ORIGINAL ARTICLE

Elevated levels of Th17 cells in children with central obesity

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Abstract

Background. It is believed that the recently discovered interleukin 17-producing Th17 cells play a role in the pathogenesis of chronic inflammation in the course of obesity and diabetes. Objectives. The purpose of our study was to complete data on this subject in children. Methods. We assessed Th17 cell levels in the peripheral blood of children diagnosed with central obesity (n = 14) and compared the results with data obtained in patients with newly diagnosed (n = 11) and long-term type 1 diabetes mellitus (n = 18), and in a control group as well (n = 24). Results. (i) Children with central obesity were characterized by higher percentages of Th17 cells as compared to children from the control group; (ii) in the peripheral blood of patients with long-term type 1 diabetes the Th17 cell counts were higher compared to the control group; (iii) total plasma cholesterol concentration correlated positively with Th17/Treg cells ratio; and (iv) among patients with long-term diabetes, disease duration correlated positively with Th17 cell count and Th17/Th1 cell ratio. Conclusion. The results of our study indicate that Th17 cells may be involved in chronic inflammation accompanying obesity and type 1 diabetes mellitus in children.

Key Words: Diabetes immunity, inflammation, Th1 cells, regulatory T cells

Introduction

Several decades of unlimited access to food in developed countries has contributed to the global epidemic of obesity, also in children [1]. The fat cells accumulated around the waist (central obesity) are a source of cytokines leading to chronic inflammation and its long-term consequences including atherosclerosis and cardiovascular complications [2]. The obesity-related immunological disturbances may involve both innate and acquired immune response, including specific action of T cells in the case of the latter one. Another condition accompanied by immunological disorders is type 1 diabetes mellitus (T1DM) known as the most common autoimmune disease of the developmental age [3]. The pathogenesis of T1DM involves the induction of cellular and humoral immune responses leading to destruction of pancreatic islet beta cells, and consequently lack of insulin and symptoms of hyperglycaemia. Previous studies have highlighted the key role of proinflammatory cells with Th1 profile predominance and deficiency of regulatory T cells (Tregs) in this process [4]. Chronic hyperglycaemia in diabetes leads to the occurrence of long-term vascular complications of inflammatory nature, being the cause of increased morbidity and mortality in this group of patients.

There is a number of evidence indicating participation of the newly discovered interleukin 17-producing Th17 cells in the pathogenesis of many inflammatory diseases including autoimmune conditions such as multiple sclerosis, psoriasis, arthritis, bowel diseases and lupus [5]. However, strictly proinflammatory function of those cells was recently questioned [6]. Perhaps depending on the environment, Th17 cells can have either pathogenic or protective role in such diseases as obesity, atherosclerosis or diabetes. In addition, interleukin 17 may be involved in inflammation associated with long-term complications of diabetes [7]. If we assume that obesity is also accompanied by chronic inflammation, the role of Th17 cells should predominate over other cell populations, including regulatory T cells [8].
The existing data do not conclusively support the involvement of Th17 cells in immune disorders that accompany obesity and diabetes, moreover, there are no such reports in children, where both these pathologies are increasingly common. The aim of our study was to evaluate the significance of Th17 cells in children diagnosed with central obesity and to compare the results with data obtained in children with newly diagnosed and long-term type 1 diabetes mellitus.

**Patients and methods**

Children with central obesity (n = 14), newly diagnosed type 1 diabetes mellitus (n = 11), long-lasting disease (n = 18) and a control group (n = 24) were enrolled in the study (for full version, see Supplementary material, available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2015.1066845). Central obesity was diagnosed on the basis of waist circumference (values above the 90th percentile [9]). Type 1 diabetes mellitus was diagnosed on the basis of the International Society for Pediatric and Adolescence Diabetes (ISPAD) criteria. The control group included children in whom we excluded diabetes mellitus (negative family history of type 1 diabetes mellitus, no signs or symptoms, normal fasting plasma glucose levels) or chronic disease with inflammatory background. The following parameters were assessed for each child: Anthropometric and questionnaire data (age, sex, height, weight, body mass index – BMI and standardized body mass index – SDS-BMI, waist circumference with its standardized value [10]), blood pressure, and additional tests described further. The following laboratory parameters were determined in all patients: Lipid profile, thyroid stimulating hormone (TSH) and albuminuria. None of the children (in all subgroups) had anti-thyroid (ATG, TPO), anti-tissue transglutaminase or antiendomysium antibodies. Carbohydrate metabolism was also assessed (glycated haemoglobin, oral glucose tolerance test with insulin concentration test) in patients with obesity. In addition, the following disease-associated parameters were obtained in group of children with diabetes: Duration of diabetes, treatment type, mean daily insulin dose, and glycated haemoglobin levels (mean for the previous year of treatment). Arterial hypertension was diagnosed when systolic or diastolic blood pressure values exceeded the 95th percentile, with regard to age and sex [11]. The project was approved by the Ethics Committee at the Medical University of Białystok. The parents/guardians consent was obtained for participation of each child in the study.

**Flow cytometry**

Blood for cytometric tests was collected at the time of performing routine laboratory tests, including in days 7–14 from the diagnosis of diabetes in the group of children with newly diagnosed disease, and on the first or second day of hospitalization in the other groups of children. Peripheral blood mononuclear cells (PBMCs) collected after density-gradient centrifugation were incubated for 6 h (37°C, 5% CO₂) in the presence of PMA, ionomycin and brefeldin A (Leukocyte Activation Coailt with GolgiPlug; BD Bioscience). PBMCs collected after cell culture were stained with the following monoclonal antibodies: anti-CD4 FITC (clone RPA-T4), anti-CD25 PE-Cy5 (clone M-A251), anti-CD127 Alexa647 (HIL-7R-M21), anti-CD161 APC (clone DX12), anti-CD196 PerCP-Cy5.5 (clone 11A9). For additional intracellular staining, cells were permeabilised and incubated with monoclonal antibodies directed at IFN-gamma (clone 25723.11) and IL-17 (clone SCPL1362) or Foxp3 (clone 259D/C7). Moreover, appropriate unstained andFMO (fluorescence-minus-one) and fully stained unstimulated (in reference to in vitro cytokine release experiment) controls were prepared according to the procedures described above in order to separate the positive signals from the background and to establish cytokine production in response to stimulation. Detailed compensation strategy is presented in Figure 1. The following cell populations were noted: CD4+ IL-17+, CD4+ CD161+ CD196+ IL-17+ (Th17), CD4+ IFN-γ (Th1), CD4+ FoxP3+ (Tregs), CD4+ CD161+ CD196+ IFN-γ+ (Th17 secreting IFN-γ). Flow cytometric data were acquired on FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with the use of CellQuest software and FlowJo software.

The one-way analysis of variance (ANOVA) was used to determine whether there were any significant differences between the medians of groups of patients. Correlation of the obtained data was performed using the Spearman’s test. P value of < 0.05 was considered statistically significant. The results are presented as medians (with quartiles) or means (± standard deviation).

**Results**

**Clinical data**

Data concerning age, sex and anthropometric parameters are presented in Table I. Children in subgroups did not differ in terms of age (mean age: 13.3 ± 3.4 years) and gender (boys: 36, i.e. 53.7%). In the group with long-term type 1 diabetes mellitus, mean disease duration was 5.0 (± 2.1) years.

Children with central obesity had higher values of standardized waist circumference and standardized body mass index (see Table I) compared to children in the control group, children with newly diagnosed type 1 diabetes mellitus, and those with long-term disease.
Figure 1. Compensation strategy applied in the current study. (A) CD4 positivity (delineated by solid line filled with gray; upper panel, left was established with the use of unstained controls (unfilled, dashed line). Commonly observed downregulation of CD4 associated with stimulation (lower panel) did not affect accurate visualization of CD4 + population. (B) Expression of CD161 APC and CD196 PerCP-Cy5.5 (dot plots in lower panel) was determined on the basis of FMO controls including CD4 FITC alone (corresponding dot plots in the upper panel). (C) For evaluation of truly positive signals detecting IFN-gamma and IL-17 intracellular release following 24-hour *in vitro* stimulation (lower dot plots), unstimulated but fully stained with CD4 FITC/CD161 APC/CD196 PerCP-Cy5.5 and IFN-gamma PE or IL-17 PE) sample was used (upper dot plots). (D) Detailed graphical demonstration of the compensation strategy. Compensation of each fluorochrome pairs was accomplished by the use of proper FMO controls prepared from both patients’ and healthy volunteers’ PBMC samples.

Table I. Age, gender and anthropometric data in the examined children.

|                      | Control       | Obesity       | Newly recognized diabetes | Long lasting diabetes |
|----------------------|---------------|---------------|----------------------------|-----------------------|
| Age (mean, SD)       | 12.8 ± 2.9    | 13.0 ± 3.5    | 12.4 ± 4.2                 | 13.7 ± 2.1            |
| Gender M/F No. %     | 13/11 54.1%/45.8% | 7/7 50%/50%   | 6/5 54.5%/45.4%            | 10/8 55.5%/44.4%     |
| Waist circumference SDS | 0.38 ± 0.9    | 2.1 ± 1.1     | -0.3 ± 1.2                 | 0.5 ± 0.7             |
| Body Mass Index-SDS  | 0.5 ± 0.4     | 1.6 ± 2.0     | -0.2 ± 0.4                 | 0.4 ± 0.5             |

*p*≤0.06 are bolded.
The data from laboratory tests including lipid and carbohydrate profile, and hormonal tests, are presented in the Supplementary material (Table I, available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2015.1066845). Children with central obesity had higher values of triglycerides and albuminuria. Glycated haemoglobin values were significantly higher in children with newly diagnosed and long-term type 1 diabetes mellitus. Arterial hypertension was seen in patients with central obesity (7/14 cases, i.e. 50%), as well as in children with long lasting diabetes (4/18, i.e. 22.2%).

Patients with newly diagnosed diabetes were treated only with pens (human insulins), whereas those with long-term disease in 10 cases (55%) remained on functional therapy using personal insulin pump; the others continued using human insulins in pens. The dose of insulin in the group of children with newly diagnosed type 1 diabetes mellitus on the day of study was 0.90.2 U/kg body weight/day, whereas in the group of children with long-term disease: 0.80.4 U/kg/day.

Flow cytometric data

Representative example of flow cytometry data derived from in vitro experiments is shown in Figure 2, and a summary of the results is presented in Table II and Figure 3. We found that after stimulation with PMA/ionomycin, CD4+ T cells with Th17 phenotype (namely CD4+CD161+CD196+) derived from children with central obesity secreted significantly larger levels of not only interleukin 17 but also interferon-gamma as compared to control group (Figure 2). Similarly, after stimulation, the levels of CD4+CD161+CD196+IL-17+ cells as well as CD4+CD161+CD196+ cells producing IFN-γ were significantly higher in long-term diabetes compared to the control group. There were no statistically significant differences in the remaining T-cell populations assessed in each group of patients.

Correlations

We looked for the relations between pro- and anti-inflammatory cells populations and anthropometric/laboratory findings in obese and diabetic children. In this paragraph we describe statistically significant correlations between the clinical parameters and data obtained from flow cytometry.

In children with central obesity, the frequencies of interferon-gamma-producing Th17 cells (CD4+CD196+CD161+IFN-γ+) after stimulation correlated positively with insulin levels 2 h after oral glucose tolerance test (p = 0.03, r = 0.73). Total cholesterol levels were positively correlated with Th17/Treg cell ratio (p = 0.03, r = 0.71).

Among children with newly diagnosed type 1 diabetes mellitus, there was a negative correlation between standardized body mass index and Th17/Th1 cell ratio (p = 0.02, r = −0.76). LDL cholesterol concentrations correlated positively with Th17/Tregs cell ratio (p = 0.008, r = 0.83).

Among patients with long-lasting type 1 diabetes, the disease duration was positively correlated with CD4+IL-17+ cell count and with the Th17/Th1 cell ratio (after adjustment for the subject’s age): p = 0.005, r = 0.79. Metabolic control (HbA1c) in this group of patients was negatively correlated with CD4+CD161+CD196+IL-17+/CD4+CD161+CD196+IFN-γ+ ratio (p = 0.04, r = −0.68).

Figure 2. Gating strategy used for delineation of CD4+ T cell subpopulations. CD4+ T cells were gated on the basis of FSC/SSC properties and positive expression of CD4. The expression of IFN-gamma and IL-17 was evaluated within population of CD4+ T cells, and within CD4+CD161+CD196+T cells.
Figure 3. Summary analysis of frequencies of IFN-gamma- and IL-17- secreting CD4+ T cells (upper panel) and CD4 + CD196 + CD161 + T cells (lower panel). Higher counts of CD4 + CD196 + IL-17 + cells were seen in children with central obesity and those with long-term diabetes mellitus compared to the children in the control group.
Discussion

In our experiment, we demonstrated higher percentages of Th17 cells in the peripheral blood of children diagnosed with central obesity. In addition, levels of IFN-γ-producing cells with CD4+CD161+CD196+ phenotype were higher in this group of patients. Moreover, counts of CD4+CD161+CD196+ cells, including those with IFN-γ expression, were likewise higher in patients with long-term type 1 diabetes. Our data appear to confirm the important role of IL-17 in immune disorders accompanying chronic inflammation in the course of obesity and diabetes. There is no data available on Th17 cells in children with central obesity, however, some authors consider IL-17 as the missing link between inflammation, autoimmune reaction, and obesity [12]. In adults, high Th17 cell counts have been reported in the peripheral blood of patients with simple obesity (without metabolic disorders), and in type 2 diabetes mellitus patients [13]. However, the reported changes were not accompanied by abnormal IL-17 serum levels. In that experiment, Th17 cells were defined as lymphocytes with CD4+IL-17+ phenotype. In our study, those cells were described more specifically as CD4+CD161+CD196+IL-17+ cells. In another study, elevated levels of IL-17 in the sera of obese female patients did not correlate with body mass index, waist circumference, HOMA index or leptin levels [14].

The mechanism initiating the Th17-type immune response in patients with persistent obesity-associated inflammation is unknown. Dendritic cells with CD11c+CD1c+ phenotype, present in the adipose tissue, may play an important role in this process [15]. There is compelling evidence that the adipose tissue collected from obese insulin-resistant subjects shows high Th17 cell counts compared to the tissue of obese subjects without signs of insulin resistance and neither to nonoverweight subjects [8]. That niche could be the source of IL-17 leading to immune disorders in obesity, and participate in the pathogenesis of insulin resistance.

Different results were reported by Goswami et al., who found that IL-17 inhibits growth of the adipose tissue [16]. In that experiment, excessive weight gain was seen in mice lacking the receptor for IL-17A. Similarly, a protective role of IL-17 in the development of atherosclerosis and atherosclerotic plaque stabilisation was suggested by Taleb et al. [17].

The Th17 subpopulation may also be involved in the pathogenesis of late complications of diabetes. The counts of Th1 and Th17 cells, as well as IL-17 and IFN-γ levels, correlated with albumin/creatinine ratio – a marker of nephropathy in patients with type 2 diabetes [18]. Tregs/Th17 ratio in favour of the Th17 cells was seen in long-term type 1 diabetes in children [19]. The authors of this report suggest that there is a connection between this abnormality and progression of microvascular complications. The mechanism through which chronic hyperglycemia leads to Th17 cell activation is unknown. An influence of Advanced Glycation End-products (AGEs) on the activation of proinflammatory Th1 and Th17 cells and suppression of regulatory T cells was shown recently [20]. It is questionable whether diabetes/hyperglycaemia or obesity are the main cause of immune disorders in type 2 diabetes. According to work of Martinez et al., after stratification of patients with type 2 diabetes by obesity, they found that only obese patients had increased Th17 cell counts [21].

Perhaps the prevalence of Th17 we have shown would be even higher if assessed in patients with type 1 diabetes and concomitant obesity. Our patients with diabetes had higher body mass index than children with newly diagnosed type 1 diabetes; however, at the time of diagnosis there is a weight loss associated with glycosuria and burning of fat associated with energy loss. Nevertheless, the course of autoimmune disease in obese mice model is faster and more dramatic [12].

Obesity-associated inflammation, including Th17 dominance, can be modified by diet, e.g. rich in polyunsaturated N-3 fatty acids (PUFA) [22]. Among the available drugs, DPP-4 inhibitors have a suppressive effect on the Th1/Th17 axis, and can be used either in type 1 or type 2 diabetes mellitus [23].

Conclusions

The results of our study indicate that both obese children and those with long-term type 1 diabetes mellitus have elevated Th17 cells counts in the peripheral blood. That fact might suggest a key role of Th17 in chronic inflammation in the course of diabetes, but moreover, indicate a connection between phenomena present in diabetes and obesity. However, further studies on the mechanism of inflammation accompanying obesity and diabetes are required to allow for establishment of therapeutic attempts.

Authors’ contributions

WL, KG and MM made substantial contribution to conception and design of project, also analyzed and interpreted the data, BGO and AB participated in data collection and drafting/revising the manuscript. All authors were involved in writing the paper and gave final approval of submitted manuscript.

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Th17 cells in children with obesity

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary data and Table I available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2015.1066845.