ИЗМЕНЕНИЯ СУБПОПУЛЯЦИОННОГО СОСТАВА Т-ХЕЛПЕРОВ 17 ТИПА И ЦИТОКИНОВОГО ПРОФИЛЯ В ЗАВИСИМОСТИ ОТ КЛИНИЧЕСКОЙ КАРТИНЫ САРКОИДОЗА

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Резюме. При саркоидозе происходит гиперактивация клеток иммунной системы, а продуцируемые ими цитокины играют важную роль в патогенезе заболевания. Клеточно-опосредованные реакции являются основными в иммунопатогенезе саркоидоза. В их развитии участвуют субпопуляции Т-хелперов (Th), в том числе Th17-типа и регулирующих их цитокинов. Были исследованы образцы плазмы крови больных саркоидозом (n = 123), 18% с острым и 82% с хроническим течением. Контрольная группа – образцы, полученные от практически здоровых лиц (n = 43). Определялось содержание субпопуляций лимфоцитов методом проточной цитофлуориметрии и концентраций цитокинов (пг/мл) методом мультиплексного анализа по технологии xMAP (Luminex). Содержание «классических» Th17 достоверно снижено в образцах больных острым саркоидозом относительно хронического: 28,3% против 33,3%, p = 0,046. Уровень «дважды-позитивных» Th17 (DP Th17) был достоверно повышен в образцах крови больных с хроническим и острым саркоидозом относительно группы контроля: 31,7% и 34,2% против 26,2%, p < 0,001; содержание «не классических» Th17.1 оказалось достоверно снижено у больных хроническим течением относительно условно здоровых лиц: 27,9% и 35,9%, p < 0,001. Анализ клинико-лабораторной значимости определения DP Th17 среди CD45RA-негативных клеток Th эффекторной памяти выявил, что при остром саркоидозе относительно группы условно здоровых лиц: чувствительность – 82%; специфичность – 71%; при хроническом: 67% и 56%

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соответственно. У больных саркоидозом относительно условно здоровых лиц отмечалось достоверно значимое повышение уровней цитокинов IL-12 (p70) – 1,3 против 0,56, p = 0,028; IL-17A – 1,5 против 0,43, p < 0,001; IFNγ – 4,1 против 1,1, p < 0,001; TNFα – 21,7 против 6,7 пг/мл, p < 0,001. Таким образом, выявление субпопуляций $CCR6^{+}$Th17 и DP Th17, а также уровни синтезируемых такими клетками цитокинов, являются важными в диагностике саркоидоза при его разном течении: показана прямая корреляционная зависимость между уровнем активности ангиотензин-превращающего фермента и относительным содержанием DP Th17-клеток памяти; у больных с прогрессирующим течением в сравнении с регрессирующим течением заболевания было достоверно снижено абсолютное содержание всех $CD45RA^{-}$Th17-клеток памяти и клеток центральной памяти; у больных с экстрапульмональными проявлениями саркоидоза отмечалось достоверно повышенное относительное содержание DP Th17 $CD45RA^{-}$ и DP Th17 эффекторной памяти; при хроническом саркоидозе были достоверно повышены концентрации IL-17A, IFNγ, IL-12 и обнаружена положительная корреляция между уровнем IFNγ и активностью ангиотензин-превращающего фермента.

Ключевые слова: саркоидоз, Т-хелперы, дифференцировка Т-хелперов, Т-хелперы 17 типа, проточная цитометрия, цитокины

SARCOIDOSIS CLINICAL PICTURE GOVERNS ALTERATIONS IN TYPE 17 T HELPER CELL SUBSET COMPOSITION AND CYTOKINE PROFILE

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Abstract. Immune cell hyperactivation along with cytokines they overproduce plays an important role in sarcoidosis and related disease pathogenesis. A central place in the immunopathogenesis of sarcoidosis is held by diverse cell-mediated reactions governed by T helper (Th) cell populations including Th17 subsets and relevant signature cytokines. We studied peripheral blood plasma samples of the patients with sarcoidosis (n = 123): 18% with acute and 82% with chronic course. The control group – samples from healthy volunteers (n = 43). T cell subset composition was assessed by flow cytometry. Cytokine concentrations (pg/mL) were measured by multiplex analysis using xMAP technology (Luminex). The level of "classical" Th17 turned out to be significantly reduced in acute *vs* chronic sarcoidosis: 28.3% *vs* 33.3% (p = 0.046). The level of "double-positive" Th17 (DP Th17) was significantly increased in chronic and acute *vs* control group: 31.7% and 34.2% *vs* 26.2% (p < 0.001 in both cases), without differences patient inter-group; "non-classical" Th17.1 were shown to have significantly reduced level only in chronic *vs* healthy subjects: 27.9% and 35.9% (p < 0.001). Clinical and laboratory diagnostic characteristics for blood DP Th17 levels in CD45RA-negative Th effector memory cells in sarcoidosis: in acute sarcoidosis *vs* healthy subjects, they were characterized by sensitivity – 82%; specificity – 71%, whereas in chronic: 67% and 56%, respectively. In patients with sarcoidosis *vs* healthy subjects were found to have significantly increased level of IL-12 (p70) – 1.3 *vs* 0.56, p = 0.028; IL-17A – 1.5 *vs* 0.43, p < 0.001; IFNγ – 4.1 *vs* 1.1, p < 0.001; TNFα – 21.7 *vs* 6.7, p < 0.001. Thus, $CCR6^{+}$ Th17 and DP Th17 subsets and relevant signature cytokines are important in diagnostics of sarcoidosis of varying clinical course: a direct correlation was shown between the level of angiotensin-converting enzyme activity and percentage of memory DP Th17; disease progression *vs* regression had significantly reduced absolute number of total $CD45RA^{-}$ memory and CM Th17; extrapulmonary manifestations had a significantly increased percentage of DP Th17 $CD45RA^{-}$ and EM DP Th17; in chronic sarcoidosis are significantly increased concentration of IL-17A, IFNγ, IL-12 and positively correlation between IFNγ and the activity of angiotensin-converting enzyme.

Keywords: sarcoidosis, $CD4^{+}$ T cells, Th cell differentiation, Th17 cell subsets, flow cytometry, cytokines

Introduction

Sarcoidosis is a multisystem inflammatory granulomatous disease of unknown etiology characterized by developing highly organized immune cell aggregates (granulomas) lacking signs of necrosis in the affected organs, most commonly found in the respiratory organs [12, 14, 16]. Sarcoid granulomas are predominantly localized in the bronchopulmonary lymph nodes as well as directly in the lung tissue, less commonly being observed in other anatomical sites. Extrapulmonary or systemic sarcoidosis manifestations are presented due to damage to the skin, eyes, joints, heart, nervous system and some other organs [4, 14, 16].

Immune cell hyperactivation along with cytokines and chemokines they overproduce plays an important role in developing sarcoid granuloma and related disease pathogenesis [13, 16]. According to current concepts, a central place in the immunopathogenesis of sarcoidosis is held by diverse cell-mediated reactions including T helper cell subsets (Th): Th1, Th17 types, as well as relatively recently described "non-classical" or "plastic" Th17 subpopulations (Th1/Th17 and Th17.1), expressing surface chemokine receptors and capable of simultaneously producing several signature cytokines [5, 13, 16, 18]. It was shown that various Th17 subsets and relevant cytokines (IL-17A, IL-22, IFNγ) also take part in granuloma formation. Currently, special attention is paid to the role of Th17 cells in the immunopathogenesis, course and outcome of sarcoidosis, which are detected in the center and along the periphery of sarcoid granulomas [3, 4, 12, 13, 16].

Alterations in cytokine profile and the effects related to IFNγ, IL-12 and TNFα may result in transition of Th1/Th17 cells to plastic IFNγ-producing Th17, called "non-classical" Th17 [8, 17, 18]. Th17 cells include IFNγ/IL-17A-producing double positive (Th1/Th17 cells) and IFNγ single positive cell subsets defined on the basis of surface expression of chemokine receptors: Th17.1 cells [3, 13] co-expressing CCR6 (Th17 lineage) and CXCR3 (Th1 lineage) [17].

While investigating immune-related pathogenesis of sarcoidosis it is important to assess cytokine and chemokine profile ensuring directed migration of immune cells from the circulation to the focus of inflammation followed by sarcoid granuloma formation [1, 2, 3, 11, 13, 14]. Moreover, both cytokines and diverse immune cell types largely account for resolution or outcomes of granulomatous process in sarcoidosis, from resorption of granulomas in spontaneous remission to developing pulmonary fibrosis in unfavorable scenario, are also largely determined by the participation of various cells and cytokines [1, 2, 4, 12, 13].

Various studies in the field often report contradictory data, therefore underlying a need to search for and identify most diagnostically significant and informative markers (T cell subset composition and cytokine profile) aligned with disease clinical signs and course. Thus, **the aim of the study** was to assess type 17 T helper cell subset composition and some cytokine levels in accordance to clinical course of sarcoidosis.

Materials and methods

Patients with sarcoidosis (n = 123) were enrolled to the study, of which 18% (22/123) with acute ("Löfgren's syndrome") and 82% (101/123) with chronic disease course ("non-Löfgren's syndrome") were examined at the Research Institute of Interstitial and Orphan Lung Diseases, Pavlov First Saint Petersburg State Medical University, Ministry of Health of Russia. Sarcoidosis in all patients examined was verified as first-onset disease, without therapeutic interventions applied at time of examination.

Informed consent to participate in the study was obtained from all patients. Studies were conducted in accordance with the Declaration of Helsinki of the World Association "Ethical principles for conducting scientific medical research involving humans" amended in 2013, "Rules of Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Health of the Russian Federation dated of 19.06.2003 No. 266 and the "Rules of Good Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Health of the Russian Federation dated 01.04.2016 No. 200n.

Patients with sarcoidosis were enrolled to main group: 49 males, 74 females, aged 20 to 67 years. Diagnosis in all patients was established based on comprehensive clinical and radiological (including chest computed tomography) study. The diagnosis of sarcoidosis was verified by histological examination after biopsy of bronchopulmonary tissue and/or mediastinal lymph nodes was collected in 67% (82/123) patients as well as according to clinical and radiological data in 33% (41/123) patients. In the control group, there were analyzed biological samples from 43 apparently healthy subjects lacking any clinical manifestations including respiratory diseases (18 males, 25 females, aged 20 to 61 years), age- and sex-matched to sarcoidosis patients.

According to the recommendations of the American Thoracic Society (ATS), the European Respiratory Society (ERS), and the World Association of Sarcoidosis and Other Granulomatosis (WASOG) [6], radiological classification was used to characterize patient groups. Patients were examined by using generally accepted methods (analyzed complaints, history of disease, life, occupational anamnesis, physical examination, palpation, percussion, auscultation, clinical blood count, general urinalysis, biochemical blood and urine tests for assessing disease course and activity). All patients underwent chest multislice computed tomography (MSCT), a comprehensive functional examination of external respiration

(CFEER), echodopplercardiography (EchodopplerCG) based on calculated systolic pressure in the pulmonary artery (SPPA, mm Hg), abdominal ultrasound examination, videothoracoscopy (VTS), if necessary, was performed with a biopsy of the lung tissue or mediastinal lymph node, fibrobronchoscopy (FBS) with endobronchial biopsy of the bronchial mucosa or transbronchial biopsy of the lung tissue, followed by histological examination in laboratories and departments of the Pavlov First Saint Petersburg State Medical University.

Based on the aforementioned data, patients were stratified as follows: group with pulmonary sarcoidosis without systemic disease manifestations (44%, 39/88) and group with respiratory sarcoidosis along with extrapulmonary manifestations (56%, 49/88).

Level of blood serum angiotensin-converting enzyme (ACE) was used to assess disease activity. Units of measurement – units of activity: ACE Unit (= 1 IU/mL). Reference values for ACE activity for subjects over 18 – 20-70 ACE Units. Accordingly, for patients with sarcoidosis elevated ACE activity level was set above 70 ACE Units.

Clinical disease course was assessed 3 months, six months and one year after establishing a verified diagnosis, in 38 patients enrolled to the study, based on the chest CT scan data, functional parameters and detected extrapulmonary manifestations. A retrospective analysis of immunological parameters was carried out depending on the clinical picture of the disease one year later by taking into account: presence or absence of spontaneous regression; severity of extrapulmonary manifestations; severity of pulmonary fibrosis.

There were investigated venous blood samples collected by puncture of peripheral vein into K3EDTA-containing vacuum tubes. T cell subset composition in peripheral blood samples was assessed by flow cytometry using a Navios diagnostic cytometer (Beckman Coulter, Inc., USA) equipped with three lasers with emittance at 405, 488, and 638 nm wavelengths. Data processing was carried out using Navios Software v. 1.2, Kaluza™ v. 2.0 (Beckman Coulter, USA). Various combinations of direct monoclonal antibodies were applied for immunophenotyping. The data on lymphocyte level are presented as follows: % – percentage out of total number of lymphocytes or examined lymphocyte subset; absolute (cell/μL, number of cells in 1 μL of peripheral blood) number of lymphocytes.

In the study, 100 μL of peripheral blood samples were stained with monoclonal antibodies in accordance with the manufacturer's recommendations; we have described the details previously [7]. Erythrocytes were removed by using VersaLyse lyse (Cat. No. A09777) adding 975 μL sample with *ex tempere* prepared 25 μL of IOTest 3 Fixative Solution (Cat. No. A07800). After incubation, the samples were washed once from unbound antibodies using phosphate buffered saline, by centrifugation for 7 minutes at 330g. The supernatant was removed, and cell pellet was resuspended in 200 μL of same solution (pH 7.2-7.4) containing 2% neutral paraformaldehyde (Cat. No. HT5011, Sigma-Aldrich, USA).

The following antibody cocktail was used to analyze peripheral blood Th cell subset composition: antibodies against CD3 (clone UCHT1) and CD4 (clone 13B8.2); Th cells were detected as $CD3^+CD4^+$ lymphocytes. In order to identify individual Th cell subsets at various differentiation stages, antibodies against surface CD45RA (clone 2H4LDH11LDB9 (2H4)) and CD62L (clone DREG56) were used. "Naive" Th cells bearing the $CD45RA^+CD62L^+$ phenotype were not used for further studies due to the lack of expressed surface chemokine receptors. CD45RA-positive terminally differentiated effector memory T cells (TEMRA) bearing $CD45RA^+CD62L^-$ phenotype were also excluded from further analysis due to almost full lack of such cell population in the peripheral blood in apparently healthy donors.

Memory Th cells were subdivided based on CD62L and CD45RA surface expression into central (CM) and effector (EM) memory T helper cells with $CD45RA^-CD62L^+$ and $CD45RA^-CD62L^-$ phenotypes, respectively. The expression levels of the following chemokine receptors were analyzed on Th cell subsets by using monoclonal antibodies: CD45RA-FITC, CD62L-PE, CXCR5- PerCP/Cy5.5, CCR6-PE/Cy7, CXCR3-APC, CD3-APC/Cy7, CD4-PacBlue, CCR4-BV510. We used antibodies against CD3, CD4, CD45RA and CD62L, respectively (Beckman Coulter, USA). Antibodies against CCR4, CCR6, CXCR3 and CXCR5, respectively (BioLegend, USA) were also used.

Polarized Th17 cell subset composition was analyzed by immunophenotyping as follows: four major Th17 cell subsets were identified based on expression patterns of surface chemokine receptors CXCR3 and CCR4, and according to the spectrum of functional properties in total pool of $CCR6^+$ cells of all CD45RA-negative, CM Th, and EM Th cells [15]. According to the phenotyping strategy, peripheral blood cells were divided into the following subsets: "classical" Th17 cells with $CCR4^+CXCR3^-$ phenotype, "double-positive" $CCR4^+CXCR3^+$ (DP Th17), "non-classical" $CCR4^-CXCR3^+$ (Th17.1) and "double-positive" $CCR4^-CXCR3^+$ (Th17.1), "double-negative" $CCR4^-CXCR3^-$ (DN Th17).

Blood plasma cytokine levels (pg/mL) were analyzed by using commercially available Milliplex MAP kits (Millipore, USA) along with magnetic microspheres (Milliplex Mag, USA), according to manufacturer's instructions. Data registration and analysis were carried out using Luminex MAGPIX instrument (Luminex, USA).

The data obtained were statistically processed by using software Statistica 8.0 (StatSoft, USA) and GraphPad Prism 5.00 for Windows (GraphPad Prism Software Inc., USA). For this, there were used standard

nonparametric methods of statistical processing. Quantitative data were presented as median (Me) and interquartile range ($Q_{0.25}$-$Q_{0.75}$). Non-parametric Mann–Whitney test, non-parametric Spearman rank correlation method and coefficient (r) calculation were applied. To determine a diagnostic relevance of the data, ROC analysis (receiver-operating-characteristic ROC curve) was used. The area under curve (AUC) of the operating characteristic, magnitude of the optimal point (criterion) of separation, sensitivity and specificity level were determined. Hypotheses were considered as statistically different with significance level set at $p < 0.05$.

Results and discussion

During the study there were analyzed parameters between main comparison groups: patients with acute onset of sarcoidosis – group 1, patients with chronic sarcoidosis – group 2, and control group – group 3.

It was found that CCR6-positive Th17 cell level was altered in EM Th cell subset composition. In particular, peripheral blood samples from patients with acute *vs* chronic sarcoidosis and control group were shown to have significantly increased absolute number of CCR6-positive Th17 cells: 73 cells/μL (60-150) *vs* 57 (38-88) and 59 cells/μL (46-75), respectively, at $p_{1-2} = 0.011$ and $p_{1-3} = 0.032$. However, no changes in percentage and absolute number of CM Th17 cells were observed (Table 1).

While assessing polarized Th17 cell subset composition in peripheral blood samples from patients with sarcoidosis, it was found that Th17 cell level was significantly altered in total CD45RA-negative memory T cell pool.

The level of "classical" Th17 cells turned out to be significantly reduced in acute *vs* chronic sarcoidosis: 28.3% (23.6-35.4) *vs* 33.3% (26.5-40.4) ($p = 0.046$). In addition, the level of DP Th17 cells was significantly increased in chronic and acute sarcoidosis *vs* control group: 31.7% (26.2-38.9) and 34.2% (29.1-42.7) *vs* 26.2% (22.6-28.3) ($p < 0.001$ in both cases), without differences between patient groups. Regarding "non-classical" Th17.1 cells, it was found that their level was significantly reduced only in chronic sarcoidosis *vs* healthy subjects: 27.9% (23.0-33.9) and 35.9% (26.5-41.3) ($p < 0.001$). A significant decline in level of DN Th17 cells was revealed in chronic and acute sarcoidosis *vs* control group: 4.7% (3.2-6.1) and 4.2% (2.7-5.3) *vs* 5.9% (4.4-7.7) ($p = 0.002$).

The data comparing the level of such Th17 cell subsets in CD45RA-negative CM and EM Th17 cells are shown in Figure 1 and Figure 2, respectively. Along with that, $CCR6^+$ CM Th cell subset level was significantly reduced for "non-classical" Th17.1 and DN Th17 cells in chronic sarcoidosis compared to control group: 22.6% (18.6-28.1) *vs* 27.5 % (21.8-34.3) ($p < 0.001$) and 5.6% (3.9-7.1) *vs* 6.7% (4.8-8.9) ($p = 0.004$), respectively. The level of CCR4-expressing DP Th17 cells in chronic sarcoidosis was significantly higher than in control group: 30.9% (26.2-37.4) *vs* 27.2% (23.6-31.6) ($p = 0.002$), whereas in acute disease it was noted to increase as compared to healthy subjects (32.3% (28.5-37.4) *vs* 27.2% (23.6-31.6), $p = 0.003$) along with decreased DN Th17 cell levels (5.3% (3.2-7.2) *vs* 6.7% (4.8-8.9) ($p = 0.017$).

While analyzing magnitude of CCR6-positive EM Th cells capable of exiting from the circulation and migrating to peripheral tissues, similar changes were observed. Patients with chronic and acute sarcoidosis *vs* control group were noted to have significantly reduced EM level of "non-classical" Th17.1 cells: 36.2% (30.3-44.9) and 40.3% (30.6-46.9) *vs* 45.9% (38.3-54.7) ($p < 0.001$ and $p = 0.017$, respectively). Moreover, patients with both chronic and acute onset sarcoidosis had a significantly increased DP Th17 cell level compared to control group: 30.9% (24.4-

TABLE 1. PERCENTAGE AND ABSOLUTE NUMBER OF PERIPHERAL BLOOD CENTRAL MEMORY (CM) AND EFFECTOR MEMORY (EM) Th17 CELL SUBSET LEVEL IN ACUTE AND CHRONIC SARCOIDOSIS COMPARED TO CONTROL SUBJECTS, Me ($Q_{0.25}$-$Q_{0.75}$)

Th cell subset		Group 1 (n = 22)	Group 2 (n = 101)	Group 3 (n = 43)	p
Th17 CM*	%	37.1 (32.8-41.3)	39.3 (33.4-46.3)	36.8 (31.6-43.3)	Th17 CM*
	cells/μL	84 (58-125)	83 (58-1110)	96 (76-143)	$p_{1-2} = 0.656$ $p_{1-3} = 0.343$ $p_{2-3} = 0.074$
Th17 EM**	%	57.5 (44.9-67.6)	58.6 (44.3-69.4)	51.3 (40.9-60.1)	Th17 EM**
	cells/μL	73 (60-150)	57 (38-88)	59 (46-75)	$p_{1-2} = 0.011$ $p_{1-3} = 0.032$ $p_{2-3} = 0.583$

Note. Significant differences according to the Mann–Whitney U test: p_{1-2}, differences between groups of patients with acute and chronic onset sarcoidosis; p_{1-3}, differences between groups of patients with acute onset sarcoidosis and apparently healthy subjects; p_{2-3}, differences between groups of patients with chronic onset sarcoidosis and apparently healthy subjects (control group). *, level within total CM Th cell population. **, level within total EM Th cell population.

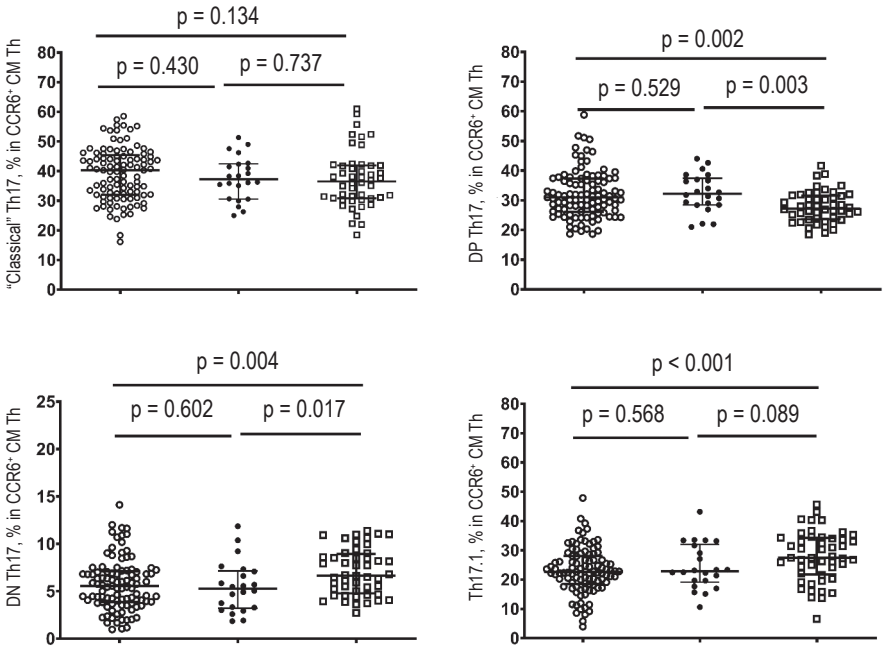

Figure 1. Major Th17 cell subset distribution within total CCR6$^+$ CD45RA$^-$CD62L$^+$ central memory (CM) Th cells in chronic (n = 101) and acute (n = 22) sarcoidosis as well as control subjects (n = 43)

Note. White circles, patients with chronic onset sarcoidosis; black circles, patients with acute onset sarcoidosis; white squares, group of apparently healthy donors. Results are presented as median and interquartile range (Me ($Q_{0.25}$-$Q_{0.75}$)). Differences between groups are presented based on nonparametric Mann–Whitney test. Phenotypes of major Th17 cell subsets are as follows: "classical" Th17 – CCR6$^+$CCR4$^+$CXCR3$^-$, "double-positive" DP Th17 – CCR6$^+$CCR4$^+$CXCR3$^+$, "non-classical" or Th17.1 – CCR6$^+$CCR4$^-$CXCR3$^+$, "double-negative" DN Th17 – CCR6$^+$CCR4$^-$CXCR3$^-$.

40.9) and 33.4% (27.1-52.9) *vs* 24.7% (20.2-27.5), respectively ($p < 0.001$).

To determine a relevance for data on percentage of peripheral blood DP Th17 cells in the examined patients, we analyzed operating characteristic curves (ROC analysis) by plotting representative ROC curves and calculating AUC. It was found that levels of DP Th17 among total CD45RA-negative memory cells in acute onset sarcoidosis *vs* control group were characterized with AUC = 0.812 ($p < 0.001$). Applying a separation criterion > 29%, the sensitivity was 82%, with specificity reaching 81%. In contrast, chronic sarcoidosis *vs* control group was associated with AUC = 0.727 ($p < 0.001$), separation criterion > 27%, sensitivity and specificity of 72% and 63%, respectively. The data on assessing DP Th17 cell level among CD45RA-negative EM Th cells in acute sarcoidosis *vs* healthy subjects were characterized by AUC = 0.807, $p < 0.001$, separation criterion > 27%, sensitivity – 82%; specificity – 71%, whereas in chronic disease: AUC = 0.709, $p < 0.001$, separation criterion > 25%, sensitivity – 67%; specificity – 56%.

A relation between level of peripheral blood Th17 cell subsets and ACE as a generally accepted laboratory parameter of disease activity was assessed. A correlation analysis revealed a direct relationship between ACE activity level and percentage of DP Th17 cells in total CD45RA-negative memory cells, DP Th17 CM and EM memory cells: r = 0.422, p = 0.001; r = 0.330, p = 0.012 and r = 0.410, p = 0.002, respectively. It was shown that chronic onset sarcoidosis was associated with positive correlations between level of ACE activity and percentage of DP Th17 CD45RA-negative, DP Th17 CM and DP Th17 EM memory cells: r = 0.498, $p < 0.001$; r = 0.366, p = 0.012, and r = 0.512, $p < 0.001$, respectively.

Previously, we reported on cytokine magnitude and highest relevance in patients with sarcoidosis aligned with the characteristics of the disease course [9, 10]. In particular, along with the quantitative characterization of plastic Th17 cells, there were analyzed levels of crucial cytokines they produce in patients with varying clinical course of sarcoidosis.

Blood plasma samples from patients with sarcoidosis *vs* healthy subjects were found to have significantly increased level of cytokines IL-12 (p70) – 1.3 pg/mL (0.56-2.0) *vs* 0.56 pg/mL (0.23-1.3), p = 0.028; IL-17A/CTLA8 – 1.5 pg/mL (0.44-3.3) *vs* 0.43 pg/mL (0.15-1.2), $p < 0.001$; IFNγ – 4.1 pg/mL (2.7-6.9) *vs* 1.1 pg/mL (0.29-2.3), $p < 0.001$; TNFα – 21.7 pg/mL (12.6-30.3) *vs* 6.7 pg/mL (3.4-10.6), ($p < 0.001$). In contrast, no difference was observed for IL-1 and IL-12 (p40) in patients *vs* control subjects: 3.0 pg/mL (1.2-10.6) *vs* 7.5 pg/mL (0.6-14.7), p = 0.391 and 11.9 pg/mL (3.9-28.4) *vs* 12.8 pg/mL (0.36-17.2), (p = 0.633), respectively.

While assessing patients with chronic *vs* acute sarcoidosis, it was shown that level of cytokines IL-17A/CTLA8 was significantly increased (1.9 pg/mL (0.67-3.9) *vs* 0.67 pg/mL (0.42-1.9), p = 0.018),

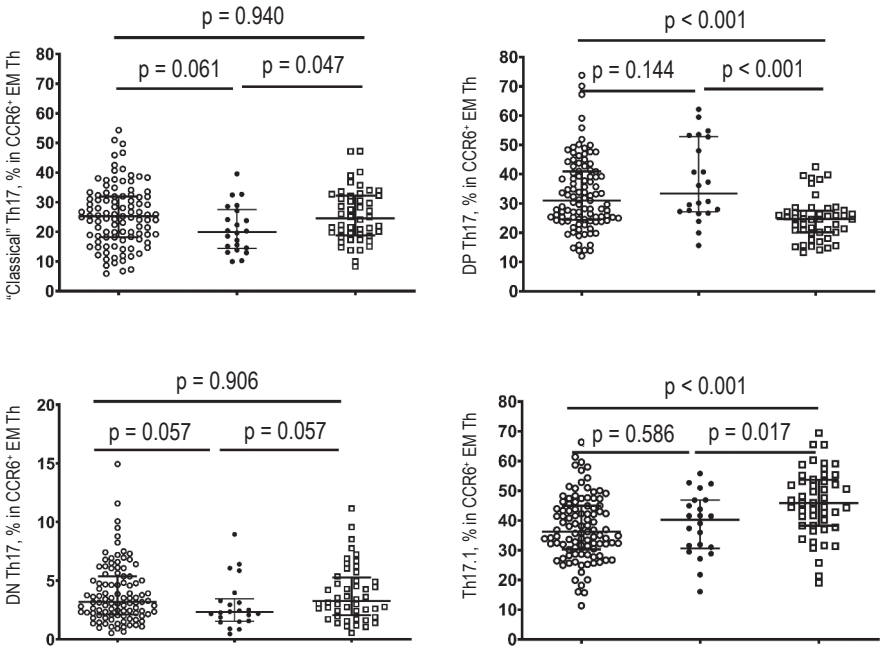

Figure 2. Major Th17 cell subset distribution within total CCR6$^+$ CD45RA$^-$CD62L$^-$ effector memory (EM) Th cells in chronic (n = 101) and acute (n = 22) sarcoidosis as well as control subjects (n = 43)

Note. As for Figure 1.

IFNγ (5.1 pg/mL (2.9-8.1) *vs* 2.7 pg/mL (2.5-4.6), p = 0.027). At the same time, IL-17A/CTLA8 were also significantly increased in chronic sarcoidosis *vs* healthy subjects: 1.9 pg/mL (0.67-3.9) and 0.43 pg/mL (0.15-1.2), at p < 0.001). The concentration of cytokine IL-12 (p70) was significantly increased only in chronic sarcoidosis *vs* control group comprising 1.3 pg/mL (0.56-2.0) *vs* 0.56 pg/mL (0.23-1.3), p = 0.037). While comparing cytokine levels based on clinical course of sarcoidosis, a positive correlation was found between level of IFNγ (pg/mL) and ACE activity (r = 0.349; p = 0.032).

Retrospectively analyzed alterations in type 17 T helper cell composition in patients with respiratory sarcoidosis aligned with changes in clinical picture during a one-year follow-up allowed to find that 31.6% (12/38) of the examined patients with a newly diagnosed disease receiving no immunosuppressive therapy had spontaneous regression of clinical and radiological signs as well as normalized functional parameters. In contrast, 68.4% of subjected (26/38 patients) receiving immunosuppressive therapy were characterized by signs of disease progression (deteriorating pulmonary changes based on chest organ CT scan data, functional parameters, and documented extrapulmonary manifestations).

Flow cytometry analysis assessing level of polarized peripheral blood Th17 subset composition in sarcoidosis aligned with clinical disease course allowed to find the following alterations: disease progression *vs* regression was associated with significantly reduced absolute number of total CD45RA$^-$Th17 and CD45RA$^-$CM T cells: 111 cells/μL (63-148) *vs* 180 cells/μL (138-315), p = 0.009 and 56 cells/μL (37-88) *vs* 110 cells/μL (66-157) (p = 0.006), respectively.

Patients with disease progression *vs* regression were found to have significantly reduced absolute number of both CD45RA$^-$Th17 and CD45RA$^-$CM cells: 111 cells/μL (63-148) *vs* 180 cells/μL (138-315), p = 0.009 and 56 cells/μL (37-88) *vs* 110 cells/μL (66-157), (p = 0.006), respectively. In case of extrapulmonary manifestations in sarcoidosis, a significantly increased percentage of DP Th17 CD45RA$^-$ and DP Th17 EM cells was observed: 38.3% (29.6-41.6) *vs* 29.4% (23.2-34.6), (p = 0.037) and 43.6% (25.4-48.9) *vs* 26.9% (21.9-30.8), (p = 0.018), respectively.

Here, we present data regarding a role for peripheral blood plastic Th17 cells in various types of clinical course of sarcoidosis. Our data suggest an important pathogenetic role played by plastic Th17 cells, not only in triggering granulomatous inflammation in sarcoidosis, but also in kinetics of granuloma formation potentially resulting in distinct outcomes of the pathological process (spontaneous disease remission in acute course and progression with formation of pulmonary fibrotic in chronic course). While comparing the patterns described here with available publications, it turned out that the data on peripheral blood Th17 cell subset composition in sarcoidosis were contradictory.

Some studies suggest about increased level of CCR6$^+$ effector Th (CD45RA$^-$CD45R0$^+$) cells in patients with sarcoidosis compared with control group, whereas others evidence that number of peripheral blood IL-17A-producing T cells is markedly reduced compared to healthy subjects [8, 12, 13,

16]. At the same time, numerous studies are noted showing increased Th17-produced cytokine and chemokine level in sarcoidosis, e.g., IL-6, IL-17, IL-22, IFNγ and CCL20 [12, 13, 18]. At the same time, bronchoalveolar lavage fluid (BALF) along with granulomatous tissue samples are not only characterized by elevated cytokine level, but also contain higher number of cells involved in cytokine production [13].

Th17 cells are detected in granulomas of patients with sarcoidosis, both during active and recurrent disease course [13, 18]. Apparently, Th1/Th17 cells play a central role in developing pulmonary inflammation in sarcoidosis, because their increased level is observed in BALF and peripheral blood samples [3, 4, 8].

A study by Broos et al. showed that the mediastinal lymph nodes of patients with sarcoidosis *vs* control group contained elevated number of $CCR6^+$ Th17, including classical Th17 and Th17.1 cells [3]. In addition, transitional $CCR6^+CXCR3^+CCR4^+$Th Th17/Th17.1 or DP $CCR6^+$ Th cell level was increased [3, 13].

While analyzing our data, we found significantly increased level of peripheral blood CM and EM Th17 memory cells providing a deeper insight into understanding granuloma formation, because among all Th17 subsets, it is DP Th17 cells that are characterized by higher potential to migrate to peripheral anatomical sites due to the high level of expressed adhesion molecules and chemokine receptors [3, 12, 18].

Many studies have been attempting to establish a role for Th17 cell subsets in developing granulomas. It was shown that cytokines IL-1 , IL-6 and IL-23, necessary for polarization towards Th17 cells, or IL-12 responsible for naive Th0-to-Th1 cell differentiation, the majority of DP Th17 cells, in *in vitro* settings, acquired the phenotype and properties of non-classical Th17 cells [3, 4, 8, 12, 18]. It is the "non-classical" Th17 or Th17.1 cell subsets, which seem to represent the major IFNγ producers in developing granulomas [17, 18]. It should be noted that BALF from patients with sarcoidosis was noted in many studies to have increased levels of ligands for surface Th17.1 cell chemokine receptors CXCR3 (e.g., CXCL10 [1]) and CCR6 (CCL20 [4]).

It may be assumed that compared with other Th17 cell subsets, this cell type is able to migrate more efficiently along the chemokine gradient to be selectively accumulated in the focus of inflammation, which is confirmed by the data on predominantly detected Th17.1 cells in the foci of granuloma formation and BALF in sarcoidosis [3].

Apart from this, it was also observed that Th17 cell subset composition was altered providing, with most valuable relevance found for level of memory DP Th17 cells, correlations with ACE activity level as well as their increased level in respiratory sarcoidosis with extrapulmonary manifestations.

It has been uncovered that cytokines such as IL-1 , IL-6, IL-12, IL-17, IL-23, TNFα, and IFNγ are involved in cell differentiation and granuloma formation. Moreover, the roles of IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13, as well as a number of anti-inflammatory cytokines (IL-1RA, IL-10), are of importance in the pathogenesis of sarcoidosis [3, 11, 12, 13, 14, 18]. While assessing the cytokine profile, we showed that patients with chronic sarcoidosis had significantly increased level of cytokines IL-17A/CTLA8, IFNγ, and IL-12 (p70).

Aligning the level of the peripheral blood pro-inflammatory cytokine IFNγ with varying types of sarcoidosis allowed to find that first diagnosed respiratory sarcoidosis prior to immunosuppressive therapy was associated with a direct correlation between IFNγ and ACE level. Such parameters mirror disease activity. It is known that IFNγ is a key mediator in developing granulomatous inflammation. Current data indicate that not only Th1 cells, but also various plastic Th17 cell types, and to a greater extent, Th17.1 cells, are capable of producing IFNγ [1, 3, 13, 18]. The study by Arger et al. revealed the role for IFNγ in developing sarcoid granulomas coupled to importance of identifying plastic Th17 cell types. Other studies show a correlation between the number of Tbet-positive Th17.1 and severity of manifestations in systemic sarcoidosis as well as the level of IFNγ-dependent chemokines: CXCL9, CXCL10, and CXCL11 [1].

Conclusion

Thus, our study allowed to find that peripheral blood plastic CCR6-positive particularly DP Th17 cell subsets as well as level of cytokines they produce driving their differentiation in sarcoidosis are important in diagnostics of sarcoidosis with varying clinical course:

– a direct correlation was shown between the level of angiotensin-converting enzyme activity and percentage of memory DP Th17 cells;

– patients with disease progression *vs* regression had significantly reduced absolute number of total CD45RA-negative memory and CM Th17 cells;

– patients with extrapulmonary manifestations of sarcoidosis had a significantly increased percentage of DP Th17 $CD45RA^-$ and effector memory DP Th17 cells;

– patients with chronic sarcoidosis were shown to have significantly increased concentration of blood plasma cytokines IL-17A/CTLA8, IFNγ, IL-12 (p70) paralleled with positively correlated level between IFNγ (pg/mL) and the activity of angiotensin-converting enzyme.

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