ОРИГІНАЛЬНІ ДОСЛІДЖЕННЯ

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OVERWEIGHT IN YOUNG PEOPLE CONTRIBUTES TO THE EXPRESSION OF STAT1 AND STAT6 GENES IN THE PERIPHERAL BLOOD MONOCYTES, STIMULATED BY IL-4*

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Підвищення маси тіла та ожиріння призводить до формування прозапального фенотипу макрофагів жирової тканини, але достеменно не відомо, яким чином реалізується баланс транскрипційних факторів STAT1 та STAT6 в моноцитах периферичної крові і як це впливає на подальший процес поляризації за умов підвищення маси. Досліджено рівень та співвідношення експресії транскрипційних факторів STAT1 та STAT6 при поляризації моноцитів периферичної крові в залежності від маси тіла. Дослідження проведено за участі 20 осіб жіночої та чоловічої статі віком від 18 до 25 років. За ІМТ проведено розподіл по групам: особи з нормальною масою тіла ІМТ 18,50-24,99 кг/м2 із 5 жінок та 5 чоловіків, особи з підвищеною масою ІМТ 25,00-29,99 кг/м2 із 5 чоловіків та 5 жінок. За стандартними методиками були виділені моноцити периферичної крові, стимульовані LPS, γIFN, та IL-4 і проведена інкубація на 3 і 7 добу. В інкубованих клітинах визначали рівень експресії генів stat1 та stat6 методом ПЛР. У супернатанті клітин на 7 добу інкубації визначали рівень IL-6 і TGFβ1, у сироватці крові рівень TGFβ1 і вчСРБ методом ІФА. Одержані результати виявили достовірне підвищення рівня IL-6 в супернатанті макрофагів, стимульованих LPS та γIFN у осіб з підвищеною масою тіла. Рівень вчСРБ у сироватці крові був також достовірно вищий у осіб з підвищеною масою тіла. Показано, що за умов формування підвищеної маси тіла відбувається достовірне підвищення рівня експресії генів stat1 і stat6 у клітинах, стимульованих IL-4. Отримані дані свідчать про наявність стану прекондиціювання моноцитів периферичної крові з активацією сигнальних мереж переважно у макрофагах, стимульованих за М2 фенотипом за умов підвищеного надходження нутрієнтів.

Ключові слова: макрофаги, поляризація макрофагів, STAT1, STAT6, індекс маси тіла, підвищена маса тіла.

Overweight and obesity lead to the formation of a pro-inflammatory phenotype of the adipose tissue macrophages, but it is not known how exactly the balance of STAT1 and STAT6 transcription factors is implemented in the peripheral blood monocytes and how this affects the further polarization process in overweight. The article examines the level and ratio of expression of the STAT1 and STAT6 transcription factors in the polarization of the peripheral blood monocytes depending on the body weight. The study enrolled 20 women and men aged from 18 to 25 years. In terms of BMI, the subjects were divided into the following groups: individuals with normal body weight (BMI 18.50-24.99 kg/m2), represented by 5 women and 5 men; overweight individuals (BMI 25.00-29.99 kg/m2), including 5 men and 5 women. Using standard methods peripheral blood monocytes stimulated by LPS and γIFN, IL-4 was isolated and incubated for 3 and 7 days. PCR method was used to determine the expression level of the stat1 and stat6 genes in incubated cells. The concentration of IL-6 and TGFβ1 was measured in the supernatant on the 7th day of incubation, and TGFβ1 and hs-CRP in the serum of the subjects. The obtained results revealed a significant increase in the level of IL-6 in the supernatant of macrophages stimulated by LPS and γIFN in overweight individuals. The level of hs-CRP in the serum was also significantly higher in overweight individuals. It has been shown that under the conditions of overweight development, there is a significant increase in the level of expression of stat1 and stat6 genes in cells stimulated by IL-4. The obtained data indicate the presence of a preconditioning state of the peripheral blood monocytes with activation of signaling networks mainly in macrophages stimulated by the M2 phenotype under conditions of increased nutrients intake.

Key words: macrophages, macrophages polarization, STAT1, STAT6, body mass index, overweight.

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Introduction

Excessive accumulation of metabolically active adipose tissue in overweight and obesity leads to chronic systemic inflammation of low intensity and significant infiltration of the tissue by macrophages [1].

Cells of the monocyte-macrophage line are characterized by significant diversity, plasticity, and flexibility, which are the key features of mononuclear phagocytes and states of their activation [2, 3]. There are two phenotypes of activated macrophages: M1 – classically, and M2 – alternatively activated, which corresponds to the division of activated T lymphocytes into Th1 and Th2 types and emphasizes the connection of macrophages with the implementation of the respective type of immune response.

During the development of overweight and obesity, the number of macrophages of the M1 subpopulation increases and correlates with inflammation in the adipose tissue, which contributes to further weight gain and possible insulin resistance. Upon activation, M1 macrophages produce pro-inflammatory cytokines and induce aerobic glycolysis. In individuals with normal body weight, macrophages of the M2 subpopulation predominate; they secrete anti-inflammatory cytokines and use oxidative metabolism to maintain homeostasis [4].

At present, it is known that the family of proteins – signal converters and activators of the STAT transcription (Signal Transducers and Activators of Transcription), are the main factors of the transcriptional polarization control.

STAT1 is associated with the polarization of macrophages by the M1 phenotype, which is reflected in the Th1 immune response, whereas STAT6 is associated with the activation of the M2 macrophages during the Th2 cell-mediated immune response. STAT1 and STAT6 can act as important antagonistic regulators that are crucial in the M1 and M2 polarization of macrophages [5, 6].

Janus kinase (JAK), an activator of the STAT transcription cascade, is the central component of the signaling cascades [7]. The JAK/STAT signaling pathway is present in all cells and can mediate cell-specific responses. JAK/STAT in the adipose tissue is involved in the paracrine link between adipocytes and immune cells of the adipose tissue, and it is of great importance in the pathogenesis of obesity [8]. In immune cells, the JAK/STAT pathway mediates the regulation of inflammation, which is associated with metabolic disorders and obesity [9].

The formation of the pro-inflammatory phenotype of the adipose tissue macrophages under conditions of gradual weight gain and obesity has been confirmed, but it is not known exactly how the balance of STAT1 and STAT6 transcription factors in the peripheral blood monocytes is implemented and how it affects further polarization in overweight.

Therefore, the aim of our study was to determine the level and ratio of the expression of the STAT1 and STAT6 transcription factors in the polarization of the peripheral blood monocytes depending on the body weight.

Materials and methods

The study enrolled 20 women and men aged from 18 to 25 years. Informed consent to participate in the study was signed with each participant. The study was conducted with the permission of the Commission on Bioethics of Ukrainian Medical Stomatological Academy. The absence of somatic pathology was the criterion for the inclusion of respondents in the study. The anamnestic data of the subjects were entered into the observation record.

Based on the anthropometric data, the body mass index (BMI) was calculated according to the formula: BMI = body weight (kg)/height (m^2) [10]. In terms of BMI, the subjects were divided into the following groups: individuals with normal body weight (BMI 18.50-24.99 kg/m^2), represented by 5 women and 5 men; overweight individuals (BMI 25.00-29.99 kg/m^2), including 5 men and 5 women.

Blood was obtained from the ulnar vein in the morning on an empty stomach in vacutainers with heparin (Vacutest Kima, Italy) and diluted with 0.9% sodium chloride solution (Yuria-Pharm, Ukraine) in a ratio of 1:1. The suspension of the peripheral blood mononuclear cells was isolated by centrifugation on a density gradient of ficoll/verografin (ρ = 1.077 g/ml^3, Granum, Ukraine) according to standard methods.

The attached monocytes were resuspended in the RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich, USA) and transferred by 0.5 ml into the wells of 24-well sterile plates at a concentration of at least 3-5×10^6 cells/ml.

To induce the polarization of monocytes, 100 ng/ml of E. coli lipopolysaccharide (LPS) (Sigma-Aldrich, USA) [11] and 100 ng/ml of γ-interferon (γIFN) (Ingaron, Pharmaclone, Russia) were added to the wells to the M1 macrophage subpopulation [12].

To induce the polarization of monocytes, 20 ng/ml of interleukin-4 (IL-4) (Sino Biological, USA) were added to the M2 subpopulation [11]. Unstimulated monocytes/macrophages served as controls.

Under sterile conditions, the cells were incubated for 7 days at 37°C in an atmosphere of 5% CO_2. Cells and supernatant on days 3 and 7 of incubation were used for the study.

The real-time PCR method was used to determine the expression level of the stat1 and stat6 genes. Isolation of total RNA from polarized macrophages was conducted using a set of reagents for isolation and purification of RNA with a magnetic sorbent (UkrGenTech, Ukraine).

The level of expression of stat1 and stat6 genes was determined using a detection amplifier "DT-light" (DNA Technology, Russia).

The sequence of primers for determining the expression of stat1 genes [13] was as follows:

direct: 5'-CCAAAGGAAGCACCAGAGCC-3';
reverse: 5'-AGAGCCCACTATCCGAGACACC-3',
stat6 [14]:
direct: 5′-CTTTCCGGAGCCACTACAAG-3′;
reverse: 5′-AGGAAGTGGTTGGTCCCTTT-3′.
The GAPDH gene was used as a reference gene:
direct: 5′-TGCACCACCAACTGCTTAGC-3′;
reverse: 5′-GGCATGGACTGTGGTCATGAG-3′.

Data analysis was conducted via the relative Ct method based on the formula $2^{-\Delta Ct}$.

The level of IL-6 (Vector-Best, Russia) and transforming growth factor β1 (TGFβ1, Affimetrix, eBioscience, Austria) was determined in the supernatant of cells on the 7th day of incubation, the level of TGFβ1 and high-sensitivity C-reactive protein (hsCRP, Vector-Best, Russia) was measured in the blood serum using the reagent kits for solid-phase enzyme-linked immunosorbent assay according to the manufacturer's instructions. The results were recorded on a microplate analyzer LabLine-026.

STATISTICA 10.0 (StatSoft Inc., USA) and GraphPad

Prism 8.00 (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical data processing. Data are presented as arithmetic mean (M) and its error (m). The Shapiro-Wilk test was used to verify the normality of the data distribution. Statistical processing was performed using the nonparametric paired Wilcoxon test and the unpaired Mann-Whitney test. Relationships between indicators were analyzed by Spearman's correlation. The differences were considered statistically significant at $p<0.05$.

Results

The obtained findings showed that in individuals with normal body weight, the expression level of stat1 was significantly higher in macrophages stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells on the 3rd day of incubation. The expression of stat1 in macrophages stimulated by IL-4 is significantly higher as opposed to macrophages stimulated by LPS and γIFN (Table 1).

Table 1

The level of expression of stat1 and stat6 genes in monocytes/macrophages on the 3rd day of incubation (M ± m)

Groups	Stat1, $2^{-\Delta Ct}$		
	Unstimulated cells	Cells stimulated by LPS and γIFN	Cells stimulated by IL-4
	n=10	n=10	n=10
Individuals with normal body weight	0.0726±0.012	0.0883±0.0155 p = 0.0051	0.1106±0.0183 p = 0.0051 p1 = 0.0077
Overweight individuals	0.0709±0.0164	0.1151±0.0250 p = 0.0051	0.1558±0.0324 p = 0.0051 p1 = 0.0051
	Stat6, $2^{-\Delta Ct}$		
	Unstimulated cells	Cells stimulated by LPS and γIFN	Cells stimulated by IL-4
Individuals with normal body weight	0.0016±0.0002	0.0029±0.0003 p = 0.0051	0.0036±0.0003 p = 0.0051 p1 = 0.0051
Overweight individuals	0.0025±0.0003	0.0046±0.0004 p = 0.0051	0.0063±0.0006 p = 0.0051 p1 =0.0012

***Notes**: here and further in table 2: p is the significance of differences between the expression parameters in cells stimulated by LPS and γIFN, IL-4 and cells without stimulation;*
p1 is the significance of differences between the expression parameters in cells stimulated by LPS and γIFN, IL-4.

In overweight individuals, a significantly higher level of stat1 expression was detected in macrophages stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells on the 3rd day of incubation. We found significantly higher levels of stat1 expression in IL-4-stimulated macrophages as opposed to LPS- and γIFN-stimulated macrophages (0.1558±0.0324 and 0.1151±0.0250).

The level of stat6 expression in individuals with normal body weight was significantly higher in macrophages stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells on the 3rd day of incubation. The level of stat6 expression in IL-4-stimulated macrophages was significantly higher as opposed to LPS- and γIFN-stimulated cells.

In overweight individuals, the expression of stat6 in macrophages, stimulated by LPS and γIFN, IL-4 was also significantly higher as compared to unstimulated cells. The expression of stat6 in macrophages stimulated by IL-4 was significantly higher as opposed to cells stimulated by LPS and γIFN.

On the 7th day of incubation, in individuals with normal body weight, a significantly higher level of stat1 expression was found in macrophages, stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells (Table 2). We also observed significantly higher levels of stat1 expression in LPS- and γIFN-stimulated macrophages as opposed to IL-4-stimulated macrophages.

Table 2

The expression level of stat1 and stat6 genes in monocytes/macrophages on the 7th day of incubation (M ± m)

Groups	Stat1, $2^{-\Delta Ct}$		
	Unstimulated cells	Cells stimulated by LPS and γIFN	Cells stimulated by IL-4
	n=10	n=10	n=10
Individuals with normal body weight	0.0919±0.0214	0.1503±0.032 p = 0.0051	0.1291±0.0319 p = 0.0051 p1= 0.0051
Overweight individuals	0.1270±0.0341	0.1771±0.0398 p = 0.0051	0.2579±0.0523 p = 0.0051 p1 = 0.0051
	Stat6, $2^{-\Delta Ct}$		
	Unstimulated cells	Cells stimulated by LPS and γIFN	Cells stimulated by IL-4
Individuals with normal body weight	0.0024±0.0003	0.0035±0.0004 p = 0.0051	0.0049±0.0005 p = 0.0051 p1=0.0051
Overweight individuals	0.0025±0.0004	0.0040±0.0006 p = 0.0051	0.0055±0.0007 p = 0.0051 p1 = 0.0051

In overweight individuals, a significantly higher level of stat1 expression was found in macrophages stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells. The level of stat1 expression in IL-4-stimulated macrophages was significantly higher as opposed to LPS- and γIFN-stimulated macrophages.

Expression of stat6 in individuals with normal body weight was significantly higher in macrophages stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells. Significantly higher levels of stat6 expression were also found in IL-4-stimulated macrophages as opposed to LPS- and γIFN-stimulated macrophages.

In overweight individuals, a higher level of stat6 expression was detected in macrophages stimulated by LPS and γIFN, IL-4 as compared to unstimulated cells (0.0040±0.0006 and 0.0055±0.0007 versus 0.0025±0.0004, respectively, p = 0.0051). The level of stat6 expression in IL-4-stimulated macrophages was significantly higher than in LPS- and γIFN-stimulated macrophages.

Further, we compared the stat1 and stat6 expression levels between the study groups.

The expression level of stat1 was significantly higher by 99.77% in macrophages stimulated by IL-4 in overweight individuals as compared to individuals with normal body weight on the 7th day of incubation (Table 3).

Table 3

The level of expression of stat1 and stat6 genes in monocytes/macrophages of the study groups (M ± m)

Parameters	Individuals with normal body weight n = 10	Overweight individuals n = 10
Stat1, $2^{-\Delta Ct}$		
Unstimulated cells, 3 days of incubation	0.0726±0.012	0.0709±0.0164 p = 0.9308
Cells stimulated by LPS and γIFN, 3 days of incubation	0.0883±0.0155	0.1151±0.0250 p = 0.3754
Cells stimulated by IL-4, 3 days of incubation	0.1106±0.0183	0.1558±0.0324 p = 0.2421
Unstimulated cells, 7 days of incubation	0.0919±0.0214	0.1270±0.0341 p = 0.3938
Cells stimulated by LPS and γIFN, 7 days of incubation	0.1503±0.0324	0.1771±0.0398 p = 0.6066
Cells stimulated by IL-4, 7 days of incubation	0.1291±0.0319	0.2579±0.0523 p = 0.0498
Stat6, $2^{-\Delta Ct}$		
Unstimulated cells, 3 days of incubation	0.0016±0.0002	0.0025±0.0003 p = 0.0337
Cells stimulated by LPS and γIFN, 3 days of incubation	0.0029±0.0003	0.0046±0.0004 p = 0.0048
Cells stimulated by IL-4, 3 days of incubation	0.0036±0.0003	0.0063±0.0006 p =0.0014
Unstimulated cells, 7 days of incubation	0.0024±0.0003	0.0025±0.0004 p = 0.9165
Cells stimulated by LPS and γIFN, 7 days of incubation	0.0035±0.0004	0.0040±0.0006 p = 0.5168
Cells stimulated by IL-4, 7 days of incubation	0.0049±0.0005	0.0055±0.0007 p = 0.4198

***Notes**: p is the significance of differences between the parameters in overweight individuals and individuals with normal body weight.*

Studies have shown a significantly higher level of stat6 expression by 56.25% in unstimulated cells, by 58.62% in macrophages stimulated by LPS and γIFN, and by 75% in macrophages stimulated by IL-4 in overweight individuals as compared to individuals with normal body weight on the 3rd day of incubation.

To determine the balance of stat1 and stat6 transcription factors in the polarization process of monocytes/macrophages in individuals with normal body weight and overweight, we calculated the ratio of the expression level of stat1/stat6 in the subjects.

According to the results, the stat1/stat6 ratio in macrophages stimulated by LPS and γIFN was significantly lower by 23.91% as compared to unstimulated cells in individuals with normal body weight after 3 days of incubation (Table 4).

Table 4

The ratio of the expression level parameters of stat1/stat6 genes (M ± m)

Groups	The stat1/stat6 ratio, unstimulated cells	The stat1/stat6 ratio, cells stimulated by LPS and γIFN	The stat1/stat6 ratio, cells stimulated by IL-4
3 days of incubation			
Individuals with normal body weight, n = 10	46.35±7.12	35.27±6.92 p = 0.0093	36.02±8.19 p = 0.0744 p1 = 0.8784
Overweight individuals n = 10	33.94±9.41	28.47±7.91 p = 0.7212	29.66±9.12 p = 0.7988 p1 = 0.8784
7 days of incubation			
Individuals with normal body weight, n = 10	55.391±20.19	45.13±10.92 p = 0.9594	31.39±9.75 p = 0.0218 p1 = 0.005
Overweight individuals n = 10	55.92±13.88	57.85±16.79 p = 0.7212	57.08±16.48 p = 0.767 p =0.7212

***Notes**: p is the significance of differences between the expression parameters in cells stimulated by LPS and γIFN, IL-4 and cells without stimulation;*

p1 is the significance of differences between the expression parameters in cells stimulated by LPS and γIFN, IL-4.

The stat1/stat6 ratio on the 7th day of incubation was significantly lower by 43.34% in IL-4-stimulated macrophages as compared to unstimulated cells and by 30.45% as opposed to LPS- and γIFN-stimulated macrophages in individuals with normal body weight.

When comparing the stat1/stat6 ratio in polarized monocytes/macrophages after 3 and 7 days of incubation, no significant differences between individuals with normal body weight and overweight were found (Table 5).

Table 5

The ratio of the expression level parameters of stat1/stat6 genes in individuals of the study groups (M ± m)

Parameters	Individuals with normal body weight, n=10	Overweight individuals, n=10
3 days of incubation		
The stat1/stat6 ratio, unstimulated cells	46.35±7.12	33.94±9.41 p = 0.306
The stat1/stat6 ratio, cells stimulated by LPS and γIFN	35.27±6.92	28.47±7.91 p = 0.526
The stat1/stat6 ratio, cells stimulated by IL-4	36.02±8.19	29.66±9.12 p = 0.610
7 days of incubation		
The stat1/stat6 ratio, unstimulated cells	55.391±20.19	55.92±13.88 p = 0.982
The stat1/stat6 ratio, cells stimulated by LPS and γIFN	45.13±10.92	57.85±16.79 p = 0.533
The stat1/stat6 ratio, cells stimulated by IL-4	31.39±9.75	57.08±16.48 p = 0.196

***Notes**: p is the significance of differences between the parameters in overweight individuals and individuals with normal body weight.*

In addition, the correlation analysis did not reveal any significant interrelations between the expression of stat1 and stat6 genes (data not shown).

Further, we measured the concentration of IL-6 and TGFβ1 in the supernatant on the 7th day of incubation, and TGFβ1 and hsCRP in the serum of the subjects.

According to the results, the level of IL-6 was significantly lower by 30.75% in the supernatant of macrophages stimulated by IL-4 as compared to unstimulated cells and by 34.97% as opposed to macrophages stimulated by LPS and γIFN in individuals with normal body weight (Table 6).

Table 6

Concentrations of IL-6 and TGFβ1 in the supernatant of cells on the 7th day of incubation (M ± m)

Groups	IL-6, pg/ml		
	Unstimulated cells	Cells stimulated by LPS and γIFN	Cells stimulated by IL-4
	n=10	n=10	n=10
Individuals with normal body weight	321.73±14.94	342.59±0.26 p = 0.202	222.80±34.54 p = 0.0125 p1 = 0.0166
Overweight individuals	321.46±18.06	343.82±0.20 p = 0.0050	299.74±19.51 p = 0.3862 p1 = 0.0069
	TGFβ1, ng/ml		
	Unstimulated cells	Cells stimulated by LPS and γIFN	Cells stimulated by IL-4
	n=10	n=10	n=10
Individuals with normal body weight	24.45±4.86	18.98±1.33 p = 0.0284	25.98±6.48 p = 0.7213 p1 = 0.9593
Overweight individuals	30.08±4.34	29.65±5.81 p = 0.7989	30.64±4.80 p = 0.7988 p1 = 0.6465

***Notes**: p is the significance of differences between the parameters in the supernatants of cells stimulated by LPS, γIFN, IL-4 and without stimulation;*

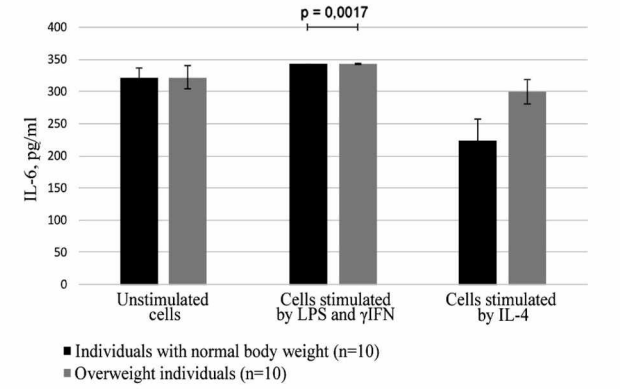
p1 is the significance of differences between the parameters in the supernatants of cells stimulated by LPS and γIFN, IL-4.

In overweight individuals, we found significantly higher levels of IL-6 by 6.96% in the supernatant of macrophages stimulated by LPS and γIFN as compared to unstimulated cells. The level of IL-6 in the supernatant of macrophages stimulated by IL-4 was significantly lower by 12.83% as compared to macrophages stimulated by LPS and γIFN.

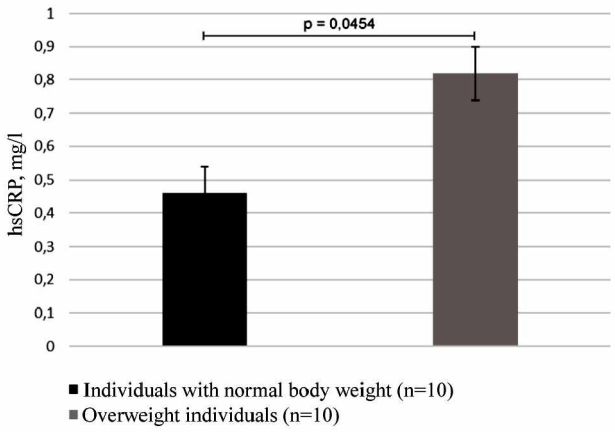
The level of TGFβ1 in the supernatant of macrophages stimulated by LPS and γIFN in individuals with normal body weight was lower by 22.37% as compared to unstimulated cells (p = 0.0284).

The obtained results revealed a significant increase in the level of IL-6 in the supernatant of macrophages stimulated by LPS and γIFN in overweight individuals as compared to those with normal body weight (Fig. 1, A).

The level of hsCRP in the serum was significantly higher by 79.62% in overweight individuals as compared to subjects with normal body weight (Fig. 1, B).



A



B

Figure 1. Concentrations of IL-6 in the supernatant of cells and hsCRP in the serum
(A) parameters of IL-6 in the supernatant of cells on the 7th day of incubation in individuals of the study groups;
(B) parameters of hsCRP in the serum of individuals of the study groups.

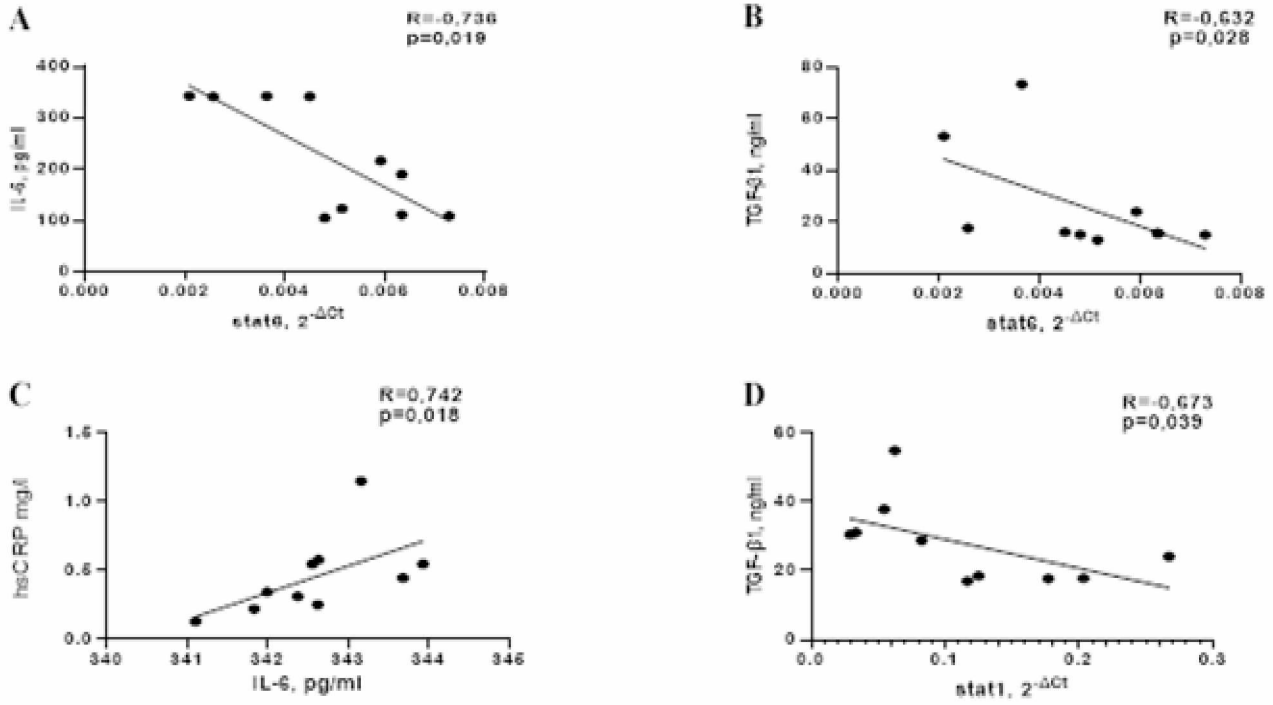
The obtained results did not reveal a significant difference between the level of TGFβ1 in the serum of the study groups.

We conducted a correlation analysis to determine the relationships between the parameters. In individuals with normal body weight, a negative relationship of high strength was found between the level of stat6 expression in macrophages stimulated by IL-4 and the level of IL-6 in the supernatant of macrophages stimulated by IL-4 (r = -0.736, p = 0.019) (Fig. 2, A), and of medium strength between the expression level of stat6 in macrophages stimulated by IL-4 and the level of TGFβ1 in the supernatant of macrophages stimulated by IL-4 (r = -0.632, p = 0.028) after 7 days of incubation (Fig. 2, B).

We detected the formation of a positive high-strength relationship between the level of IL-6 in the supernatant of macrophages stimulated by LPS and γIFN after 7 days of incubation and the level of hsCRP (r = 0.742, p = 0.018) in the serum of individuals with normal body weight (Fig. 2, C).

In overweight individuals, negative relationships of medium strength were found between the level of stat1 expression in macrophages stimulated by LPS and γIFN for 3 days of incubation and TGFβ1 in the serum (r = -0.673, p = 0.039) (Fig. 2, D), and negative relationships of high strength were detected between the expression level of stat1 in macrophages stimulated by IL-4 for 3 days of incubation and TGFβ1 in the serum (r = -0.758, p= 0.015) (Fig. 2, E).

Medium-strength relationships were found between the expression level of stat6 in unstimulated cells for 3 days of incubation and TGFβ1 in the serum (r = -0.669, p= 0.040) (Fig. 2, F).



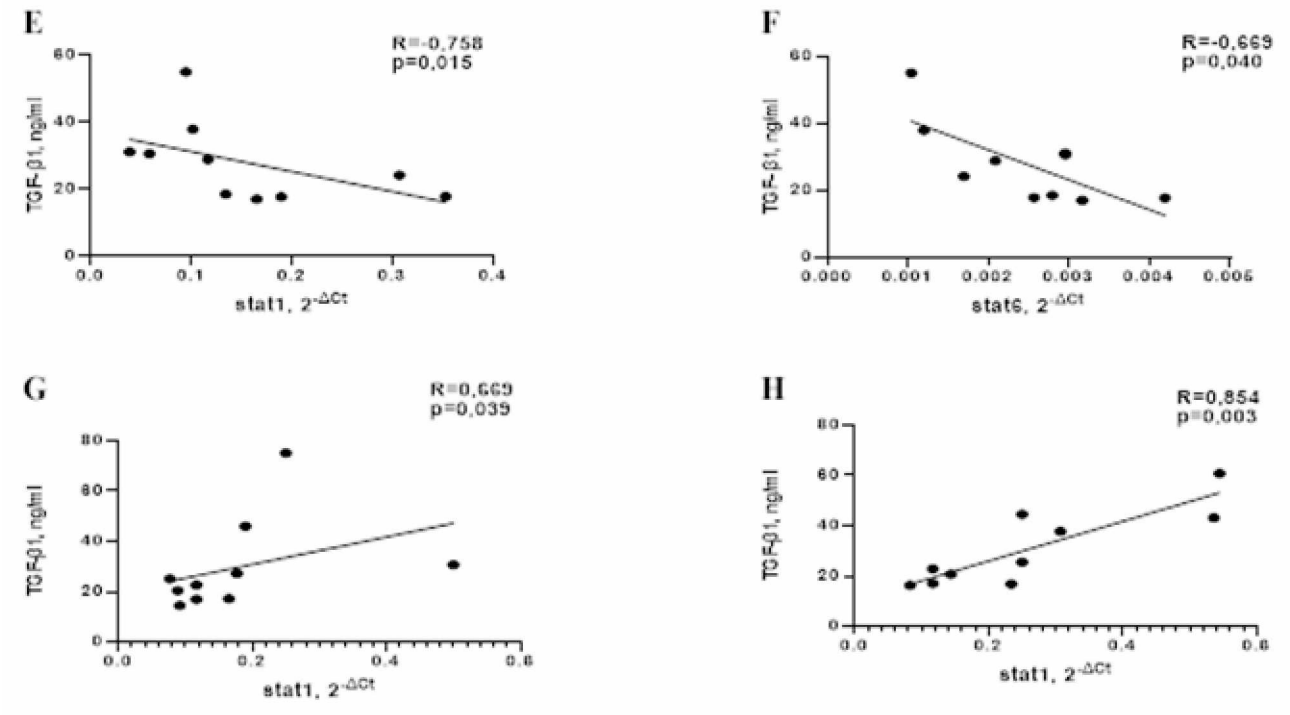


Figure 2. Correlation analysis of relationships: in subjects with normal body weight:
(A) expression of stat6 and IL-6 levels in the supernatant upon stimulation by IL-4 for 7 days;
(B) expression of stat6 and TGFβ1 levels in the supernatant upon stimulation by IL-4 for 7 days;
(C) IL-6 in the supernatant upon stimulation by LPS and γIFN for 7 days and the level of hsCRP in the serum;in overweight individuals:
(D) expression of stat1 upon stimulation by LPS and γIFN for 3 days and TGFβ1 level in the serum;
(E) expression of stat1 upon stimulation by IL-4 for 3 days and TGFβ1 level in the serum;
(F) expression of stat6 in unstimulated cells and TGFβ1 level in the serum;
(G) expression of stat1 and TGFβ1 levels in the supernatant upon stimulation by LPS and γIFN for 7 days;
(H) expression of stat1 and TGFβ1 level in the supernatant upon stimulation by IL-4 for 7 days.

Furthermore, in overweight individuals, we found the formation of a positive medium-strength relationship between the level of expression of stat1 in macrophages stimulated by LPS and γIFN, and the level of TGFβ1 in the supernatant of macrophages stimulated by LPS and γIFN (r = 0.669, p = 0.039) for 7 days of incubation Fig. 2, G). A positive high-strength relationship was found between the level of stat1 expression in the IL-4-stimulated macrophages and the level of TGFβ1 in the supernatant of the IL-4-stimulated cells (r = 0.854, p=0.003) for 7 days of incubation (Fig. 2, H).

Discussion

Overweight and obesity, caused by excessive nutrients intake, increase the macrophages infiltration in the fat depot [15]. The functioning of immune cells in the adipose tissue during the low-intensity inflammatory process is implemented with the involvement and polarization of macrophages, the formation of a macrophage-like phenotype of preadipocytes [16]. In hypertrophied adipose tissue, cytokines and chemokines mediate the impact on regulatory pathways, and this is particularly relevant for cytokines that are activated and released by monocytes/macrophages.

Previous studies have considered the ability of transcription factors of the STATs family to modulate the function of adipocytes by transcriptional regulation of specific gene targets in response to stimulation. It is also known that canonical signaling via the IFN-regulatory factor (IRF)/STAT is a central way to modulate the polarization of macrophages. Activation of IRF/STAT signaling pathways by IFN and TLR-mediated signals induces the STAT1-mediated M1 phenotype polarization, and activation of STAT6-mediated IRF/STAT signaling pathways by IL-4 and IL-13 stimulates the M2 phenotype formation [17]. However, the established antagonism between STAT1 and STAT6 as some of the main transcription factors of M1/M2 macrophages polarization has not been thoroughly studied yet [5].

Stimuli of the microenvironment mediate the direction of polarization of monocytes by the pro-inflammatory phenotype in the development of obesity. However, the features of the transcription profile of monocytes before recruitment into the adipose tissue with a gradual increase in body weight are not known.

We conducted an *in vitro* study on the peripheral blood monocytes to determine the level of expression of the stat1 and stat6 genes under conditions of stimulation by the pro- and anti-inflammatory phenotype and the production of cytokines directly by cells and in the serum.

We determined that the largest increase in the expression level of both studied genes was observed under conditions of stimulation of monocytes by IL-4. Moreover, in the dynamics of incubation, differences in groups were found. Hence, in individuals with normal body weight on the 7th day of incubation, the level of stat1 expression was significantly higher under conditions of stimulation of cells by LPS and γIFN. In overweight individuals, the highest level of stat1 expression was maintained in IL-4-stimulated cells.

The expression of the stat6 gene in the dynamics of incubation was significantly higher in macrophages stimulated by IL-4 in individuals of both study groups.

The correlation analysis did not reveal significant relationships between the expression of stat1 and stat6 genes.

The obtained data show that in overweight individuals, a significant increase in the expression level of both stat1 and stat6 genes occurs in cells stimulated by the M2 phenotype. The activation of signaling networks that mediate the formation of both pro- and anti-inflammatory phenotypes may be a reflection of the state of preconditioning of monocytes against the background of increased nutrients intake. Further development of the polarization direction depends on the development of low-intensity inflammation in the adipose tissue.

To determine the possible direction of polarization, we calculated the ratio of the expression level of the stat1/stat6 genes. In individuals with normal body weight, the expression ratio of stat1/stat6 in the incubation dynamics varied. Compared with unstimulated cells, this value was significantly reduced under the conditions of stimulation by LPS and γIFN on the 3rd day and by IL-4 on the 7th day of incubation. Such changes indicate the state of preconditioning of the peripheral blood monocytes with the formation of a subpopulation of macrophages by anti-inflammatory phenotype under the conditions of activation of signaling networks in terms of both STAT1 (3 days) and STAT6 (7 days).

In contrast to individuals with normal body weight, in overweight individuals, the stat1/stat6 ratio in the incubation dynamics did not change significantly. There were also no significant differences in the ratio between the groups.

It should be noted that many functional and almost all molecular studies of macrophages mainly focus on primary macrophages, macrophage cell lines, and the *in vitro* action of single highly polarizing ligands: LPS, γIFN, and IL-4. However, *in vivo* macrophages are often simultaneously exposed to a wide range of stimuli, the integration of which over time determines the continuum of different transcriptional and functional outcomes [18].

The study by Piccolo V. et al. (2017) has demonstrated that the IL-4-STAT6 signaling pathway can partially inhibit the IFNγ-induced macrophage transcription program after co-activation by IL-4 and INFγ [19]. The results provide evidence that the IL-4-STAT6 signaling pathway induces epigenetic changes that persist after the release of STAT6 from DNA, leading to impaired activation of inflammatory enhancers. These studies suggest that there are complex bidirectional interactions between different polarization signals, which determine the overall sensitivity and response of macrophages to environmental stimuli [20].

It is possible that the M2 phenotype of macrophages, which is dominant under conditions of normal body weight, becomes a kind of target under altered conditions of energy imbalance. It is in these cells that the active interaction of signaling networks takes place, which mediates the formation of the pro- and anti-inflammatory phenotype. In particular, such an interaction can occur at the level of PPARγ and the main pro-inflammatory transcription factor NF-κB [20, 21].

Adipocytes, which play an important role in the accumulation of lipids, energy homeostasis, and insulin sensitivity, produce and secrete numerous enzymes, hormones, cytokines, and growth factors that modulate appetite, lipid and glucose homeostasis, and insulin sensitivity. Some of them, such as leptin, prolactin, and IL-6 are activators of the JAK/STAT signaling pathway [8].

We determined the levels of IL-6 and TGFβ1, which are secreted by M1 and M2 subpopulations of macrophages, respectively. According to our data, the production of IL-6 by macrophages in the cells of individuals in both groups was predominant under the conditions of LPS and γIFN stimulation. IL-6 production was significantly higher in overweight individuals. These results are confirmed by the formation of negative correlations between the level of stat6 expression and the level of IL-6 in cells stimulated by IL-4 for 7 days in individuals with normal weight.

Similar results were obtained by Smith T.D. et al. [22]. According to this study, macrophages stimulated by LPS and γIFN demonstrated the highest secretion of inflammatory cytokines, including IL-6, as compared to the levels in the supernatants of unstimulated cells or cells exposed to IL-4.

A study of the level of IL-6 in the serum of overweight and obese people showed its significant increase as compared to subjects with normal body weight [23]. Similar results were obtained by Roytblat L. et al. (2000) in obese patients [24].

Representatives of the TGFβ family play a fundamental role in the regulation of basic biological processes such as growth, embryonic development, tissue homeostasis, and immune system regulation [25]. They perform a critical biological role in promoting alternative macrophage activation [26]. A study of TGFβ1 levels in individuals with normal body weight showed a significant decrease under stimulation of cells by LPS and γIFN, and negative relationships between stat6 expression and TGFβ1 levels in the supernatant of IL-4-stimulated cells.

No significant changes in TGFβ1 levels during incubation were detected in overweight individuals. However, positive relationships were found, which linked the expression of stat1 in cells stimulated by LPS and γIFN, IL-4 for 7 days, and the level of TGFβ1 in the supernatant of cells stimulated by the corresponding phenotype.

In the serum of individuals in the study groups, no significant difference between the level of TGFβ1 was detected. However, in overweight individuals, negative relationships were found between the stat6 expression in unstimulated cells, the stat1 expression in cells stimulated by LPS and γIFN, IL-4 for 3 days, as well as the serum TGFβ1 levels.

The parameter of hsCRP in the blood serum was significantly higher in overweight individuals as compared to the group of subjects with normal body weight. A positive relationship was also found between the level of IL-6 in the supernatant of LPS- and γIFN-stimulated cells and hsCRP in the serum of individuals with normal body weight.

Higher levels of hsCRP in the serum of overweight individuals as compared to individuals with normal body weight have also been detected by other researchers [27].

Studies by J.P. Bastard et al. (2000) have shown significantly higher serum IL-6 levels in overweight and obese individuals, and hsCRP levels in obese individuals as compared to normal-weight individuals [28]. A significant increase in the serum hsCRP was found in overweight and obese women as compared to individuals with normal body weight [29].

Thus, research has shown that under conditions of excess nutrients intake and the development of overweight, there is a significant increase in the expression of stat1 and stat6 genes in cells stimulated by

the M2 phenotype. Activation of signaling networks that mediate the formation of the pro- and anti-inflammatory phenotypes is a possible reflection of the state of preconditioning of the peripheral blood monocytes against the background of increased nutrients intake. Further development of the polarization direction depends on the development of low-intensity inflammation in the adipose tissue, the signs of which, according to the level of cytokines and hsCRP, are present in overweight individuals. Under changed conditions of energy imbalance, the M2 macrophage phenotype implements the processes of interaction of signal networks, which are responsible for the formation of the pro- and anti-inflammatory phenotype. A possible level of interaction is located between PPARγ and NF-κB transcription factor.

Conclusions:

1. Stimulation of the peripheral blood monocytes by LPS and γIFN, IL-4 leads to a significant increase in the level of expression of stat1 and stat6 genes in both groups. The highest level of expression of both genes was detected in macrophages stimulated by IL-4.

2. Overweight increases the expression level of stat1 under conditions of stimulation by IL-4 on the 7th day of incubation and stat6 with stimulation by both phenotypes on the 3rd day of incubation.

3. The expression ratio of stat1/stat6 in individuals with normal body weight in the dynamics of incubation is reduced on the 3rd day under conditions of stimulation by LPS and γIFN, and on the 7th day of stimulation by IL-4. In overweight individuals, the value of the ratio does not change significantly.

4. The obtained data indicate the presence of a preconditioning state of the peripheral blood monocytes with activation of signaling networks mainly in macrophages stimulated by the M2 phenotype under conditions of increased nutrients intake.

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