® Postepy Hig Med Dosw (online), 2019; 73: 159-164
e-ISSN 1732-2693

www.phmd.pl
Original Article

Received: 17.12.2018
Accepted: 15.02.2019
Published: 18.03.2019

Disrupted Treg/Th17 balance in patients with recurrent furunculosis*

Zaburzenia równowagi pomiędzy komórkami Treg i Th17 u pacjentów z rozpoznaniem nawracającej czyraczności

Danuta Nowicka[1, A D E F], **Ewelina Grywalska**[2, B C D F], **Agnieszka Grafka**[3, B], **Elżbieta Fitas**[2, B F], **Agata Surdacka**[2, B B], **Jacek Roliński**[2, A G]

[1]Department of Dermatology, Venereology and Allergology, Wrocław Medical University, Wroclaw, Poland
[2]Department of Clinical Immunology and Immunotherapy, Medical University of Lublin, Lublin, Poland
[3]St. John's Cancer Center, Lublin, Poland

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Aim: Tregs and Th17 functioning may be impaired in patients with recurrent furunculosis (RF); therefore, we analyzed changes in CD4+ lymphocyte T subsets (Treg/Th17) in patients with RF, and assessed the relationships between increased susceptibility to infections and Treg/Th17 status in RF patients and healthy subjects.

Material/Methods: Peripheral blood samples from 30 patients with RF and 20 healthy age – and sex-matched subjects were examined. The percentage and number of Th17 cells, Tregs, and other basic lymphocyte subsets were examined.

Results: Tregs and CD3+CD4+ count was significantly lower in patients with RF ($p < 0.0001$ and $p = 0.0003$), while Th17 and CD19+CD25+ cell count was significantly higher ($p = 0.0450$ and $p = 0.0119$) in comparison with controls. Strong positive correlations occurred between the following subsets of cells: Th17 and Th CD3+CD4+($r = 0.55$, $p<0.05$); CD3+CD4+T17 and CD3+CD4+ lymphocytes ($r = 0.66$, $p <0.05$); CD3+CD4+ lymphocytes and CD3+CD25+ lymphocytes ($r = 0.69$, $p <0.05$), and between lymphocytes B and CD19+CD25+ lymphocytes ($r = 0.81$).

Conclusions: Proportions of subpopulations of lymphocytes in patients with RF differ to those in healthy subjects. It is possible that a decrease in the percentage and number of Tregs together with a decrease in the number of CD3+CD4+ and increase in the percentage and number of Th17 may contribute to the pathogenesis of RF.

Keywords: **staphylococcal infection • staphylococcosis • recurrent furunculosis • immune response • skin infection • *in vitro* study**

GICID 01.3001.0013.1136
DOI: 10.5604/01.3001.0013.1136
Word count: 2298
Tables: 1
Figures: 1
References: 22

*This study was supported by the National Science Center, Poland [grant numbers: UMO-2011/01/N/NZ6/01762 and UMO-2016/21/B/NZ6/02279], and grant no. DS460 of the Medical University of Lublin]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author's address: Danuta Nowicka MD, PhD, Department of Dermatology, Wroclaw Medical University, Wroclaw, Poland; T. Chalubinskiego str 1; 50-368 Wroclaw, Poland; e-mail: danuta.nowicka@umed.wroc.pl

Abbreviations: **IL** – interleukin; **PBMC** – peripheral blood mononuclear cells; Th17 – T helper 17 cell; **Treg** – regulatory T cell.

INTRODUCTION

Regulatory T cells (Tregs) constitute only approximately 10% of a population of CD4+ cells in the peripheral blood, but they play an important role in many physiologic and pathologic processes such as autoimmune reactions, infections, and carcinogenesis. They can be identified with a flow cytometer based on the presence of CD4 and CD25 molecules on their surface as well as intracellular expression of FOXP3 antigen [12]. The presence of CD25 antigen is equivalent to the interleukin (IL)-2R receptor alpha-chain. Thanks to that, Tregs need IL-2 for proliferation, functioning and survival; however, they do not produce IL-2 themselves [5].

Expression of FOXP3 is characteristic for Tregs which differentiate in the thymus (induced regulatory-iTreg) in response to own antigens and for those native T-cells which differentiate based on their peripheral activation, outside of the thymus, in the vivo or in vitro (peripherally induced regulatory-pTreg) [1, 22]. The presence of FOXP3 in Tregs is responsible for their dual activity: at low concentration of exogenous IL-2, Tregs suppress other cells from the CD4+ subpopulation, while at a high concentration, this activity becomes abrogated [13]. The activity of Tregs affects helper cells – CD4+, suppressor cells – CD4+CD8+ and Tregs with reciprocal functioning – Th 17 cells. Th17 cells secrete IL-17A and they affect development of inflammatory and autoimmune processes as well as development of cancer diseases [4]. In sepsis, infection triggers the systemic inflammatory response which is accompanied by defective immune responses. When sepsis is caused by superantigen-producing organisms, an additional mechanism is activated in which direct superantigen-stimulation of CD25 – FOX P3 – T cells results in more T cells with regulatory function [18].

Furunculosis, as well as chronic and recurrent folliculitis caused by *Staphylococcus aureus* stains, engage the immune system, both specific and non-specific responses [15]. It seems interesting what the exact mechanisms predispose to the development of this chronic disease which frequently appear in inpatient and outpatient settings [9].

It was proven that Th17 cells are important for immune response to extracellular bacterial pathogens and to some fungi and viruses. Their role was detected in *S. aureus, Streptococcus pneumoniae* and *Klebsiella pneumoniae* infections among others [6, 16].

The aim of the present study was to analyze possible changes in CD4+ lymphocyte T subsets, especially Treg and Th17, in patients with recurrent furunculosis, and to assess the relations between increased susceptibility to infections and Treg/Th17 status in this group in comparison to healthy controls.

MATERIALS AND METHODS

Study and control groups

Between 2014 and 2017, we recruited 30 dermatological patients (15 men and 15 women) and 20 healthy age – and sex-matched volunteers who served as a control group. All patients were being treated for recurrent furunculosis at the Outpatient Immunology Clinic of the Medical University of Lublin. The number of furuncles varied from 4 to 20 per year. Duration of the disease was from 1 to 7 years. The current study is a part of a series on explaining pathogenesis of recurrent furunculosis [14].

The study group included 30 patients aged between 19 and 44 years (average 31.1 years). Recurrent furunculosis started at the average age of 27.7 ±2.8 years (range 17-42 years). The duration of the disease varied from 1 to 7 years. Remission period lasted from 4 to 20 years. Patients received from 4 to 20 antibiotic therapy courses and underwent from 4 to 12 surgeries. The control group included 20 healthy, age – and sex-matched volunteers with the mean age 31.95± 4.1 years (18-48).

Each patient denied undergoing immunosuppressive or immunomodulative treatment. None of the patients presented with signs of infection at the moment of enrollment and at least 1 month before the study. None suffered from autoimmune diseases, diabetes, cancer, and allergy. None reported receiving blood transfusions in the past.

This study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The research was approved by the Local Ethical Committee at the Medical University of Lublin. All patients gave their written informed consent prior to entering the study.

Blood collection

From each patient and volunteer, 5 mL of peripheral blood was drawn from the basilic vein for the frequencies of selected lymphocyte subsets and collected to tubes with the anticoagulant EDTA. Percentages of lymphocyte subsets were assessed on fresh peripheral blood samples.

Isolation of peripheral blood cells and the detection of Th17 and Treg cells

Isolation of peripheral blood cells and the detection of Th17 and Treg cells were performed as described previously [13]. Peripheral blood mononuclear cells (PBMCs) were aseptically separated by standard density gradient centrifugation (Gradisol L, Aqua Medic, Lodz, Poland). In order to detect Th17 cells, they were resuspended in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), 2 mM l-glutamine, 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, US), and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Mononuclear cells were stimulated for 5 h at 37°C in 5% CO_2 with 25 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma Chemical C., St. Louis, MO, USA), 1 µg/mL of ionomycin (Sigma Aldrich, St. Louis, MO, USA) and 10 µg/mL of brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) in order to block the intracellular transport processes, which results in the accumulation of cytokine proteins on the Golgi complex. Following stimulation, PBMCs were collected, washed with phosphate-buffered saline (PBS) solution, and added to cell culture media at a final concentration of 106 cells/mL. Leukocyte viability was tested with 1% trypan blue exclusion method. Finally, they were stained with anti-CD3 CyChrome and anti-CD4 fluorescein-isothiocyanate (FITC) conjugated monoclonal antibodies (Becton Dickinson, San Diego, CA, USA). The cell membranes were permeabilized for 15 min at 4°C with a Cytofix/Cytoperm Kit (BD Pharmingen, San Jose, CA, USA). Next, mononuclear cells were washed twice with PBS. The permeabilized cells were stained with a PE-conjugated anti-human IL-17A monoclonal antibody (eBioscience, San Diego, CA, USA). After this procedure, cells were washed twice with PBS.

In order to detect Treg CD4(+)CD25(+)Foxp3(+) cells, the cell surface and intracellular antigens were determined on the fresh cells at the time of the sample submission by cell staining according to the manufacturer's protocols. For each, 5 µL of an appropriate solution of anti-Human CD25 phycoerythrin (PE) and anti-Human CD4 PE-Cy5-conjugated antibodies (BioLegend, San Diego, CA, USA) was added to 500 µL of cell suspension, incubated for 30 min at 4°C in the dark, centrifuged, washed twice by adding 1 mL of cold PBS, 1% sodium azide, and 1% FCS to each tube, and next centrifuged again at 400 × g for 10 min. The standard incubation was carried out with antibodies directed against surface markers followed by the incubation by fixation and permeabilization with FoxP3 Fix/Perm Buffer and FoxP3 Perm Buffer (BioLegend, San Diego, CA, USA). Next, antibodies were directed against the intracellular protein FoxP3–anti-Human FoxP3 (Pacific Blue) monoclonal antibody (BioLegend, San Diego, CA, USA) by subsequent incubation. At the end, the supernatant was separated and washed. Each sample was suspended in 200 µL of PBS.

Immediately after preparation, cells were measured with the Becton Dickinson Canto II flow cytometer (Becton Dickinson, San Diego, CA, USA) and analyzed with FACSDiva™ Software (Becton Dickinson, San Diego, CA, USA). The percentage of CD45+ cells stained with the antibody was calculated based on the comparison with the control samples. To increase precision of measurements, background fluorescence was determined using isotype-matched directly conjugated mouse anti IgG1/IgG2α monoclonal antibodies. Cell aggregates and debris were excluded from analysis using forward scatter versus side scatter for gating live cell population [10].

Assessment of basic lymphocyte subsets

Three-color immunofluorescence analyzes were performed using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with a 488 nm argon laser. A minimum of 10,000 events were acquired and analyzed using CellQuest Software. Mean fluorescence intensity (MFI) and the percentage of cells expressing surface markers were analyzed. The cells were phenotypically characterized by incubation (20 min in the dark at room temperature) with a combination of relevant fluorescein isothiocyanate (FITC)–phycoerythrin (PE)–and CyChrome-labeled monoclonal antibodies. Immunofluorescence studies were performed using a combination of the following mAbs: CD3 FITC/CD19 PE, CD8 FITC/CD4 PE, CD25 CyChrome, purchased from Becton Dickinson (San Diego, CA, USA) [8].

STATISTICAL ANALYSIS

For statistical analysis, the R Project for Statistical Computing v. 3.4 was used. Descriptive statistics were calculated. The Student's t-test, F-test, and the Brown-Forsythe were used to carry out a comparison between groups of variables. Associations between cell counts in patients with recurrent furunculosis were assessed with Pearson coefficient for dependent variables. Differences were considered statistically significant at $p<0.05$.

RESULTS

In the group of patients with recurrent furuncles, Tregs count was significantly lower in comparison with the Tregs count in healthy controls ($p=0.000001$) and similarly to this, CD3+ CD4+T helper lymphocyte count was also significantly lower ($p=0.0002$), while Th17 count was

significantly higher than in the control group (p=0.04), despite the lack of difference in B lymphocyte count between both groups. Additionally, no significant difference in CD3+ CD25+T lymphocyte count between the study group and the control group was found. Results are presented in Table 1 and Figure 1.

Results of cytometry of peripheral blood mononuclear cells are presented in Table 1 and Figure 1.

The study revealed that Tregs count was positively correlated with Th17 cells (r=0.21, p<0.05) and with CD3+CD4+(r=0.55, p<0.05), while moderately with CD3+CD25+ cells count. Those cells correlated with B lymphocytes (r=-0.42, p<0.05) and CD19+CD25+ cells (r=-0.19, p<0.05).

T17 lymphocyte count correlated with CD3+CD4+ lymphocyte count (r=0.66, p<0.05), moderately with CD3+CD25+ lymphocyte count (r=0.45, p<0.05) and with CD19+CD25+ lymphocyte count and B lymphocyte count (r=0.10 and r=0.01, p<0.05, respectively). Additionally, positive correlation was found between CD3+CD4+ lymphocyte count and CD3+CD25+ lymphocyte count (r=0.69, p<0.05), while positive between CD3+CD4+ lymphocyte count and CD19+CD25+ lymphocyte count (r=0.14, p<0.05).

Lymphocyte B count was correlated with CD19+CD25+ lymphocyte count (r=0.81, p<0.05), with both CD3+CD4+ lymphocyte count (r=0.14, p<0.05) and CD3+CD25+ lymphocyte count (r=0.10, p<0.05). Also, CD3+CD25+ lymphocyte count was positively correlated with CD19+CD25+ lymphocyte count (r=0.17, p<0.05).

DISCUSSION

Tregs are responsible for silencing the immune response and inducing immune tolerance [17, 19]. Significant reduction in their number in patients with recurrent furunculosis may explain frequent clinical manifestations of infection. A marked predisposition to inflammatory processes due to the lack of inhibiting factors such as a decrease in the number of Tregs is stimulated by an increase in the number of Th17 cells which produce pro-inflammatory cytokine IL-17 [3, 21]. Also, a significant decrease in the number of CD3+CD4+ T helper lymphocytes observed in the group of patients with recurrent furunculosis weakens the killing capacity in the scope of specific cellular immunity. Interestingly in patients with recurrent furunculosis, a significantly elevated percentage of T and B lymphocytes with the presence of IL-2 receptors on their surface that is CD3+CD25+ and CD19+CD25+ cells was observed.

A CD25+ molecule is mainly expressed on resting Tregs – Treg FOXP3+ and temporarily on activated T and B cells [18, 20]. In our study, although the number of Th and B lymphocytes was lower in patients with furunculosis than in healthy subjects, the expression of CD25 molecule appeared on a greater number of cells. Additionally in patients with recurrent furunculosis, the CD25+ molecule on resting Tregs FOXP3+ was expressed to a small extent, which does not preserve proper function of Tregs because their percentage and number is significantly smaller than in healthy subjects.

It was experimentally proved that an increase in CD25+ expression may appear on allogeneic CD45+ lymphocytes as an effect of the culturing of these cells with human fibroblasts [11]. The number of CD3+CD25+ lymphocytes is positively correlated with the number of both Tregs and Th17 with similar strength, but this relationship is the strongest with CD3+CD4+ cells. This suggests that they may support processes of both inhibiting and stimulating of inflammatory reaction as well as assist acquired cellular immunity due to strong positive correlation with the number of CD3+CD4+ cells. This correlation is even strongest than this between CD3+CD4+ cells and T17 cells. Experiments showed that it is possible to induce the expression of CD25 antigen on CD4+ CD25 – T helper lymphocytes by 24-hour stimulation of peripheral blood mononuclear cells with *S. aureus* 161:2-cell free supernatant or staphylococcal enterotoxin A. Also, expression of FOXP3 on CD4 Th cells with the possibility to produce IL-10, INF-γ and IL-17A to a smaller extent may be triggered [2]. The experience of Bjorkander's team and other researchers indicate that the possibility for changing the cell phenotype under stimulation by microbiological

Table 1. Comparison of the mean cell counts (G/l) measured with cytometry of peripheral blood mononuclear cells between the study group and the control group

Parameter	Study group (mean ± SD)	Control group (mean ± SD)	P value
Regulatory T-cells	0.0130±0.0071	0.0500± 0.0202	<0.0001
Th17	0.0828±0.0651	0.0489± 0.0368	0.0450
CD3+CD4+	0.4269±0.1770	0.6452± 0.2141	0.0003
CD3+CD25+	0.1384±0.0767	0.1132± 0.0577	0.2167
B lymphocytes	0.2272±0.0971	0.2168± 0.1127	0.7294
CD19+CD25+	0.0093±0.0098	0.0033± 0.0034	0.0119

SD, standard deviation; significant difference marked in bold

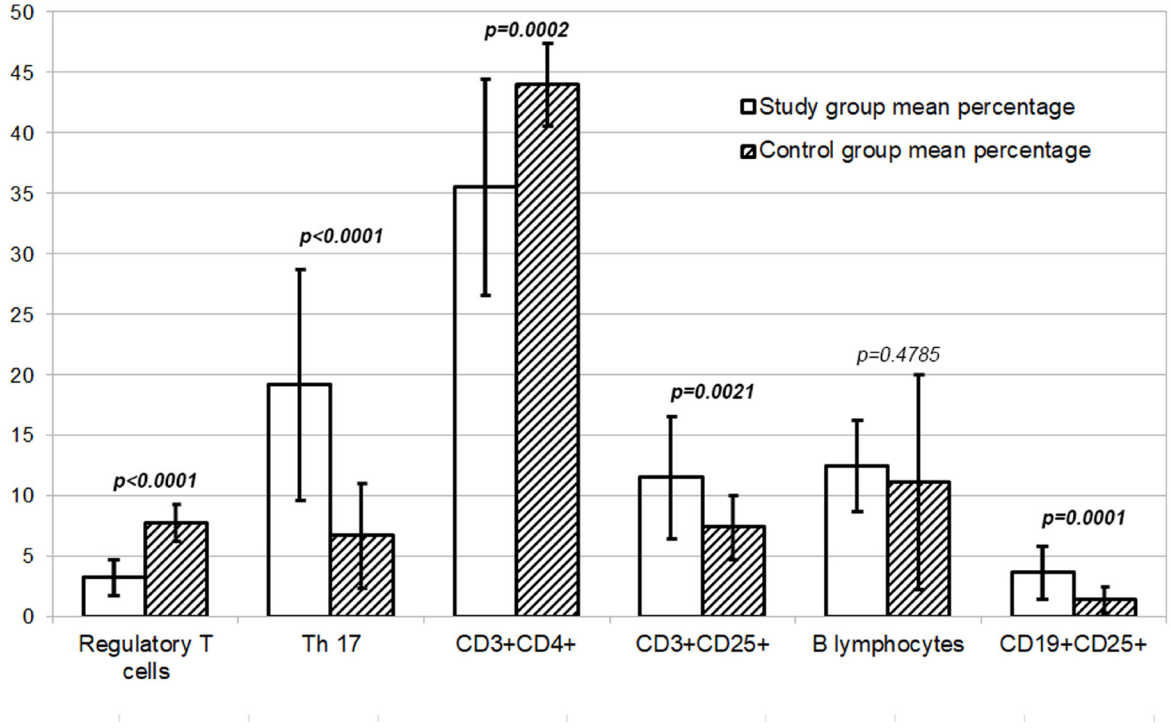

Fig. 1. Comparison of the mean percentage of cells between the study group and the control group

factors exists [2, 7]. This fact should also explain intensive expression of CD25 antigen on T and B lymphocytes in patients with recurrent furunculosis. Although the number of B lymphocytes was similar in patients with furunculosis and healthy subjects, both the percentage and number of CD19+CD25 + cells are higher in the group of patients with recurrent furunculosis. Negative, though weak correlation indicates that relationships between those two populations of cells are affected by considerable decrease in the percentage and number of Tregs.

It is not possible to answer the question why in recurrent furunculosis, Tregs are presented in such a small percentage and number. The cause of a decrease in stimulation of proper Tregs production is unknown. However, it seems that changes in Tregs number along with a decrease in Th CD3+CD4+ and an increase in the percentage and number of Th17 are a very important link in the pathogenesis of recurrent furunculosis.

CONCLUSIONS

Proportions of subpopulations of lymphocytes in patients with recurrent furunculosis in remission stage differ to those in healthy subjects. It is possible that a decrease in the percentage and number of Tregs together with a decrease in the number of Th CD3+CD4+ and increase in the percentage and number of Th17 may contribute to the pathogenesis of recurrent furunculosis.

REFERENCES

[1] Abbas A.K., Benoist C., Bluestone J.A., Campbell D.J., Ghosh S., Hori S., Jiang S., Kuchroo V.K., Mathis D., Roncarolo M.G., Rudensky A., Sakaguchi S., Shevach E.M., Vignali D.A., Ziegler S.F.: Regulatory T cells: recommendations to simplify the nomenclature. Nat. Immunol., 2013; 14: 307-308

[2] Bjorkander S., Hell L., Johansson M.A., Forsberg M.M., Lasaviciute G., Roos S., Holmlund U., Sverremark-Ekstrom E.: *Staphylococcus aureus*-derived factors induce IL-10, IFN-γ and IL-17A-expressing FOXP3$^+$CD161$^+$ T-helper cells in a partly monocyte-dependent manner. Sci. Rep., 2016; 6: 22083

[3] Byrd A.L., Deming C., Cassidy S.K., Harrison O.J., Ng W.I., Conlan S., NISC Comparative Sequencing Program, Belkaid Y., Segre J.A., Kong H.H.: *Staphylococcus aureus* and *Staphylococcus epidermidis* strain diversity underlying pediatric atopic dermatitis. Sci. Transl. Med., 2017; 9: eaaI4651

[4] Chen K., Kolls J.K.: Interluekin-17A (IL17A). Gene, 2017; 614: 8-14

[5] Chinen T., Kannan A.K., Levine A.G., Fan X., Klein U., Zheng Y., Gasteiger G., Feng Y., Fontenot J.D., Rudensky A.Y.: An essential role for the IL-2 receptor in Treg cell function. Nat. Immunol., 2016; 17: 1322-1333

[6] Cruciani M., Etna M.P., Camilli R., Giacomini E., Percario Z.A., Severa M., Sandini S., Rizzo F., Brandi V., Balsamo G., Polticelli F., Affabris E.,Pantosti A., Bagnoli F., Coccia E.M.: *Staphylococcus aureus* Esx factors control human dendritic cell functions conditioning Th1/Th17 response. Front. Cell. Infect. Microbiol., 2017; 7: 330

[7] Duhen T., Duhen R., Lanzavecchia A., Sallusto F., Campbell D.J.: Functionally distinct subsets of human FOXP3^{+} Treg cells that phenotypically mirror effector Th cells. Blood, 2012; 119: 4430-4440

[8] Grywalska E., Surdacka A., Miturski A., Kwaśniewski W., Malec A., Olender A., Wallner G., Roliński J.: Characterisation of lymphocyte subsets in asplenic patients – preliminary report. Centr. Eur. J. Immunol., 2010; 35: 239-244

[9] Ibler K.S., Kromann C.B.: Recurrent furunculosis – challenges and management: a review. Clin. Cosmet. Investig, Dermatol., 2014; 7: 59-64

[10] Klatka M., Grywalska E., Partyka M., Charytanowicz M., Kiszczak-Bochynska E., Rolinski J.: Th17 and Treg cells in adolescents with Graves' disease. Impact of treatment with methimazole on these cell subsets. Autoimmunity, 2014; 47: 201-211

[11] Lu Q., Yu M., Shen C., Chen X., Feng T., Yao Y., Li J., Li H., Tu W.: Negligible immunogenicity of induced pluripotent stem cells derived from human skin fibroblasts. PLoS One, 2014; 9: e114949

[12] Min B.: Heterogeneity and stability in Foxp3+ regulatory T cells. J. Interferon Cytokine Res., 2017; 37: 386-397

[13] Moon B.I., Kim T.H., Seoh J.Y.: Functional modulation of regulatory T cells by IL-2. PLoS One, 2015; 10: e0141864

[14] Nowicka D., Grywalska E., Fitas E., Mielnik M., Rolinski J.: NK and NKT-like cells in patients with recurrent furunculosis. Arch. Immunol. Ther. Exp., 2018; 66: 315-319

[15] Nurjadi D., Kain M., Marcinek P., Gaile M., Heeg K., Zanger P.: Ratio of T-helper type 1 (Th1) to Th17 cytokines in whole blood is associated with human β-defensin 3 expression in skin and persistent *Staphylococcus aureus* nasal carriage. J. Infect. Dis., 2016; 214: 1744-1751

[16] Sardana K., Verma G.: *Propionibacterium acnes* and the Th1/Th17 axis, implications in acne pathogenesis and treatment. Indian J. Dermatol., 2017; 62: 392-394

[17] Szulc-Dabrowska L., Gierynska M., Depczynska D., Schollenberger A., Toka F.N.: Th17 lymphocytes in bacterial infections. Postępy Hig. Med. Dośw., 2015; 69: 398-417

[18] Taylor A.L., Llewelyn M.J.: Superantigen-induced proliferation of human CD4^{+}CD25 – T cells is followed by a switch to a functional regulatory phenotype. J. Immunol., 2010; 185: 6591-6598

[19] Tilahun A.Y., Chowdhary V.R., David C.S., Rajagopalan G.: Systemic inflammatory response elicited by superantigen destabilizes T regulatory cells, rendering them ineffective during toxic shock syndrome. J. Immunol., 2014; 193: 2919-2930

[20] Triplett T.A., Curti B.D., Bonafede P.R., Miller W.L., Walker E.B., Weinberg A.D.: Defining a functionally distinct subset of human memory CD4^{+} T cells that are CD25^{POS} and FOXP3^{NEG}. Eur. J. Immunol., 2012; 42: 1893-1905

[21] Zhao Y., Zhou M., Gao Y., Liu H., Yang W., Yue J., Chen D.: Shifted T helper cell polarization in a murine *Staphylococcus aureus* mastitis model. PLoS One, 2015; 10: e0134797

[22] Zhu J.: T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine, 2015; 75: 14-24

The authors have no potential conflicts of interest to declare.