and disease [47]. In its many centuries of coevolution with the human host, Mtb has developed several mechanisms to evade the immune system, including exploiting TLR-2 activation. Some proteins secreted by Mtb (such as hsp60 and PPE18) can induce production of the anti-inflammatory cytokine IL-10 in a TLR-2 dependent manner by human macrophages [48]. An important virulent factor of Mtb, the protein ESAT-6 is also reported to bind to TLR-2 and inhibit production of IL-12, a cytokine that is crucial for eliminating Mtb [49]. Furthermore, several Mtb lipoproteins can interact with TLR-2 and inhibit MHC-II antigen presentation [50,51]. Taken together, the findings suggest that metabolic changes due to DM may facilitate Mtb growth in monocytes through increased TLR-2 expression.

A different result was observed when monocytes were infected with *M. abscessus* subsp. *massiliense*, as bacterial growth was not changed among the tested groups (Fig. 7). As demonstrated by Kim et al., [52], *M. abscessus* subsp. *massiliense* can interact with TLR-2, although this interaction did not result in advantage for *M. abscessus* subsp. *massiliense*, as with Mtb. These results suggest that *M. abscessus* subsp. *massiliense* lack some evasion mechanism present in Mtb, due to the different composition of their cell walls; demonstrating that there could be a mycobacterial factor involved in the growth inhibition, besides the differences among monocytes. Also, the different environment in which these cells were (the patient) before *in vitro* challenge may also influence the outcome of this assay.

Although the conditions in which we cultured the monocytes for infection with both bacteria were the same, the cells came from different patients with different inflammatory and metabolic states (specially OBDM patients) that could have influenced the monocytes to be less able to control the infection by Mtb. We believe that the increase in TLR-2 and CD206 could contribute to the diminished capacity of OBDM and TB macrophages to control the multiplication of Mtb, and not to *M. abscessus* subsp. *massiliense* due to the better adaptation of Mtb to evade cellular microbicide responses trough TLR-2 manipulation [50,51]. Further, a degree of immune exhaustion could be present in the OBDM cells due to the chronic inflammatory state where these cells were generated [41], that could render the macrophages functionally less capable of eliminating a more pathogenic bacteria like Mtb. These findings are in line with other studies that demonstrate lesser capacity of DM derived macrophages to eliminate several strains of Mtb [40].

It has been observed that in OB, alterations in adipokine metabolism result in dysregulation in the production of adiponectin, which is reduced in obese individuals [53]. It is known that adiponectin is related to activation of dendritic cells, leading to polarization of TCD4+ into Th1 and Th17 cells [54]. Many papers already described the production and the function of adiponectin in obesity [53] and diabetes [55]; in our work the elevated serum levels of adiponectin of TB patients was expected as it is described that this adipokine is a modulator of inflammation and has been considered a serum marker for severity of tuberculosis infection [26,27]. It is also suggested that Mtb use the adipose tissue to hide from the immune system and persist in the host, generating an increased production of adiponectin by the adipose tissue [27]. The presence of elevated levels of adiponectin in both obesity and tuberculosis reinforces the possible connection of these two diseases.

The authors recognize that the number of patients in this study was not ideal, but as all the evaluations were repeated three times with consistent results, we believe that the results obtained are accurate. Another limitation of this study is that it was not possible to test the OB patients for latent tuberculosis infection due to a shortage of PPD availability in Brazil. The sex differences in the studied groups may also be an issue, because in our cohorts' women predominated among obese individuals, while men prevailed among TB patients, a reflection of the disease's epidemiology. Also, the control group included individuals younger than the study groups. Nonetheless, alterations in monocyte populations, especially non-classical monocytes, have been shown to be independent of sex and age in obesity [10]. The authors also recognize the limitations in not performing a sorting assay to better define if non-classical monocytes could be responsible for TB susceptibility.

The results presented herein suggest that the non-classical monocyte population may be a link between OBDM and TB and may be one of the factors that contribute to the association of these two diseases, due to various phenotypical and functional similarities found in these cells in both diseases. Although, some differences are still present (cytokine production) that may be relevant to confirm this association. We conclude that obesity induced diabetes and tuberculosis present several immune similarities, especially in the population of circulating non-classical monocytes, and these similarities are another step in the understanding of the association of these two diseases. Nevertheless, further studies should be conducted to clarify the differences found and to determine the mechanisms involved in this phenomenon.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

This work was supported by the National Council for Scientific and Technological Development, Brasília, DF, Brazil [grant number 303675/2015-2] and Fundação de Amparo à Pesquisa do Estado de Goiás, Goiânia, GO, Brazil [grant number 05-2012].

Acknowledgments

This work is part of DPR PhD thesis at Tropical Medicine and Public Health Graduate Program from Comissão de aperfeiçoamento de Pessoal de nível superior with financial support of CNPq.

The authors thank the Laboratório Profa. Margarida Dobler Komma - IPTSP - UFG for biochemistry measurements, the Laboratório multiusuário de cultura de células – Escola de Veterinária e Zootecnia – UFG for flow cytometer use, the Secretaria municipal de Saúde de Goiânia, and the clinical research unit of Hospital das Clínicas-UFG. Dr. Erika Aparecida da Silveira is the principal investigator of the project DietBra Trial, which examines nutritional aspects of severe obesity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.11.003.

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