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Alterations in innate and adaptive immune leukocytes are involved in paediatric obesity

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What is already known about this subject

- Circulating immune cells such as monocytes and T cells infiltrate adipose tissue in the context of obesity.
- Plasma leptin and free fatty acid levels are usually elevated in obesity.
- Apart from the involvement of the innate immune response in obesity, the role of adaptive immunity is now beginning to be unravelled.

What this study adds

- Circulating monocytes and T cells are committed to a pro-inflammatory state regardless of their entry into the adipose tissue of obese children.
- Leptin and linoleic acid effects on monocytes contribute to the promotion of inflammation in obesity.
- An increased Th1 and cytotoxic commitment and a higher leptin-mediated up-regulation of interferon gamma in $CD4^+$ and $CD8^+$ T cells are observed in obese children.

Summary

Background: Adipose tissue is the main source of the cytokines and adipokines that are increased in the context of obesity. The production of reactive oxygen species (ROS) and cytokines by circulating immune cells can be regulated by these pro-inflammatory factors even before infiltration into adipose tissue.

Objective: To investigate the alterations that can occur in circulating monocytes and lymphocytes in paediatric obesity.

Methods: In this study, 54 paediatric obese patients and 30 age-matched metabolically healthy individuals were enrolled. Intracellular cytokines were analyzed after phorbol myristate acetate (PMA) or leptin plus PMA stimulation of lymphocytes and monocytes by flow cytometry. ROS generation was measured using dichlorofluorescein-diacetate. Both a 'stimulation index' and a 'fold of increase' were calculated for statistical purposes.

Results: Both interferon gamma (IFN-γ) production by circulating $CD4^+$ and $CD8^+$ lymphocytes and ROS production by monocytes following PMA stimulation were increased in obese patients. Leptin induced an increased production of IFN-γ in both subsets of T cells and tumour necrosis factor alpha in monocytes, and linoleic acid induced a higher ROS production in monocytes.

Conclusions: The distinct functional responses of circulating cells suggest that alterations in both innate and adaptive immune cells are involved in the maintenance of low-grade inflammation in paediatric obesity.

Keywords: Monocytes, oxidative stress, T cells, Th1/Th2 cytokines.

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Introduction

Obesity and its associated metabolic conditions, including insulin resistance, have reached epidemic proportions (1).Paediatric obesity is associated with a range of adverse consequences during both childhood and adulthood partly due to the development of obesity-related morbidities such as type 2 diabetes, dyslipidemia, hypertension, atherosclerosis and non-alcoholic fatty liver disease (2).

While inflammation mediated by the innate immunological response is known to have a critical role in obesity-related insulin resistance, the role of the adaptive immunological system is now beginning to be unravelled (3–5).

The increase in adipose depots favours adipocyte death, which is considered as the key initial event leading to macrophage, infiltration preceded by neutrophils and $CD4^+$ and $CD8^+$ T cell recruitment, and the establishment of insulin resistance within the adipose tissue (6,7). Insulin resistance and an increase in reactive oxygen species (ROS) release also result from the interaction between macrophages and adipocytes (8,9). Physiological ROS act as a signalling molecule to trigger pro-inflammatory cytokine production, while an overproduction of ROS has been linked to oxidative stress and inflammation (10).

Plasma levels of free fatty acids have been demonstrated to correlate with macrophage activation in obesity (11) and to enhance ROS production by neutrophils (12,13). Furthermore, a high-fat, high-carbohydrate meal induces a more intense inflammatory stress response, with a higher ROS generation in obese patients than normal-weight individuals (14).

In obese children, a low and sustained pro-inflammatory state is evidenced by increased plasma levels of C-reactive protein, tumour necrosis factor alpha (TNF-α), interleukin (IL)-6, resistin and leptin, and by signs of oxidative stress (15–17). Leptin not only modulates T cell differentiation towards a Th1 profile responsible for pro-inflammatory cytokine release but also activates monocytes through a Janus kinase–signal transducer and activator of transcription (JAK–STAT) signalling pathway (1,18,19).

In this context, we aimed to investigate the potential immunological alterations involved in paediatric obesity. To this end, we tested the hypotheses that leptin, free fatty acids and cytokines might induce a distinct activation at both arms of the immune response in obese patients in contrast to lean individuals.

Material and methods

Subjects

A total of 54 paediatric obese patients (<18 years; OB) from the Pediatric Nutrition Service of the Clinical Hospital 'José de San Martin' in Buenos Aires, Argentina were enrolled. Obese and overweight patients were determined based on cut-off points for body mass index (BMI; kg m^{-2}) by gender and age (20). Because there is an increasingly changing pattern of childhood BMI (21) and the consequences of early excess fat affect overweight and obese children in the same way (22), patients of both weight-associated categories were considered collectively. We excluded obese patients who have taken medications that can cause weight gain. None of the patients included in this study suffer from asthma or diabetes.

A total of 30 age-matched [median age: 11 years (range 2–17)], metabolically healthy individuals (CO) from the same hospital were included as lean controls. All subjects were enrolled in the study after written informed consent was obtained from their parents. The study was approved by the local Ethics Committee based on the internationally endorsed standards of the Helsinki Declaration. Control individuals were not taking any medication and their BMI was appropriate for normal weight. A clinical evaluation including anthropometric data (weight and height) was performed to calculate the BMI *z*-score. Laboratory tests for the liver enzymes aspartate aminotransferase and alanine aminotransferase, triglycerides, total cholesterol, glucose (Glu) and insulin were performed. A calculation for the homeostasis model assessment (HOMA) index was performed on all patients (Table 1).

Reagents

Ficoll-Hypaque was purchased from Pharmacia Biotech (Uppsala, Sweden). Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from Life Technologies (Gaithersburg, MD, USA). Gentamicin, glutamine, gelatin, ionomycin, phorbol myristate acetate (PMA), Brefeldin A, leptin, palmitic acid, linoleic acid, human recombinant TNF-α, IL-6 and 2′7"-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DCFH-DA was made daily by dissolving in phosphate buffered saline (PBS)-glucose (Glu) containing 5 mM Glu, 0.1% gelatin and 10% methanol. A stock solution of PMA was prepared in dimethyl sulfoxide (DMSO) at 25 μg mL^{-1} and stored at –20°C. mAbs for flow cytometry, and the fixation kit and permeabilization buffer for intracellular staining were

Table 1 Demographical and biochemical characteristics of obese patients and controls

	OB	CO
Gender	25 F/29 M	10 F/20 M
Age	10 (2.00–17.00)	11 (3.00–15.00)
BMI (kg m^{-2})	43 OB/11 OVER	0 OB/0 OVER
BMI *z*-score	2.37 (1.99–2.94)	0.95 (0.76–1.12)
HOMA	2.18 (1.04–6.24)*	1.81 (1.57–2.40)
Insulin (mUI mL^{-1})	10.39 (8.19–15.36)*	8.87 (7.32–10.50)
Glucose (mg dL^{-1})	84.83 (81.00–90.00)	83.00 (81.50–88.50)
TC (mg dL^{-1})	156.5 (79.00–397.00)	153.5 (134.8–185.00)
TG (mg dL^{-1})	94.0 (43.00–164.00)	78.5 (37.00–241.00)
AST (UI L^{-1})	22.00 (19.25–24.00)	22.00 (17.50–260.00)
ALT (UI L^{-1})	18.00 (14.00–21.70)	12.00 (9.50–17.00)

Values are expressed as median (25th–75th percentile). The total number of patients with elevated values is shown in brackets. *$P < 0.05$ vs. CO.
To determine whether patients were overweight or obese, Cole's BMI cut-off points were used. The HOMA cut-off value was set at 3.5. Normal ALT and AST levels are ≤32 and ≤48 IU L^{-1}, respectively. The reference ranges for TC and TG are <181 mg dL^{-1} and <119 mg dL^{-1}, respectively. Normal Glu and Ins levels are 65–110 mg dL^{-1} and 5–20 mUI mL^{-1}, respectively.
ALT, alanine aminotransferases (UI L^{-1}); AST, aspartate aminotransferases (UI L^{-1}); BMI, body mass index (kg m^{-2}); BMI *z*-score, body mass index *z*-score; Glu, glucose (mg dL^{-1}); HOMA, homeostasis model assessment; Ins, insulin (mUI mL^{-1}); OB, obesity; OVER, overweight; TC, total cholesterol (mg dL^{-1}); TG, triglycerides (mg dL^{-1}).

purchased from Becton–Dickinson (BD; Mountain View, CA, USA).

Cell isolation

Blood samples were collected by venous puncture into heparin-containing tubes at the time of diagnosis and immediately processed to obtain peripheral blood mononuclear cells (PBMCs). PBMCs were prepared by Ficoll-Hypaque density gradient centrifugation at 2000 rpm at room temperature for 20 min. PBMCs were washed twice in PBS, counted and resuspended in RPMI 1640 with 10% foetal bovine serum, 2 mmol L^{-1} L-glutamine and 50 μg mL^{-1} gentamicin.

Effects of leptin on cytokine production

To evaluate the effect of leptin on cytokine production by T cells, PBMCs were incubated in the presence of 10 nM leptin or medium alone for 19 h at 37°C (Fig. 1, pathway B) and re-stimulated with 25 ng mL^{-1} of PMA and 1 mM ionomycin in the presence of 1 mM brefeldin A at 37°C for 4 h. To evaluate the effect of leptin on cytokine production by monocytes, PBMCs were incubated in the presence of 100 nM leptin or medium alone for 6 hr at 37°C (Fig. 1, pathway C), as described previously (23,24)).

Monoclonal antibodies and intracellular cytokine detection

After stimulation, cell-surface staining was performed using anti-CD4, anti-CD8 or anti-CD14 Peridinin chlorophyll protein (PerCP) at 4°C for 20 min. The cells were then fixed at 4°C for 20 min and washed with permeabilization buffer. PE-conjugated anti-IFN)-γ, anti-IL-4, anti-TNF-α or the corresponding isotype controls were added, and the cells were incubated at 4°C for 40 min in the presence of 50 μL permeabilization buffer.

DCFH-DA oxidation

PBMCs (10^6 cells per mL) were stimulated with 100 ng mL^{-1} PMA (Fig. 1, pathway E), 500 μM palmitic acid or 200 μM linoleic acid at 37°C for 60 min (Fig. 1, pathway F). After two washes with PBS, cells were incubated with 10 μL DCFH-DA in PBS Glu (final concentration: 5 μM) with shaking at 37° for 15 min. Basal ROS production by circulating cells was evaluated by analyzing phosphate buffer saline-treated cellular suspensions. Following stimulation, PBMCs were incubated with anti-CD14 PE for 20 min at 4°C (Fig. 1, pathways D, E and F).

In vitro stimulation with proinflammatory cytokines

PBMCs (10^6 cells per mL) were pre-incubated with TNF-α or IL-6 (1200 pg mL^{-1}) at 37°C for 2 h. PMA (100 ng mL^{-1}) or PBS was added, and the suspensions were incubated in the dark at 37°C for another 30 min. Then, DCFH-DA was added and the protocol was performed as described above (Fig. 1, pathway D).

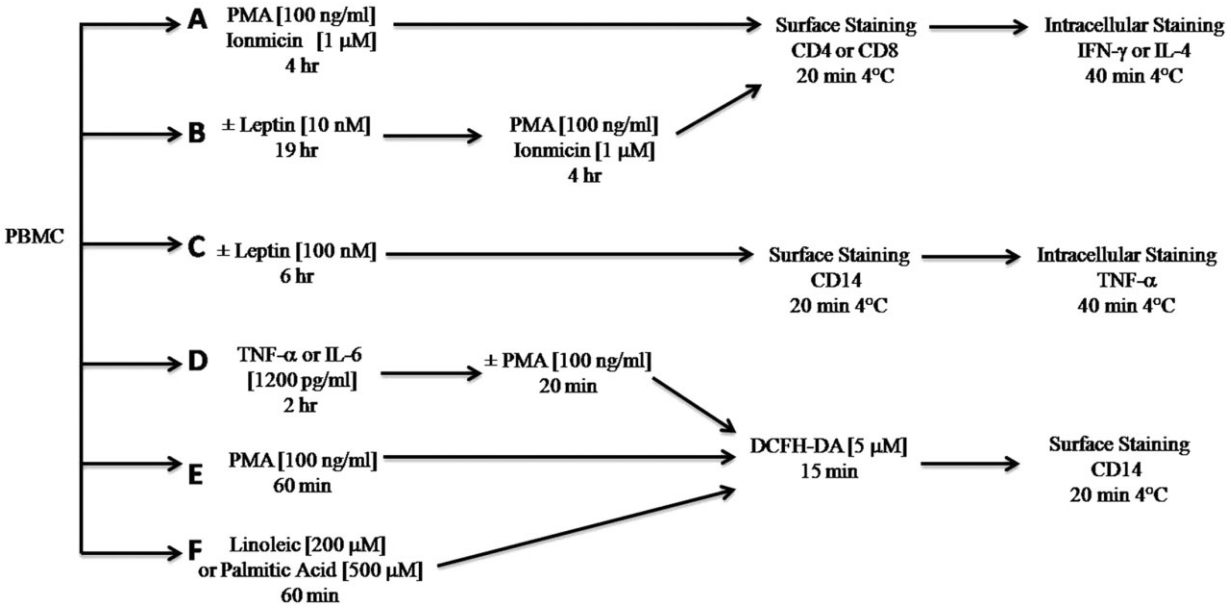

Figure 1 Schematic diagram of the experimental design using peripheral blood mononuclear cells (PBMCs). The studies performed on T cells are shown in pathways (a and b). Studies performed on monocytes are shown in pathways (c–f). PMA, phorbol myristate acetate; DCFH-DA, 2′7"-dichlorofluorescin diacetate.

Flow cytometric analysis

Gates were separately set on lymphocytes or monocytes according to their respective forward- (FSC) and side-scatter (SSC) properties and used to analyze cells stained with either control isotypes or the different fluorochrome-conjugated mAbs. For lymphocyte analysis, cells were gated on the basis of SSC and CD4 (or CD8) expression. The expression of IFN-γ or IL-4 was analyzed in the selected CD4 (or CD8) populations. For monocyte analysis, cells were gated in an FSC/SSC dot plot (region R1). Within R1, CD14-expressing cells were further gated in an SSC/FL2-PE dot plot (R2). Finally, the R2 population was analyzed with regard to its DCF fluorescence (FL1-FITC histogram) or TNF-α production (dot plot FL2 vs. FL3). Isotype-matched negative control mAbs were used in all cases to assess background fluorescence intensity. Acquisition and analysis were performed on a FACS Calibur flow cytometer (BD) using the FlowJo software version 5.7.2 (Tree Star Inc., Ashland, Oregon, USA).

Statistical analysis

GraphPad Prism© software (GraphPad, San Diego, CA, USA) was used for all the analyses. Normality was assessed by the Kolmogorov-Smirnov test. When the Mann–Whitney *U*-test was used to compare data between groups, values were expressed as median and interquartile ranges (25th–75th percentile). When the two-factor repeated-measures analysis of variance (ANOVA) was used, values were expressed as mean ± standard mean error (SEM) and the *P*-value for the interaction or for the main effects are informed. Spearman's rank correlation coefficients were used to test the association between parameters measured in obese patients. The one-sample *t*-test was used as indicated. The level of significance was fixed in all cases at $P < 0.05$.

Results

Circulating Th1, Th2 and cytotoxic precursors

TCR-mediated T cell priming commits lymphocytes to a Th1, Th2 or cytotoxic phenotype. The commitment of circulating lymphocytes was assessed by stimulating PBMCs under neutral conditions and performing an analysis of intracellular cytokine production (Fig. 1, pathway A). The percentages of CD4+ and CD8+ cells secreting IFN-γ in obese patients showed 1.81 and 2.00 times greater median values than controls, respectively [11.55 (9.13–13.85) vs. 6.28 (5.10–8.42), $P = 0.017$ and 15.75 (11.57–19.28) vs. 7.84 (5.25–8.07), $P = 0.003$, respectively, OB: $n = 10$, CO: $n = 8$]. A representative analysis of an obese patient and a control is shown in Fig. 2a. We found a significant but modest decrease in the

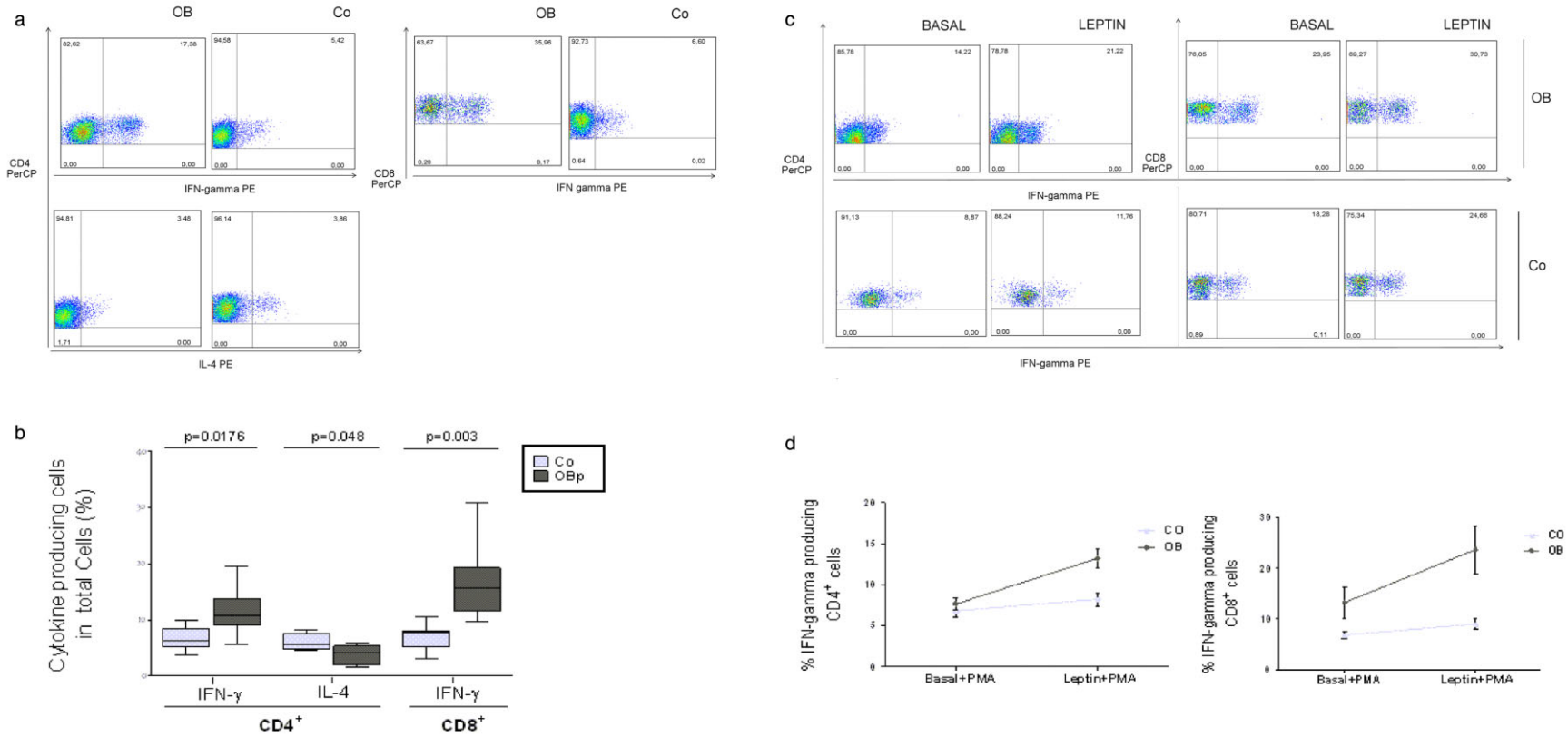

Figure 2 Percentages of interferon gamma (IFN-γ)- and IL-4-producing CD4⁺ and CD8⁺ cells in peripheral blood. Cells were gated on CD4 or CD8 vs. side scatter dot plots. (a) Representative dot plots of CD4⁺ and CD8⁺ cells showing intracellular IFN-γ and IL-4 in an obese patient (OB) and a control (CO). (b) The percentages of IFN-γ producing/total CD4⁺ and IFN-γ producing/total CD8⁺ cells were higher in obese patients (OB, $n = 10$) compared to controls (CO, $n = 8$). Lower percentages of IL-4 producing/total CD4+ cells were found in OB ($n = 10$) compared to CO ($n = 8$). The box and whiskers show non-parametric statistics: median, lower and upper quartiles, and confidence interval around the median. A two-tailed Mann–Whitney *U*-test was used to determine significant differences between the two groups. (c) Representative dot plots of CD4⁺ and CD8⁺ cells showing intracellular IFN-γ after incubation in the absence or presence of leptin in an obese patient (OB) and a control (CO). (d) An interaction between obesity status and leptin stimulation was found for the percentages of CD4⁺IFN-γ- producing cells (left) and CD8⁺IFN-γ- producing cells (right). The lines connect the mean values for 'leptin+PMA' and 'basal+PMA' in OB and CO, respectively. A two-factors repeated-measures ANOVA test was used to determine the influences of obesity status on leptin stimulation of lymphocytes. A value of $P < 0.050$ was considered statistically significant.

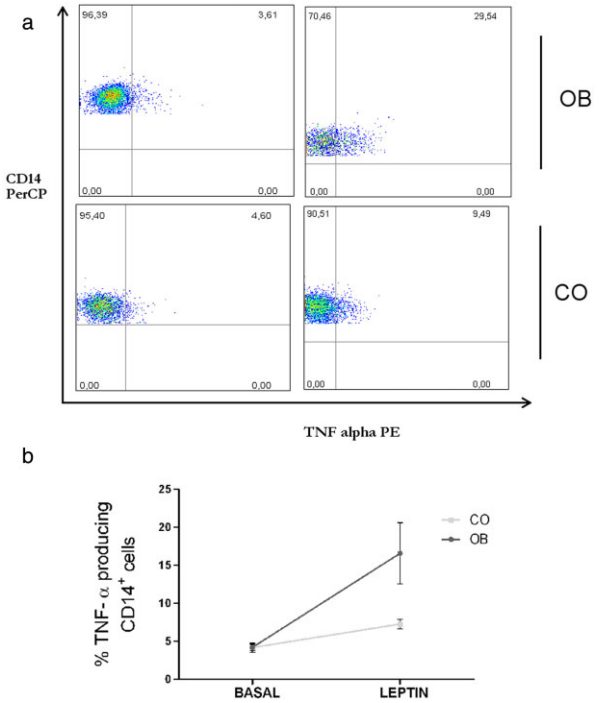

Figure 3 Percentage of TNF-α-producing CD14⁺ cells in peripheral blood after leptin stimulation. Monocytes were selected in a FSC vs. SSC dot plot, and CD14⁺ monocytes were further gated in a SSC-H vs. CD14-PE dot plot. (a) Representative dot plots of CD14⁺ cells showing intracellular TNF-α in an obese patient (OB) and a control (CO) in the absence (basal)/presence of leptin. (b) An interaction between obesity status and leptin stimulation was found for CD14⁺TNF-α⁺ cells. The lines connect the mean values for 'leptin' and 'basal' in OB and CO, respectively. A two-factor repeated-measures ANOVA test was used to determine the influences of obesity status on leptin stimulation of monocytes. A value of $P < 0.050$ was considered statistically significant.

percentage of CD4⁺ cells secreting IL-4 in obese patients compared with controls [5.65 (4.79–7.62) vs. 4.07 (1.92–5.39), $P = 0.048$; OB: $n = 10$, CO: $n = 8$] (Fig. 2c).

Effect of leptin on Th1 and Th2 cytokines production in circulating lymphocytes

Human leptin has an effect on circulating T lymphocytes. Therefore, we evaluated the effect of leptin on IFN-γ production by CD4⁺ or CD8⁺ cells (Fig. 1, pathway B); and we observed that the effect of leptin stimulation differed by obesity status [interaction: $P < 0.001$ (CD4) and $P < 0.01$ (CD8)]. In the absence or presence of leptin incubation, respectively, the mean percentages of CD4⁺IFN-γ⁺ cells were 6.83 ± 0.83 and 8.26 ± 0.79 in the CO group, $n = 6$; 7.67 ± 0.74 and 13.22 ± 1.22 in the OB group, $n = 6$. The mean percentages of CD8⁺IFN-γ⁺ cells were 6.86 ± 0.73 and 8.98 ± 1.03 (absence and presence of leptin stimulation) in the CO group, $n = 6$; 13.23 ± 2.99 and 23.67 ± 4.72 in the OB group, $n = 6$ (Fig. 2b and d).

Effect of leptin on TNF-α production in monocytes

We also evaluated the effect of leptin on TNF-α production by CD14⁺ cells (Fig. 1, pathway C) and we found that the effect of leptin stimulation differed by obesity status (interaction: $P < 0.05$; Fig. 3b). The mean percentages of CD14⁺TNF⁺ cells were 4.17 ± 0.60 and 7.27 ± 0.62 (absence and presence of leptin stimulation) in the CO group, $n = 6$; 4.20 ± 0.40 and 16.56 ± 4.00 in the OB group, $n = 6$. CD14+ cells from a representative OB and a representative CO showing intracellular TNF-α are shown (Fig. 3a).

Oxidative stress in monocytes

The generation of ROS is considered to be an early event in the activation of phagocytic cells. Therefore, we examined the activation of peripheral monocytes by determining their ROS production using the fluorescence marker DCFH-DA. A stimulation index (SI) was defined as the ratio between DCF fluorescence intensity (DCF-FI) in stimulated and non-stimulated (basal) monocytes.

When monocytes were stimulated with PMA (Fig. 1, pathway E), obese patients showed an increased production of ROS compared to CO as revealed by SI [5.77 (3.23–6.85) vs. 2.24 (1.63–3.11), $P = 0.002$; OB: $n = 15$, CO: $n = 13$] (Fig. 4b and d). Furthermore, we found a positive correlation between SI and HOMA in OB patients ($r = 0.74$, $P = 0.022$) (Fig. 4e). To clarify the likelihood that the proposed association is mediated by greater BMI within the obese category rather than ROS production of the monocytes, we tested the correlation between BMI and ROS in OB patients. We discarded that BMI acts as a confounder factor within this association because no correlation between BMI and SI values was found ($r = 0.36$, $P = 0.29$). In addition to the effect of PMA on ROS production in monocytes from OB, we found a distinctly higher SI in response to linoleic acid stimulation [6.20 (3.51–9.41) vs. 3.24 (1.87–4.70), $P = 0.023$; OB: $n = 10$, CO: $n = 10$] but not to palmitic acid in obese patients compared with controls [3.66 (1.99–4.17) vs. 3.43

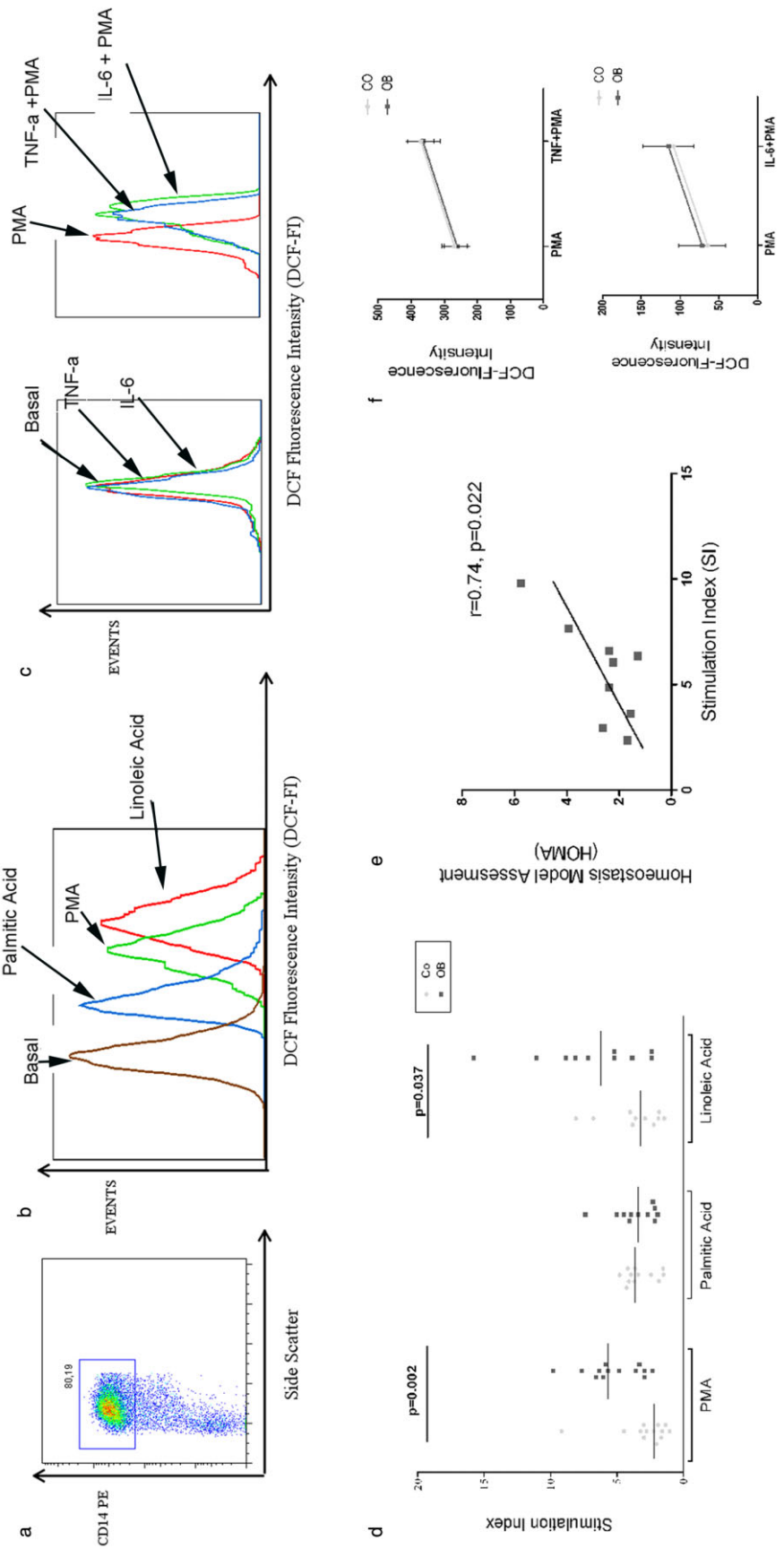

Figure 4 Stimulation index in peripheral blood monocytes after challenge with different stimuli. (a) Monocytes were selected in a FSC vs. SSC dot plot, and $CD14^+$ monocytes were further gated in a SSC vs. CD14-PE dot plot. The resulting population was analyzed regarding its DCF fluorescence intensity (DCF-FI) in the FL1-FITC histogram for (b) PMA and fatty acids or (c) TNF-α and IL-6 stimulation with or without re-stimulation with PMA. A stimulation index (SI) for monocytes was defined by the ratio between DCF-FI in stimulated and non-stimulated (basal) monocytes. (d) The SI was higher in obese patients than controls when monocytes were stimulated with PMA (OB, $n = 15$; CO, $n = 13$) and linoleic acid (OB, $n = 10$; Co, $n = 10$). No differences were found between the two group after stimulation with palmitic acid (OB, $n = 11$; CO, $n = 12$). (e) A positive correlation was found between SI and HOMA in PMA-stimulated monocytes ($n = 9$). Horizontal bars show the median values. A two-tailed Mann–Whitney *U*-test was used to determine significant differences between the two groups. A value of $P < 0.05$ was considered statistically significant. Spearman's rank correlation coefficients test was used to determine correlation between two parameters. (F) TNF-α or IL-6 stimulation influence ROS production in $CD14^+$ cells in both groups while obesity status has no effect. The lines connect the mean values for 'cytokine-stimulated+PMA' and 'basal+PMA' in OB and CO, respectively. A two-factor repeated-measures ANOVA test was used to determine the influences of obesity status on cytokine stimulation of monocytes.

(2.18–4.50), $P = 0.073$; OB: $n = 11$, CO: $n = 12$] (Fig. 4b and d).

Additionally, we found that both TNF-α and IL-6 were not sufficient to stimulate ROS production on their own by performing a one-sample *t*-test using an expected value of one ($P = 0.8$ and $P = 0.9$, respectively) [Fig. 1 (pathway D) and Fig. 4c (left histogram)]. Therefore, we investigated the role of these cytokines by determining whether they differentially enhanced PMA-stimulated ROS production in control and obese patients. Although TNF-α and IL-6 followed by PMA stimulation have influence in ROS production by monocytes in both OB and CO (Fig. 1, pathway D; IL-6: $P < 0.05$; TNF-α: $P < 0.001$), no influence was observed by obesity status as analyzed by a two-factor repeated-measures ANOVA (Fig. 4f). The mean DCF-FI were 266.85 ± 39.50 and 363.89 ± 51.97 (absence or presence of TNF-α stimulation) in the CO group, $n = 8$; 268.76 ± 37.80 and 370.10 ± 39.44 in the OB group, $n = 6$. The mean DCF-FI were 64.74 ± 10.50 and 108.93 ± 27.11 (absence or presence of IL-6 stimulation) in the CO group, $n = 8$; 71.85 ± 29.62 and 115.27 ± 32.65 in the OB group, $n = 6$.

Discussion

Childhood obesity is complicated by numerous co-morbid conditions (2).In agreement with our previous results regarding paediatric non-alcoholic steatohepatitis (25), our present data reveal that the circulatory system of obese children is also composed of immune cells committed to pro-inflammatory functions. Our findings of Th1 and cytotoxic biased CD4 and CD8 T-cell populations, respectively, enhanced ROS production by circulating monocytes after both PMA and linoleic acid stimulation; and increased TNF-α production following leptin stimulation are in agreement with the role of immune cells and adipokines in the promotion of inflammation in paediatric obesity (26,27).

It has been demonstrated that plasma TNF-α and IL-6 levels are elevated in paediatric obese patients (28). Although TNF-α and IL-6 did not seem to promote a distinctly enhanced ROS production in monocytes from obese patients, we cannot rule out their involvement in obesity-associated inflammation because both cytokines were able to induce ROS production in our *in vitro* assays. The increased response of circulating monocytes to linoleic acid and PMA, a ubiquitous activator of protein kinase C (PKC) (29), may be a consequence of previous *in vivo* exposure to high levels of pro-inflammatory cytokines.

It is known that Glu induces $p47^{phox}$ expression in monocytes (30) and promotes superoxide release due to the activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase by PKC (31). Although our two groups of patients show similar fasting Glu values, we cannot eliminate the possibility that undetected alterations in Glu metabolism, which are frequently found in overweight/obese children and adolescents (32), could increase the activity of pre-existing NADPH oxidase and underlie the higher production of ROS in monocytes from obese patients.

The positive correlation between ROS generation and HOMA levels in obese patients confirms the close relationship between oxidative stress and insulin resistance in obesity (33).We ruled out the potential influence of BMI of obese patients on this correlation using a Spearman's correlation test performed between ROS production and BMI levels. Our results indicated that BMI is an unlikely confounder factor for the relationship between ROS production and HOMA.

The role of circulating $CD4^+$ and $CD8^+$ T cells in obesity has not been clearly determined in humans or in animal models. In obese adult patients, the presence of $CD4^+$ and $CD8^+$ T cells in adipose tissue has been demonstrated and has a positive correlation between the total numbers of circulating $CD4^+$ and body mass index (7,34). IFN-γ, the main Th1 cytokine, promotes the synthesis of leucocyte adhesion molecules and chemokines by adipocytes and, as a result, macrophage recruitment into adipose tissue (35). IFN-γ, secreted by $CD4^+$ Th1 and $CD8^+$ T cells, has been demonstrated not only to decrease insulin signalling but also to stimulate M1 pro-inflammatory macrophage maintenance (36). Furthermore, adipose tissue infiltrating-$CD8^+$ T cells promote the recruitment of M1 (37). Consequently, the increased Th1 and cytotoxic commitment of $CD4^+$ and $CD8^+$ T cells observed here might suggest the involvement of peripheral T cells in the initiation of obesity-associated inflammatory processes through IFN-γ release following adipose tissue infiltration. Because both IFN-γ and TNF-α can attenuate insulin signalling (38), we provide additional evidence of the pro-inflammatory role of leptin in obesity and suggest the existence of a putative loop incorporating leptin, IFN-γ and TNF-α that might perpetuate the obesity-associated pro-inflammatory state. However, activated circulating leucocytes may not necessarily lead to tissue infiltration.

Obese patients may exhibit central leptin resistance potentially mediated by an increased hypothalamic expression of suppressor of cytokine signalling

3 (Socs3), a feedback inhibitor of the JAK–STAT pathway. Although leptin signalling in monocytes is not fully understood, it has been demonstrated that Socs3 expression is increased in the circulating mononuclear cells of obese adults (39), potentially mediating leptin resistance. Whether the pro-inflammatory effect of leptin observed *ex vivo* might be modulated *in vivo* either by this or another mechanism still remains an open question.

Whether a lifestyle change could cause a reversal of the increased cytokine production and/or ROS generation observed after *ex vivo* stimulation of circulating immune cells deserves additional research.

In summary, paediatric obesity is characterized by the presence of both circulating monocytes and T cells committed to a pro-inflammatory state, regardless of their entry into adipose tissue. The higher pro-inflammatory state of circulating immune cells is conferred by leptin and linoleic acid, an increased Th1 and cytotoxic commitment, and a higher leptin-mediated up-regulation of IFN-γ production by $CD4^+$ and $CD8^+$ circulating T cells.

Conflict of interest statement

No conflict of interest was declared.

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