

## CLINICAL STUDY

## Increased T-helper interferon- $\gamma$ -secreting cells in obese children

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### Abstract

**Objective:** Leptin, an adipocyte-secreted hormone, has emerged as a potential candidate for the link between obesity and the proinflammatory state. Specifically, leptin modulates T-helper (Th) cells toward a Th1 phenotype, with the secretion of proinflammatory cytokines. The aim of this study was to evaluate the Th1/Th2 balance in obese children and its relation with hormonal and metabolic features.

**Study design:** In 50 obese children and 20 control children, we measured the CD4-positive Th cells that secrete interferon (IFN)- $\gamma$  or interleukin (IL)-2 (taken as an index of Th1 cells), and IL-4 (taken as an index of Th2 cells) as well as serum glucose, insulin, insulin resistance (IR) index (as homeostasis model assessment model (HOMA)), lipid profile, aminotransferases, leptin and ghrelin. Obese children also underwent dual energy X-ray absorptiometry scan measurements, and liver ultrasound scanning.

**Results:** Geometric mean percentages of IL-2- and IL-4-CD4 secreting cells in obese children were not significantly different from those found in control children. However, the geometric mean percentage of CD4-positive T cells secreting IFN- $\gamma$  was significantly higher in the obese than in the control ( $P < 0.0001$ , *t*-test) group. Within the entire group of study children, the percentage of IFN- $\gamma$ -positive cells was positively associated with leptin ( $P = 0.002$ ), insulin ( $P < 0.00005$ ), and HOMA-IR values ( $P < 0.00005$ ). However, when these associations were restricted to the group of obese subjects, insulin and HOMA-IR values, but not leptin, retained statistical significance. Yet, in the obese group, the percentage of IFN- $\gamma$ -positive cells was associated with nonalcoholic steatohepatitis (NASH) ( $P = 0.001$ ), but not with body mass index-standard deviation score and total body fat mass.

**Conclusions:** In obese children, a shift to Th1-cytokine profile dominated by the production of IFN- $\gamma$  is related to insulin resistance as well as to NASH independently of anthropometric features and other metabolic characteristics. The prevalent Th1 pattern of secreted cytokines may be regarded as a mechanism contributing to inflammation in obesity.

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### Introduction

Obesity may be a low-grade, systemic, inflammatory disease. Obese children and adults have high circulating levels of both acute phase reactant proteins and inflammatory cytokines, which are closely associated with cardiovascular risk factors and cardiovascular and noncardiovascular causes of death (1). This may explain the increased risk of diabetes, heart disease and many other chronic diseases in the obese.

Among cells of the immune system, T cells play a major role in the inflammatory response. Key regulators of this response are a subset of T cells

called CD4-positive T helper (Th) cells, which can be further differentiated into two subtypes, Th1 and Th2 cells, by the different cytokines they produce (2). The appropriate regulation of Th cell immunity is critical in the control and prevention of diverse diseases. Leptin, an adipocyte-secreted hormone with structural similarity to cytokines, has emerged as a potential candidate for the link between obesity and the proinflammatory state. Studies in animal (3) and human (4) models of congenital complete leptin deficiency, which are characterized by morbid obesity and immune dysfunction, suggest a potentially important role for leptin in regulating immune function. More specifically,

leptin modulates Th cells toward a Th1 phenotype, with the secretion of proinflammatory cytokines, such as interferon- $\gamma$  (IFN- $\gamma$ ), and away from Th2 regulatory cytokine production (4). Ghrelin, the recently discovered orexigenic hormone of gastric origin, which exerts antagonistic effects to those of leptin on food intake at the hypothalamic level, also functions as a counterregulatory signal in the immune system, controlling leptin-induced expression of inflammatory mediators (5). To our knowledge, the Th1/Th2 balance and its relation to serum leptin and ghrelin concentrations has not yet been investigated in obese children.

In the present study, we measured the CD4-positive Th cells that secrete IFN- $\gamma$  or interleukin (IL)-2 (taken as an index of Th 1 cells), and IL-4 (taken as an index of Th2 cells) in peripheral blood mononuclear cells (PBMC) of a population of children with primary obesity as well as of healthy children. Additionally, we tested the possible roles of hormonal and metabolic factors in modulating the Th1/Th2 balance.

## Materials and methods

### Subjects

Over a 15-month period, obese children were enrolled at the Department of Pediatrics, La Sapienza University of Rome, if they met the following criteria:

1. body mass index (BMI) over 95th percentile for age and sex
2. no history of known chronic disease
3. no evidence of recent acute illness
4. no history of alcohol consumption or smoking (where appropriate)
5. no use of steatogenic medications or drugs deemed to affect immune competence
6. negative family and personal history of atopy.

At enrollment, their visit included a history, physical examination (including measurements of weight, standing height and BMI, and determination of the stage of puberty according to the criteria of Tanner (6, 7)), laboratory tests, measurements of body composition (including determinations of lean body mass and total fat mass), and liver ultrasound scanning.

The degree of obesity was quantified by Cole's least mean square method (8), which normalizes the skewed distribution of BMI and expresses BMI as a standard deviation score (SDS). Measurements of body composition were made with a total body scanner (Hologic QDR-4500W, Waltham, MA, USA, which uses fan-beam scanning) in array mode. This equipment uses a switched pulse stable dual energy X-ray (DEXA) operating at 100 and 140 kV. The data were analyzed by software Version 11.2. The liver ultrasonographic evaluation used an Aplio XV (Toshiba America Medical Systems, Tustin, CA, USA). The degree of hepatic steatosis (moderate or severe) was assessed by the fall of the

ultrasonic beam with depth and the different echogenicity between liver and kidney (9). We have not considered as steatosis the pattern of slight increase of the liver echogenicity and echo discrepancy between liver and kidney that other authors have classified as mild steatosis, because this figure may be equivocal in obesity (9). Combination of hepatic steatosis with a persistent (> 6 months) increase of aminotransferase levels 1.5 times above normal values indicated a diagnosis of presumptive nonalcoholic steatohepatitis (NASH) (10). In these patients, causes of chronic hepatitis, including hepatitis B, hepatitis C, alpha-1 antitrypsin deficiency, autoimmune hepatitis, Wilson's disease, drug toxicity and total parenteral nutrition, were excluded.

Over the same study period, healthy children with BMI appropriate for sex and age, attending checkups at the Department of Pediatrics, La Sapienza University of Rome, were recruited to the study if they had 1. no history of allergic and immune disorders, 2. no family history of atopy, 3. no history of alcohol consumption or smoking (where appropriate), 4. normal aminotransferase levels and 5. negative serology for hepatitis B and C.

The study was approved by the hospital ethics committee, and informed consent was obtained from subjects' parents prior to assessment.

Blood samples were collected from each subject after an overnight fast for the measurement of intracellular cytokines, and biochemical and hormonal analyses (including triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, glucose, aminotransferases, insulin, leptin and ghrelin). The homeostasis model assessment (HOMA) of insulin resistance (IR) was calculated as the product of the fasting serum insulin level ( $\mu\text{U}/\text{ml}$ ) and the fasting serum glucose level ( $\text{mmol}/\text{l}$ ), divided by 22.5 (11).

### Intracellular cytokine detection

PBMC were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. After isolation, one aliquot of PBMC was stained for the detection of cell-surface antigens, while another aliquot was transferred to culture flasks and stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 1  $\mu\text{M}$  ionomycin (Sigma), in the presence of 1  $\mu\text{M}$  monensin (Sigma) for 4 h at 37 °C in 5% CO<sub>2</sub> (12, 13).

**Antibodies** The following phycoerythrin (PE)-conjugated or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAb) were used: FITC-anti-CD3/PE-anti-CD4, FITC-anti CD8/PE-anti CD16, FITC-anti CD2/PE anti-CD19 (Immunotech, Marseille, France); FITC-anti-CD4 combined with either PE-antihuman IL-2, or PE-antihuman IFN- $\gamma$ , or PE-antihuman IL-4 (Sigma). Fluorochrome-conjugated, isotype-matched IgG1, IgG2a and IgG2b were used as controls to detect the nonspecific binding.

**Cell-surface staining** After incubation with surface-specific mAb for 30 min at 4°C, PBMC were washed, resuspended in PBS and immediately analyzed in a flow cytometer. A similar percentage of CD4 T-cell subset was obtained in unstimulated and stimulated cells.

**Intracellular cytokine staining** The stimulated cells were fixed for 15 min at room temperature with 2% paraformaldehyde in PBS (pH 7.4), and then washed once with cold PBS-A (PBS with 0.5% BSA and 0.1% sodium azide) and once with saponin buffer (PBS, 0.1% saponin, 0.1% sodium azide and 5% fetal calf serum). Fluorochrome-conjugated antibodies or the corresponding isotypes were added to the cells, and incubated at room temperature in the dark for 90 min, with occasional shaking. Cells were then washed two times with saponin buffer and resuspended in PBS.

**Fluorescence analysis** Analysis gates were set on lymphocytes according to forward- and side-scatter properties with an Epics Profile XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). This gate was used to collect the events stained with the isotypes or the different fluorochrome-conjugated mAb. The isotype controls were used to exclude the nonspecific staining of the cells.

### Hormone assays

Immunoreactive ghrelin levels were assessed by RIA (Phoenix Pharmaceuticals, Belmont, CA, USA; sensitivity, 10 pg/ml; inter- and intra-assay CV, 9.0–13.6% and 4.5–5.3% respectively) (14). RIA was also used to measure human leptin (DRG Diagnostica, Marburg, Germany; detection limit, 0.5 ng/ml; inter- and intra-assay CV, 3.0–6.2% and 3.4–8.3% respectively), and insulin (CIS Bio International, Schering S.A., Gif-Sur-Yvette Cedex, France).

### Statistical analysis

Statistical analyses were performed with the SPSS package. The data are expressed either as frequencies or as means with 95% confidence intervals (CI). The measured leptin, ghrelin, insulin, total cholesterol, HDL cholesterol, triglycerides, HOMA-IR values, and percentages of lymphocyte subset counts, and IL-2-, IL-4- and IFN- $\gamma$ -secreting cells were distributed with a long tail to the right (positive skew), but their logarithms were approximately normally distributed. Thus, mean values with 95% CI are reported as geometric means for leptin, ghrelin, insulin, total cholesterol, HDL cholesterol, triglycerides and HOMA-IR values, as well as for percentages of lymphocyte subset counts, and IL-2-, IL-4- and IFN- $\gamma$ -secreting cells. The differences between obese and control children in quantitative variables

were evaluated by *t*-test or Mann–Whitney U-test, as appropriate. Proportions were compared by the chi-square test. Multiple linear regression was used to evaluate associations with the logarithm of the percentage of IFN- $\gamma$ -secreting cells. For comparison of the mean percentage of IFN- $\gamma$ -secreting cells between the obese and control groups of children, the power was calculated after the results were obtained. With sample sizes  $n = 50$  and  $n = 20$  for the two study groups, and assuming the observed standard deviations, this study had a power of 94% to detect the observed difference statistically significantly at the 5% level.

## Results

Clinical and laboratory characteristics for the 50 obese children, as well as the 20 control children, are summarized in Table 1. Thirteen (26%) of the obese children had a diagnosis of NASH. Six (12%) and seven (14%) of the 13 children were found by sonography to have moderate and severe degree of fatty liver respectively. None of the 50 obese children had type 2 diabetes mellitus. Compared with controls, obese children had significantly higher geometric mean values of total cholesterol, triglycerides, leptin, insulin and HOMA-IR, and lower geometric mean values of HDL cholesterol and ghrelin.

Total T and B cells, Th cells, suppressor T cells and natural killer cells did not statistically differ between obese and control subjects (Table 2A). Yet, geometric mean percentages of IL-2- and IL-4-CD4-secreting cells in obese children were not significantly different from those found in control children ( $P = 0.19$  and  $0.15$  respectively) (Table 2B). However, the geometric mean percentage of CD4-positive T cells secreting IFN- $\gamma$  was significantly higher in the obese (13.6 (95% CI, 10.9–16.9)) than in the control (4.9 (95% CI, 2.9–8.3);  $P < 0.0001$ ) group (Table 2). Within the entire group of study children, the logarithm of the percentage of IFN- $\gamma$ -positive cells was positively associated with log leptin ( $P = 0.002$ ), log insulin ( $P < 0.0005$ ) and log HOMA-IR values ( $P < 0.0005$ ), but not with log ghrelin ( $P = 0.69$ ), log cholesterol ( $P = 0.74$ ), log HDL cholesterol ( $P = 0.10$ ) and log triglycerides ( $P = 0.80$ ). However, when these associations were restricted to the group of obese subjects, log insulin ( $P = 0.003$ ) and log HOMA-IR values ( $P = 0.002$ ), but not leptin ( $P = 0.69$ ), retained statistical significance. Yet, in the obese group, the percentage of IFN- $\gamma$ -positive cells was associated with NASH ( $P = 0.001$ ), but not with SDS-BMI ( $P = 0.80$ ), total body fat mass ( $P = 0.59$ ) and systolic blood pressure ( $P = 0.15$ ). The effect of NASH, as well as of insulin and HOMA-IR values, on the percentage of IFN- $\gamma$ -positive cells remained statistically significant after adjustment for gender, age, SDS-BMI and pubertal status (Tables 3 and 4). As

**Table 1** Anthropometric and metabolic characteristics of obese and control children.

Characteristic	Obese children	Control children	P-value* (n = 50) (n = 20)
Age (years)			
Mean (95% CI)	9.9 (9.0–10.9)	8.6 (7.8–10.4)	0.152
Sex – n (%)			
Female	16 (32)	9 (45)	
Male	34 (68)	11 (55)	0.31
Pubertal status – n (%)			
Prepubertal	20 (40)	7 (35)	
Pubertal	30 (60)	13 (65)	0.47
BMI – kg/m <sup>2</sup>			
Mean (95% CI)	25 (24–26)	16 (14–17)	<0.0001
SDS-BMI	2.25 (2.15–2.34)	0.13 (–0.27–0.31)	<0.0001
Systolic blood pressure – mmHg			
Mean (95% CI)	110 (105–115)	99 (90–105)	<0.01
Ghrelin – ng/ml			
Geometric mean (95% CI)	181 (156–210)	250 (183–340)	<0.05
Leptin – ng/ml			
Geometric mean (95% CI)	17.3 (15–20)	5.4 (3.8–7.5)	<0.0001
Total cholesterol – mg/dl			
Geometric mean (95% CI)	161 (146–164)	118 (114–121)	<0.001
HDL cholesterol – mg/dl			
Geometric mean (95% CI)	37 (35–39)	42 (40–44)	<0.05
Triglycerides – mg/dl			
Geometric mean (95% CI)	79 (70–96)	55 (51–60)	<0.05
Insulin – µU/ml			
Geometric mean (95% CI)	20 (17–24)	7.4 (6–9)	<0.0001
HOMA-IR values			
Geometric mean (95% CI)	4.2 (3.6–4.9)	1.6 (1.4–1.8)	<0.0001

\* Student's *t*-test or Mann–Whitney test for continuous variables and chi-square test for categorical variables. CI: confidence intervals; BMI: body mass index; SDS-BMI: standard deviation score-BMI; HDL: high-density lipoprotein; homeostasis model assessment (HOMA) of insulin resistance (IR).

NASH is the hepatic component of the metabolic syndrome, when the effects of insulin and HOMA-IR values were adjusted for the presence of NASH, the statistical significance of all three variables was reduced.

## Discussion

The main findings of this study are, first, that the systemic immune response in obese children deviates toward secretion of IFN- $\gamma$ , a proinflammatory Th1 cytokine, and, second, that increased expression of this cytokine is related to IR as well as to NASH independently of anthropometric features and other study metabolic characteristics.

Th1 cells are considered proatherogenic because IFN- $\gamma$  secreted by these cells has been demonstrated to have a variety of proatherogenic effects. IFN- $\gamma$  is an important immune-activating cytokine that can prime macrophages for activation and induce inflammatory responses such as those observed in delayed-type hypersensitivity and granulomatous lesions (15). Cell-culture studies have shown it to be a powerful growth inhibitor for endothelial cells and smooth muscle cells (SMCs) (16, 17). *In vivo* studies in rats have shown that the proliferative response of SMCs after arterial injury is inhibited by IFN- $\gamma$  (18), which also inhibits smooth muscle contractility and collagen synthesis (17, 19). It was therefore proposed that IFN- $\gamma$ -producing T cells could play an important role in plaque destabilization by redu-

cing the fibrous cap (20). Further support for a proatherogenic role came from studies of compound knockout mice lacking the IFN- $\gamma$  receptor (i.e. IFN- $\gamma$ R 0 mice) as well as the apoE gene (i.e. apoE 0 mice) (21).

**Table 2A** Lymphocyte subset counts in obese and control children.

	Obese children	Control children	P value*
T cells (CD3 <sup>+</sup> )	71 (69–73)	70 (66–74)	0.54
Helper T (CD4 <sup>+</sup> )	39 (36–42)	39 (36–43)	0.86
Suppressor T (CD8 <sup>+</sup> )	27 (25–29)	25 (22–28)	0.35
B cells (CD19 <sup>+</sup> )	10 (9–11)	11 (9–14)	0.24
Natural killer cells (CD56 <sup>+</sup> )	10 (8–11)	8 (5–10)	0.19

**Table 2B** Population of total CD4-positive T cells that secrete IL-2, IFN- $\gamma$  and IL-4.

	Cytokine-producing cells: geometric mean % (95% CI)		
	Obese children	Control children	P value*
IL-2	16.2 (13.0–19.5)	12.7 (9.8–16.4)	0.19
IFN- $\gamma$	13.6 (10.9–16.9)	4.9 (2.9–8.3)	<0.0001
IL-4	1.2 (0.5–1.4)	0.4 (0.2–1.0)	0.15

\* Student's *t*-test. IL: interleukin; IFN- $\gamma$ : interferon-gamma; CI: confidence intervals. Values are shown as geometric mean percentage (95 confidence interval).

**Table 3** Linear regression analysis of relationship between CD4-positive cells secreting IFN- $\gamma$  and clinical characteristics in obese children.

Characteristic	Regression coefficient	95% CI	P
Age	0.014	-0.05-0.08	0.67
Sex	-0.217	-0.69-0.25	0.36
Tanner stage	-0.065	-0.57-0.44	0.79
BMI	-0.001	-0.86-0.08	0.96
SDS-BMI	-0.045	-0.68-0.59	0.88
Systolic blood pressure	0.010*	-0.04-0.26	0.15
Fat mass	-0.009*	-0.05-0.10	0.65
NASH	0.744*	0.36-1.18	0.001

\* Values were adjusted for sex, age, SDS-BMI and Tanner stage. CI: confidence intervals; BMI: body mass index; SDS-BMI: standard deviation score-BMI; NASH: non-alcoholic steatohepatitis.

When compared with the apoE 0 mouse, an atherosclerosis-prone mouse strain, the apoE 0/IFN- $\gamma$ R 0 mouse exhibited a substantial reduction in atherosclerotic lesion size, a 60% reduction in lesion lipid accumulation, and a decrease in lesion cellularity, but a marked increase in lesion collagen content. All these data imply that IFN- $\gamma$  is a proatherogenic cytokine.

This is of interest because there is substantial evidence that obesity in childhood lays the metabolic groundwork for adult cardiovascular disease (22). A 55-year follow-up study showed that adults who were overweight in adolescence had an increased risk of morbidity and mortality from cardiovascular diseases, independently of adult weight (23). In the present study, the multiple cardiovascular risk factors we evaluated included great degree of body fatness, and high values for BMI, systolic blood pressure, serum triglycerides, cholesterol and insulinemia, each of which is a factor in the IR syndrome – a multifaceted syndrome that is associated with type 2 diabetes and atherosclerotic cardiovascular disease in the adult population. An independent effect of IR on cardiovascular risk has also been suggested in children (24, 25). Consistent with this possibility, our study shows that insulin resistance per se may be associated with increased IFN- $\gamma$  production in obese children and

**Table 4** Linear regression analysis of relationship between CD4-positive cells secreting IFN- $\gamma$  and laboratory variables in obese children.

Variable	Regression coefficient	95% CI	P
Total cholesterol	-0.74	-1.4-0.04	0.39
HDL cholesterol	0.65	-0.59-1.89	0.29
Triglycerides	0.166	-0.55-0.21	0.38
Insulin	0.64	0.26-1.03	0.001
HOMA-IR	0.69	0.31-1.07	0.001
Leptin	-0.099	-0.63-0.43	0.70
Ghrelin	0.31	-0.13-0.76	0.16

Values were adjusted for sex, age, SDS-BMI and Tanner stage. CI: confidence intervals; HDL: high-density lipoprotein; homeostasis model assessment (HOMA) of insulin resistance (IR).

may therefore be an early step in the development of atherosclerosis in this patient population.

The pathogenesis of liver disease associated with obesity is poorly understood. Several observations have suggested that obesity might predispose individuals to liver disease by increasing hepatic sensitivity to endotoxin (26). Hepatic T cells respond to bacterial lipopolysaccharide (LPS) by producing preferentially Th1-type cytokines, resulting in an excess of hepatotoxic Th1 cytokines, especially tumor necrosis factor (TNF) (27). Given that obese and lean male rodents experience similar induction of TNF but dramatically different degrees of liver injury after treatment with LPS, obesity is thought to promote liver injury by a mechanism that involves sensitization of hepatocytes to TNF toxicity (26). The molecular basis for obesity-related sensitization remains uncertain but might reflect, at least in part, relative overexpression of IFN- $\gamma$  after LPS exposure. IFN- $\gamma$  is known to augment TNF toxicity in isolated mouse hepatocytes (28) and is increased in many experimental models of TNF-related liver injury (29). Our observation that cellular production of IFN- $\gamma$  is increased in obese children with liver injury is provocative because it suggests that early in life intrinsic obesity-related alterations of immune function might provide an important mechanism for obesity-related liver damage.

In our obese children, circulating levels of leptin were high but did not correlate with increased percentage of IFN- $\gamma$ -positive cells. Thus, in contrast to previous observational studies that were not free from potential bias due to uncontrolled confounding (30–32), our data do not provide evidence for a proinflammatory role for leptin. It may be argued that true associations between leptin (or ghrelin) and the inflammatory status associated with obesity may have been missed because of the cross-sectional study design and/or the moderate sample sizes. However, it may be of interest that recent interventional studies in adult men have not supported an etiopathogenic role for leptin in proinflammatory states associated with leptin excess such as obesity (33, 34). In particular, in three interventional studies involving administration of recombinant methionyl human leptin to lean subjects, otherwise healthy obese subjects, and obese diabetic subjects, increasing circulating leptin levels over a wide spectrum of values (from low physiologic to high pharmacologic) did not alter serum levels of inflammatory markers and other cytokines important in the Th cell response (34).

Several recent studies suggest a relation between inflammatory markers and the development of obesity-related disorders such as cardiovascular diseases and type 2 diabetes mellitus (1, 35, 36). The present study demonstrates that a shift to Th1-cytokine profile dominated by the production of IFN- $\gamma$ , a proatherogenic, proinflammatory cytokine, is already present in youngsters with obesity. The prevalent Th1 pattern of secreted cytokines may be regarded as an

early step contributing to inflammation with subsequent increased risk of cardiovascular disease in such patient populations.

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