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ORIGINAL ARTICLE

# Generation of Functional T-Regulatory Cells in Children with Metabolic Syndrome

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**Abstract** Recent research implies a role of decreased number and/or function of T-regulatory cells (Tregs) in low-grade inflammation associated with obesity and atherosclerosis. The enhancement of atheroprotective immunity by the expansion of Tregs could serve as a therapeutic strategy in obesity-related immunological disturbances. The aim of our study was an attempt to generate Treg cells in children with risk factors for the development of cardiovascular disease and to compare the results to those obtained in healthy subjects. The study group consisted of 30 children with metabolic syndrome (MS) and 30 controls. Conventional $CD4^{+}CD25^{-}$ cells separated from the peripheral blood were converted into Treg cells with the use of CD3/CD28 antibodies and interleukin (IL)-2/transforming growth factor (TGF)-$\beta$ stimulation. The expression of critical Treg molecules and cytokines was assessed at mRNA and protein levels. The percentages of Treg cells in the peripheral blood were significantly lower in the children with MS compared to the healthy subjects. After the culture with CD3/CD28 and IL-2/TGF-$\beta$ we detected a significant increase in the expression of Tregs marker transcription factor FoxP3. The Tregs induced from the children with MS varied from the ones obtained in the controls in the expression of some molecules at mRNA level (e.g. IL-27, LGAL, KLF10 and NRP1) yet not in proliferation studies. For the first time, we have demonstrated the possibility of generating functional Treg cells in children with MS. The results of our study could be used in the design of therapeutic interventions in obesity associated immunologic disturbances.

**Keywords** Children · Immunology · Metabolic syndrome · Obesity

**Abbreviations**

| | |
|---|---|
| FoxP3 | Forkhead winged/helix transcription factor 3 |
| IFN | Interferon |
| IL | Interleukin |
| iTreg | Induced T-regulatory cell |
| MS | Metabolic syndrome |
| RT-PCR | Real-time polymerase chain reaction |
| TGF | Transforming growth factor |
| Tregs | T-regulatory cells |

## Introduction

Overweight and obesity, defined as an excessive development of adipose tissue, are one of the crucial current public health concerns. The prevalence of obesity in children has increased in recent years, probably due to the interactions between dietary intake, physical activity, environment and

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genetic susceptibility (Daniels et al. 2005). It is associated with a number of comorbidities including type 2 diabetes mellitus, metabolic syndrome (MS), orthopedic, psychological, hepatic and pulmonary conditions. MS is defined as a cluster of cardiovascular risk factors including abdominal obesity, dyslipidemia, hypertension and hyperglycemia (Zimmet et al. 2007). The incidence of MS in children is also increasing, ranging from 1 % in general population up to 40 % in obese children (Tailor et al. 2010). The presence of MS in childhood is associated with accelerated atherogenesis in adulthood (Magnussen et al. 2010). Current approaches to the prevention and treatment of obesity in children including dietary management, physical activity, pharmacological and surgical treatment are not fully effective (Connelly et al. 2007). Therefore, the support for the research on the development of new treatment interventions in this condition is necessary.

Obesity promotes insulin resistance by low-grade inflammation through the activation of adipocytes, macrophages and type 1 T-helper (Th1) cells (Gustafson et al. 2007). Atherosclerosis is regarded as chronic inflammatory disease where immune cells contribute to both disease initiation and progression (Kleemann et al. 2008). The link between obesity-induced inflammation and its complications including atherosclerosis and cardiovascular diseases has been confirmed in numerous studies (review in Lumeng and Saltiel 2011). On the other hand, besides classical Th1/Th2 paradigm of T cell subsets, new subpopulations involved in the immune response have recently been discovered (e.g. T-regulatory cells (Tregs) and $Th_{17}$ cells). Treg cells represent a small subset of lymphocytes which play a role in the maintenance of immunological tolerance and prevent the autoimmune response by suppressing the activity of effector T cells (Shevach 2009). Some recent research suggests a potential role of Treg cells in low-grade inflammation associated with obesity and the formation and progression of atherosclerosis (Feuerer et al. 2009). Since Tregs are able to suppress immune responses, their diminished number and/or function could be responsible for the ongoing inflammation that characterizes MS and atherosclerosis (Mor et al. 2007). Enhancement of atheroprotective immunity by the expansion of regulatory T cells could constitute a future therapeutic strategy (van Es et al. 2010). Besides some encouraging preliminary results, there are some questions to be answered in this field. These controversies include the actual deficiency in the number/function of Tregs in patients with obesity, the simple protocol for generation of Tregs for the future therapy and, a little volume of blood available for the research and clinical studies in pediatric patients.

With these notions in view, we attempted to generate Treg cells from the small amounts of lymphocytes separated from the peripheral blood of children fulfilling the criteria of MS. We also tested the expression of classical and novel markers and functional properties of induced Treg cells and compared the obtained results between obese and healthy children. The experiment could be helpful for designing future interventions conducted to redress the balance within obesity-related immunological disturbances.

## Materials and Methods

### Patients

The study group consisted of 30 children with MS (14 boys, 16 girls; median age 16 years). Thirty non-obese, healthy individuals (control group) were enrolled into the study (15 boys, 15 girls; median age 16.5 years). Children from the control group had no signs of autoimmune, chronic, inflammatory and neoplastic disease. Their weight, height, waist circumferences were measured, and standardized body mass index (SDS-BMI) was calculated. The values obtained from the clinical examination were compared with the international reference data (including percentile curves; Cole et al. 2000). Blood pressure was measured three times using a mercury sphygmomanometer with an appropriate cuff size according to the American Heart Association guidelines. MS was diagnosed in compliance with the International Diabetes Federation 2007 criteria (abdominal obesity and two of the following: hypertension, dyslipidemia, hyperglycemia/diabetes; Zimmet et al. 2007). The children from both the study and control groups did not receive any treatment. Blood samples from the patients and controls were obtained under the protocols approved by the Medical University of Bialystok Institutional Review Board. An informed consent was given by the participating children and their parents.

### Laboratory Investigations

Blood samples were obtained from the patients while they were fasting for the measurement of the levels of glucose, insulin, lipids, a thyroid stimulating hormone (TSH) and a cortisol profile. An oral glucose-tolerance test was then performed with the administration of 1.75 g of glucose per kilogram of body weight (maximal dose 75 g).

### Flow Cytometry, Antibodies and FoxP3 Staining

Mononuclear cells were isolated from 9 ml of peripheral blood by centrifugation over Histopaque (Sigma-Aldrich, St. Louis, MO, USA). A flow cytometric analysis of T cell

subpopulations was performed, as previously described (Łuczyński et al. 2010), with the use of the following markers (and respective isotype control antibodies): anti-CD4, anti-CD25, anti-CD127 and FoxP3 (used as a marker of Treg cells) purchased from Beckman Coulter (Brea, CA, USA), Beckton Dickinson (Franklin Lakes, New Jersey, USA) and eBioscience (San Diego, CA, USA). The samples were analyzed with 5-color flow cytometer Beckman Cytomics FC 500 MPL using CXP software ver 2.0 (Beckman Coulter, Brea, CA, USA). A minimum of $10^5$ events were acquired for each analysis. The percentages of positive cells were calculated. The following subpopulations were identified: $CD4^+$, $CD4^+CD25^{high}$, $CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{high}CD127^{low/-}FoxP3^+$.

## Separation of $CD4^+CD25^-$ Cells

$CD4^+CD25^-$ T cells were isolated from the mononuclear cells according to the producer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, the isolation of the cells was performed in a two-step procedure. First, non-$CD4^+$ cells were indirectly magnetically labeled and subsequently depleted by separation over a MACS® LD Column. Secondly the cells were directly labeled with CD25 MicroBeads and $CD4^+CD25^-$ cells were isolated by negative selection. The purity of the isolation was assessed with flow cytometry (anti-CD4 and anti-CD25 antibodies). Only the samples with more than 90 % of $CD4^+$ $CD25^-$ cells were used in further experiments.

## Tregs Generation

Treg cells were generated de novo according to the following procedure: $5 \times 10^5$ $CD4^+CD25^-$ cells/well were cultured for 4 weeks in "medium" (i.e. RPMI 1640 and 10 % FBS) at 37 °C and 5 % $CO_2$, with (or without = control culture) Treg-expander (anti-CD3/anti-CD28, Dynal/Invitrogen, Carlsbad, CA, USA) at the concentration of 4 beads/cell, transforming growth factor (TGF)-$\beta$ (5 ng/ml; Invitrogen, Carlsbad, CA, USA) and recombinant human interleukin (IL)-2 (Sigma-Aldrich, St. Louis, MO, USA)—300 U/ml for the first week and 100 U/ml for the next 3 weeks. The percentage of $CD4^+CD25^{high}CD127^{low/-}FoxP3^+$ cells was noted before and after the culture. The idea of the experiment is presented in Fig. 1.

## RNA Extraction and cDNA Synthesis

Total RNA from the cells before (from mononuclear cells and $CD4^+CD25^-$ cells) and after the culture was isolated and purified with the use of RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using SuperScript™ First-Strand Synthesis System for real-time polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

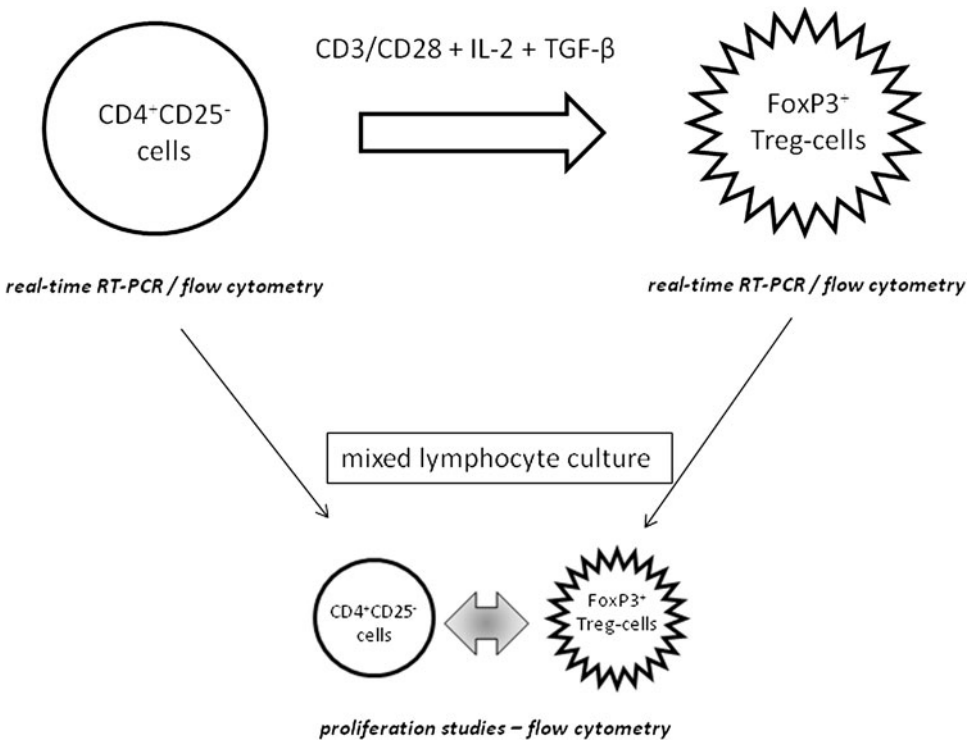


**Fig. 1** The idea of the experiment is that the conventional $CD4^+CD25^-$ cells were converted into Treg $FoxP3^+$ cells with the use of CD3/CD28 antibodies and IL-2/TGF-$\beta$ stimulation. The expression of critical Treg molecules and cytokines was assessed at mRNA and protein levels before and after the culture. The suppressor function of induced Tregs was assessed in mixed lymphocyte culture

### Real-Time PCR

The levels of transcripts were measured by RT-PCR using human genes QuantiTect Assays and QuantiTect Hs_GAPDH Assay (Qiagen, Valencia, CA, USA) as a normaliser. The following genes were assessed: 1) cytokines: IL-10, IL-23, IL-27, EBI3 (IL-35), TGF-$\beta$ and interferon (IFN)-$\gamma$; 2) critical Treg molecules: OX40, 4-1BB, ICOS, GITR, CTLA-4, CD39, CD73, galectin-1, LAG-3, neuropilin-1, GARP and granzyme B; 3) transcription factors and the other molecules: FoxP3, STAT1, STAT3, SOCS2, SOCS3, SMAD3, T-box 21, ITCH and TIEG-1. RT-PCR was performed in duplicate in 20 µl, using the QuantiTect SYBR Green PCR Master Mix (Qiagen) applying the manufacturer's instructions and conducted in the Chromo4 Real-time PCR Detector (BIO-RAD, USA). A standard curve construction was generated by a series of four dilutions of cDNA of the control group sample in reaction to the house-keeping gene, GAPDH. On the basis of these curves, the levels of total chosen gene transcripts were calculated after their normalization to GAPDH. The value of threshold cycle (CT) was determined by the first cycle number at which the fluorescence was higher than the set threshold value. To calculate our data, we used a comparative CT method for relative quantification i.e. $2^{-\Delta\Delta CT}$ method, following Livak and Schmittgen (2001). The relative values (a comparison between the control and examined samples or before and after the culture) below 0.75 were regarded as a decreased expression or downregulation, between 0.75 and 1 as a comparable expression and above one as an increased expression or upregulation (Glisic et al. 2010).

### Proliferation Assay/Mixed Lymphocyte Reaction

4 weeks after the onset of the experiment, blood samples (5 ml) from the patients and controls were taken again for proliferation assays used as a measure of a suppressive function of de novo generated Treg cells (induced Treg cells: iTregs). $CD4^+CD25^-$ responder cells were again separated and cultured at $3 \times 10^5$/well in the following conditions: with medium alone (=negative control), with concavalin A (ConA = positive control; 5 µg/ml) and with (or without) de novo generated Tregs (at a ratio 1/8). The experiments were performed in triplicate. After a 5-day culture, bromodeoxyuridine (BrdU; Beckton Dickinson, Franklin Lakes, New Jersey, USA) was added (10 µL/1 mL of cells) and after the activation intracellular staining with anti-BrdU (FITC) was performed. The percentage of $CD4^+CD25^-BrdU^+$ cells in flow cytometry as the indicator of T cell proliferation was recorded. The data were compared with respect to negative control, positive control (ConA) and mixed lymphocyte culture with ConA and de novo generated Tregs from the obese and healthy children.

### Statistical Analysis

The results were analyzed in Statistica 9.0 for Windows (StatSoft, Poland). Owing to asymmetric data distribution, non-parametric tests were used. The results are expressed by medians. Significance levels were calculated in accordance with non-parametric Wilcoxon test (differences between before and after the culture) and Mann–Whitney $U$ test (differences between the control and examined group). The correlations between the clinical parameters and flow cytometry/RT-PCR results were assessed with Spearman's Rank Correlation Test. The level of $p < 0.05$ was regarded as significant. The graphs were prepared in GraphPad Prism 5.0 and Excel for Windows 2007.

## Results

To design the future immunotherapeutic intervention in patients with obesity, we studied the possibility of generating functional Treg cells from the conventional lymphocytes separated from the peripheral blood of children fulfilling the criteria of MS. Subsequently, we compared the results with those obtained in the healthy subjects.

### Results from Physical Examination and Laboratory Investigations

We did not observe any differences in sex, age and height between the examined and control group ($p > 0.05$). The children with MS were characterized by significantly higher weight, standardized body mass index and waist circumference (medians: weight 74.0 vs. 49.2 kg, SDS-BMI 1.8 vs. 0.7, waist 92.3 vs. 71.5 cm; $p = 0.001$). With regard to blood pressure, higher systolic and diastolic values in the group of the children with MS compared to healthy subjects were observed (median values: systolic 135 vs. 115, diastolic 85 vs. 66 mmHg; $p < 0.001$). These statistical differences were recorded after the adjustment in percentile curves appropriate for age, sex and height. The laboratory investigations displayed the normal values of TSH level and a cortisol profile in both the control and examined groups ($p > 0.05$; data not shown). As expected, we observed higher glycemia and insulinemia before (fasting glycaemia: 4.9 vs. 4.2 mmol/l; $p = 0.01$; fasting insulinemia: 81.6 vs. 57.0 pmol/l; $p = 0.01$, respectively) and after (120 min: 6.2 vs. 5.6 mmol/l; 196.6 vs. 83.4 pmol/l; $p < 0.001$, respectively) an oral glucose-tolerance test in the children with MS as compared to control subjects. Total, LDL-cholesterol and triglycerides serum concentrations were also higher in children with MS and HDL-cholesterol concentration was lower in this group

(medians: total 4.26 vs. 4.01, LDL 1.3 vs. 0.9, triglycerides 2.35 vs. 2.0, HDL 1.20 vs. 1.25 mmol/l; $p < 0.01$).

### Treg Cells in Peripheral Blood of Control and Obese Children

The number and percentage of lymphocytes and $CD4^+$ cells were similar in both the examined and control children (lymphocytes: 2.1 vs. 2.4 G/l, 31.9 vs. 32.3 %, $CD4^+$ cells: 28.9 vs. 30.0 %; $p > 0.05$). The percentages of $CD4^+CD25^{high}$, $CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{high}CD127^{low}FoxP3^+$ cells were significantly lower in the children with MS compared to the healthy subjects (medians: 2.7 vs. 1.6 %, 1.2 vs. 0.8 %, 0.95 vs. 0.7 %, respectively; $p < 0.05$). Regarding mRNA levels for FoxP3, the expression in the mononuclear cells before $CD4^+CD25^-$ cell separation was lower in the examined children compared to the control ones (relative expression 0.75; see Fig. 2).

### Conventional T Lymphocytes Can Be Converted into FoxP3 Positive Treg Cells

The $CD4^+CD25^-$ cells before the culture showed a low expression of FoxP3 (<2 % in flow cytometry and 0.1 relative amount of mRNA compared to control GAPDH gene, no difference between the examined and control groups). A significant increase in the expression of FoxP3 at mRNA and protein level was detected in induced Tregs when compared with freshly isolated $CD4^+CD25^-$ T cells in the control and obese children (more than 60 % of the cells expressed FoxP3 at the protein level; $p = 0.001$ and a relative increase in mRNA expression was higher than 4). No difference was observed in the percentage of FoxP3 positive cells after the culture between the examined and control groups (medians 71.2 vs. 73.7 %; $p > 0.05$). After the stimulation, the mRNA level for FoxP3 was lower in the cultures from the children with MS in contrast to the control subjects (relative expression 0.68). However, the increase during the culture in both groups was comparable (relative change in the FoxP3 expression: 0.91).

### Changes in Gene expression in T Cells before and after the Culture

Before and after the culture the cells were assessed for the expression of molecules important in Treg cell function. The mRNA for the majority of 28 assessed genes were present in all the samples except for: (1) IL-27 mRNA present in 40 i.e. 66.6 % of the samples before the culture and in 44 i.e. 73.3 % of the samples after the culture and (2) EBI3 (IL-35) which was present in 45 i.e. 75 % of the samples before the culture and in all the samples after the culture. In the freshly isolated $CD4^+$ $CD25^-$ cells higher amounts of mRNA for IL-27 and STAT3 in the cells separated from the children with MS compared to the healthy patients were noted. We also observed lower levels of mRNA for SOCS2 and SOCS3, SMAD3, EBI3, TBX21, TGF-$\beta$, CD134, GZMB, CD39, LGAL, LAG3, ITCH, KLF10 and NRP1 in the children from the examined group compared to the control one. We did not find any differences in the expression of the other assessed genes. The relative expression of selected genes in $CD4^+$ $CD25^-$ cells is presented in Fig. 3.

We noted upregulation of the following genes: FoxP3, EBI3, IFN-$\beta$, CD134, CD137, GZMB, CD39, CD73 and LAG3, and the downregulation of: IL-23, STAT1, SOCS2, SOCS3, ICOS1, CTLA4-4, TBX21, TGF-$\beta$, ITCH, KLF10 and NRP1 in the samples separated from the control children after the stimulation with Treg-expander and IL-2/TGF-$\beta$. We also observed the upregulation after the culture of following genes: FoxP3, EBI3, IFN-$\gamma$, CD134, CD137,

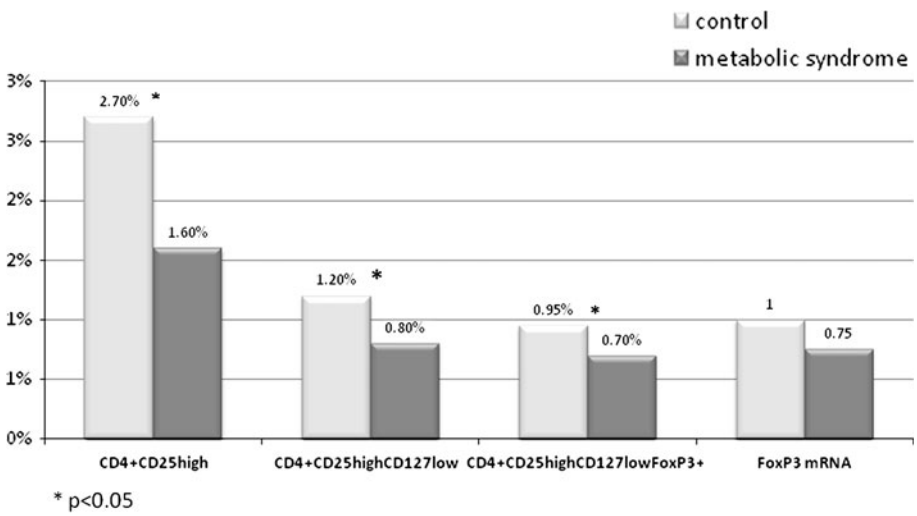


**Fig. 2** The percentages of Treg cells and the expression of their marker transcription factor FoxP3 in the peripheral blood of the obese and healthy children


**Fig. 3** The relative expression of mRNA for selected genes in $CD4^+CD25^-$ cells before the culture. The *bars* represent the relation between the children with metabolic syndrome compared to the healthy subjects. The values above "1" on the *Y* axis show the relative higher expression in the examined versus control children and values below "1" indicate the relative lower expression. Only statistically significant differences are shown

GZMB, CD39, CD73, LGAL, LAG3 and the downregulation of: IL-23, STAT1, STAT3, SOCS2, SOCS3, ICOS1, CTLA-4, IL-10, TGF-$\beta$, TBX21, ITCH, KLF10 and NRP1 in the cells isolated from the children with MS. After the comparison of the changes in the mRNA levels during the culture between the control and examined children, we showed a higher expression of mRNA for SMAD3, CD73, LGAL, ITCH, KLF10 and NRP1 in the cells cultured from the blood of the children with MS compared to the control subjects. We also noted a lower expression of mRNA for IL-23, IL-27, STAT3, ICOS1, tumor necrosis factor 18, IFN-$\gamma$, IL-10 and LRRC32 after the culture of the cells taken from the patients with MS compared to the healthy controls. These relations are demonstrated in Fig. 4.

### Induced Treg Cells Show Suppressive Activity

We sought to establish whether the induced Treg cells are able to suppress autologous T cell proliferation. Freshly isolated autologous $CD4^+$ $CD25^-$ naïve T cells were cultured with medium alone (negative control culture), concavalin A (positive control culture) and with ConA and iTregs (examined culture) in 20 cases (10 children with MS and 10 control patients). After 4 days of the lymphocyte culture, $CD4^+$ $CD25^-$ cells were assessed for BrdU expression (as a marker of proliferation) with the use of flow cytometry. The representative results from these three types of cultures are shown in Fig. 5. The proliferation of non-stimulated cells (negative control) was very low (median 1.2 %), in contrast, the expression of Brdu in ConA-stimulated cells (positive control) was always higher than 50 % (median: 74.3 %). The proliferation of ConA-stimulated $CD4^+$ $CD25^-$ cells in the mixed lymphocyte culture with iTregs was decreased to 20–30 % (median from all the cultures: 22.9 %). These results were statistically significant ($p < 0.01$). There was no difference between the results obtained in the control and examined subjects. These findings confirmed the suppressive activity of induced Treg cells in both obese and healthy children.

We found a correlation neither between the clinical data and results from flow cytometry/RT-PCR nor between results from the flow cytometry and RT-PCR.

## Discussion

Obesity, MS and atherosclerosis are recognized as inflammatory diseases and T cells are proven to be involved in the pathogenesis of these conditions. The recent experimental and clinical research conducted in both mice and human stress influence of Treg cells on adipose tissue inflammation (Cipolletta et al. 2011). We revealed lower percentages of Tregs in the peripheral blood of children fulfilling the criteria of central obesity and MS. We also managed to generate Treg cells from conventional lymphocytes. The Tregs induced from the blood of the obese children differed from those obtained from the control subjects at the mRNA level but not in proliferation studies. To our knowledge, this is the first attempt to generate functional Treg cells in children with present risk factors for developing cardiovascular disease.

One of the crucial issues in this field of research is to identify the location of obesity-related inflammation: the peripheral blood (assuming a generalized process), fat tissue (abdominal obesity as the key element of MS) or the atherosclerotic plaque. Various results are also obtained in different species or groups of examined subjects. The first results concerning the role of Tregs in obesity and

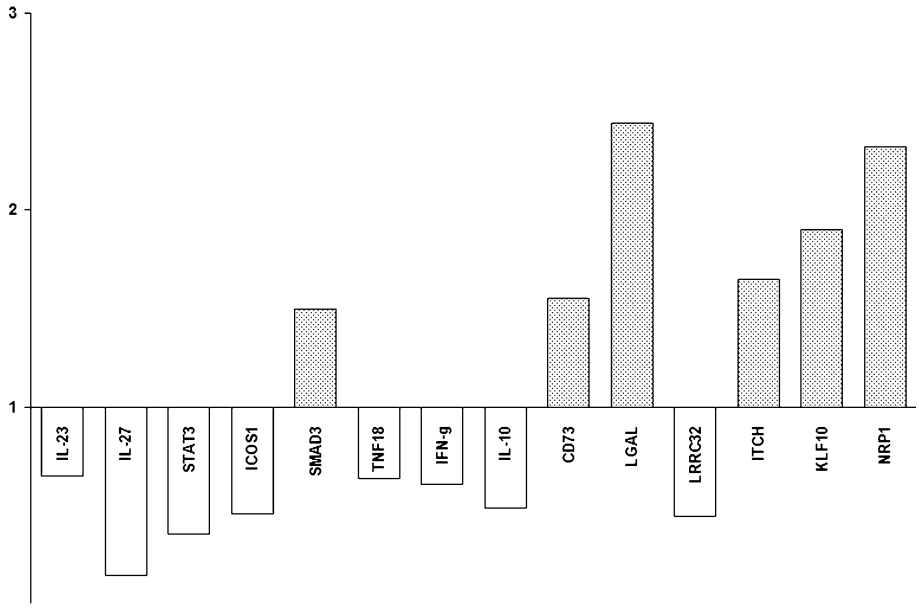


**Fig. 4** The relative expression of mRNA for selected genes after the culture. The *bars* demonstrate the change at the mRNA level in the cultures obtained from the children with metabolic syndrome compared to the cultures from the control patients. The values above "1" on the *Y* axis show a relative higher increase in the expression in the examined versus control children and values below "1" indicate a relative decrease in the expression after the culture. Only statistically significant differences are shown

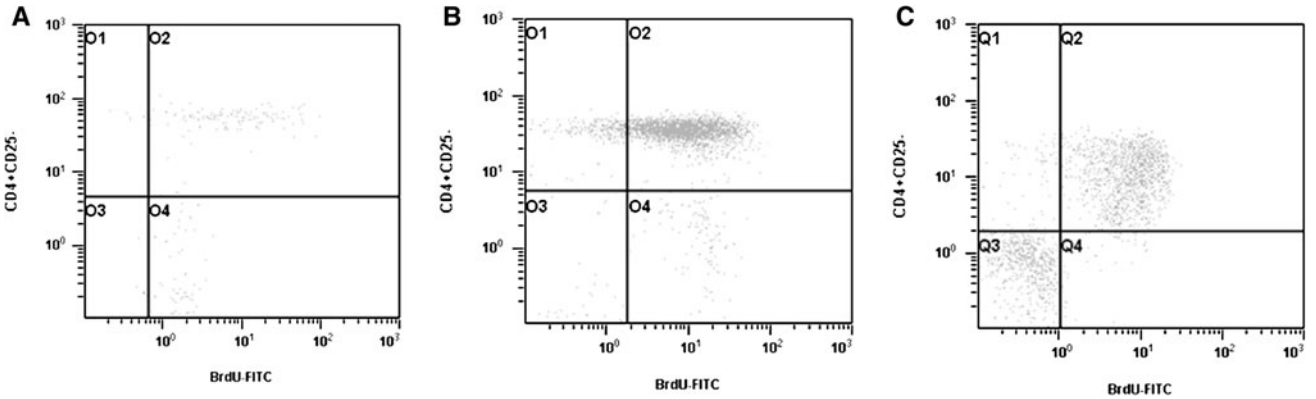


**Fig. 5** The example of the results from the proliferation assays. **a** the bromodeoxyuridine (BrdU) incorporation in non-stimulated $CD4^+CD25^-$ cells (1.2 %) = negative control, **b** the expression of BrDU in concavalin A (ConA)-stimulated $CD4^+CD25^-$ T cells (62.5 %) = positive control, **c** autologous $CD4^+CD25^-$ cells cultured with iTreg cells and ConA (20.1 %)

atherosclerosis were shown in a classical apolipoprotein E-deficient (Apo $E^{-/-}$) mice model (Xie et al. 2010). Very few experiments have been conducted in humans. Only one study was carried out in children with obesity and did not show any differences in Treg cells between obese and control patients (Svec et al. 2007). Our previous results exposed some disturbances in the expression of critical Treg molecules in children with MS compared to Tregs separated from healthy subjects (Łuczyński et al. 2010). According to other authors, obese adults as well as patients with coronary atherosclerosis are characterized by a lower number of Tregs in the peripheral blood than lean patients (Mor et al. 2006; Zhao et al. 2011). The depletion of Treg cells was also observed in visceral adipose tissue in obese humans. However, we do not know if this phenomenon was accompanied by similar changes in the peripheral blood (Deiuliis et al. 2011). As far as plaque is concerned, the human atherosclerotic tissue contained low numbers of $FoxP3^+$ Treg cells as well (de Boer et al. 2007). Unfortunately, Tregs cannot be used as an indicator of the extent or severity of atherosclerosis in humans (Ammirati et al. 2010).

We suggest that the modulation of obesity-related immune dysregulation by the activation of Tregs should represent a novel approach to the prevention and/or treatment of cardiovascular diseases. In one of the first experiments, the transfer of $CD4^+$ cells into obese mice reduced the predominance of Th1 over Treg cells, reversed weight gain and insulin resistance (Winer et al. 2009). As atherosclerosis is regarded as autoimmune-like disease,

some researchers propose the use of a prototype vaccine based on a peptide sequence derived from apolipoprotein B as the immunotherapy in this condition. Immunization of Apo $E^{-/-}$ mice with such a vaccine was associated not only with the activation of Treg cells but also the reduction of atherosclerotic lesions (Wigren et al. 2011). Other authors induced Tregs by the oral administration of anti-CD3 antibody and $\beta$-glucosylceramide resulting in the normalization of immunological imbalance in leptin-deficient obese mice (Ilan et al. 2010).

In our experiment, we showed some changes at the mRNA levels of genes vital in Tregs function. The exact mechanism of the suppressive role of Tregs in obesity-related inflammation remains vague. According to Lin et al. (2010) both cell-to-cell contact and the production of cytokines (IL-10, TGF-$\beta$) are essential in the suppression of macrophage foam-cell formation in atherosclerosis. The RT-PCR technique allows assessing the changes at the mRNA level for many genes in one small sample. However, the results are sometimes perplexing. The higher/lower amounts of mRNA do not always reflect the functional protein levels. In our experiments, we observed the disturbances at the mRNA levels of many genes significant for Treg functions. Nevertheless, in proliferation studies Treg cells induced from children with MS showed a similar suppressive activity to iTregs from healthy subjects. Other authors have been confronted with similar problems. For example, as reported by Taleb et al. (2009) a loss of SOCS3 molecule in T cells isolated from the atherosclerotic lesions resulted in the increase in IL-10 and, surprisingly, IL-17 production. The increased levels of STAT3 and IL-17 were associated with the stabilization of the plaque. These results not only identify new pathways in vascular inflammation, but also caused by the superior complexity of laboratory experiments.

The results from RT-PCR revealed that the induced Tregs expressed more IL-35 in both the examined and control groups, but the IL-10 expression was only upregulated in the cultures provided in the healthy subjects. The production of immunosuppressive cytokines by Tregs and its functional meaning is still controversial. Core-specific regulatory T cells cloned from patients with hepatitis C acted via secretion of IL-10 and IL-35 rather than a cell-contact mechanism (Langhans et al. 2010). IL-35 is recognized as a part of anti-inflammatory orchestration with a potential therapeutic tool in chronic inflammation, autoimmunity and other immunological diseases, but its role in human Tregs is questionable (Bardel et al. 2008). Our results concerning no change in the expression of the LRRC32 (GARP) confirm Probst-Kepper et al. (2009) observations which discovered that only natural but not iTregs up-regulate this molecule.

As the current literature lacks reports on Tregs generation in patients with obesity, we looked for similar experiments in other immune-dependent diseases. Classical markers of Tregs besides transcription factor FoxP3 include: CD25, GITR, CTLA-4 and ICOS1 (Yi et al. 2006). Also new molecules have lately been found as more or less exclusive for this cell population: neuropilin-1, CD39, CD73, ITCH, TIEG, KLF10, GARP (LRRC32), galectin-1, SOCS2 and LAG-3. The conditions of our experiment were chosen on the basis of the literature and our previous experience including Tregs generation in patients with type 1 diabetes. In spite of the fact that IL-2 is critical for the suppressive capacity of Tregs, in other successful studies the naïve T cells were converted into Tregs with the use of CD3/CD28 and TGF-$\beta$ (Barron et al. 2010). The Tregs induced in this manner produced like ours TGF-$\beta$, IL-10 and IFN-$\gamma$ and were fully functional. The inducible Treg cells from the patients with a new onset of type 1 diabetes showed higher suppressive activity compared with the Tregs generated in the control subjects (Glisic et al. 2010). This was accompanied, in contrast to our results, by the upregulation of ITCH molecule during the culture. In another study, the Tregs induced from the patients with autoimmune hepatitis underwent greater growth and were more resistant to apoptosis than the expanded Tregs (Longhi et al. 2008). In both the examined and control groups, we noted the upregulation of CD39 and CD73 molecules after the culture with Treg-expander and IL-2. These molecules play an important role in the inhibitory function of Treg cells via the extracellular adenosine generation (Alam et al. 2009, Dwyer et al. 2010). The accompanying upregulation of granzyme B (GZMB) evident in our experiment also indicates that cytotoxicity of these cells is enhanced and contributes to their suppressive function (Czystowska et al. 2010).

The limitations of our study include: the assessment of some molecules at mRNA rather than protein level (excluding a gold marker of Tregs-transcription factor FoxP3), the lack of comparison of Tregs generated from $CD4^{+}CD25^{+}$ cells (due to intention of taking small amounts of blood for experiments), no assessment of early indicators of atherosclerosis in children including intima media thickness of carotid arteries and/or endothelial dysfunction measured by flow mediated dilation of brachial arteries (assessed in our previous reports).

In conclusion, for the first time have we demonstrated the possibility of generating functional Treg cells in children with MS. The results of our study could be implemented in the design of future therapeutic interventions in obesity associated immunologic disturbances.

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**Conflict of Interest** We have no conflict of interest to declare.

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