

# DISSEMINATED PURULENT PERITONITIS OUTCOME AFFECTS NKT CELL PHENOTYPE

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**Abstract.** The aim of our study was to investigate the main characteristics of peripheral blood NKT cell phenotype in patients with disseminated purulent peritonitis (DPP) in dynamics of postoperative period, depending on the disease outcome. Fifty-two patients with acute surgical diseases and injuries of the abdominal organs complicated by DPP, and 68 healthy individuals in control group, were examined. Blood sampling was performed before surgery (preoperative period), as well as on the day 7, 14 and 21 of postoperative period. All patients with DPP were divided into two groups depending on disease outcome in postoperative period: patients with favorable disease outcome (n = 34); and patients with unfavorable outcome (n = 18). Study of the phenotype of blood NKT lymphocytes was performed by flow cytometry using direct immunofluorescence of whole peripheral blood samples with monoclonal antibodies. The low relative and absolute level of NKT cells was observed in DPP patients regardless of outcome disease in preoperative period. At the same time, the absolute level of NKT cells returned to normal only in patients with favorable DPP outcome and only by day 21 after surgery. Patients with favorable DPP outcome by the end of examination period had normalized quantity of mature NKT-lymphocytes and significantly decreased level of cytotoxic cells which was apparently associated with migration of such cell subsets to site of inflammation. A reduced level of non-classical (expressing CD8 marker) mature and cytokine-producing NKT cells was detected only in patients with favorable DPP outcome in preoperative period which returned to normal by the end of postoperative period. At the same time, patients with unfavorable disease outcome had reduced quantity of NKT cells of these subsets by day 21 of postoperative treatment. Patients with favorable outcome had high level of mature and cytotoxic CD11b<sup>+</sup> NKT cells already in the preoperative period, while patients with unfavorable DPP outcome had increased level of cytotoxic CD11b<sup>+</sup> NKT cells only by day 21 after surgery. The proportion of NKT cells expressing activation markers (CD28 and CD57) was reduced in patients in preoperative period that returned to normal immediately after surgery with favorable outcome, while it recovered with unfavorable outcome closer to the end of postoperative examination. The defined features of NKT cell phenotype in patients with unfavorable DPP outcome characterize disturbances in subset ratio and mechanisms of functioning of this cell fraction. This determines a need to develop immunotherapeutic methods aimed at stimulating immunoregulatory activity of NKT cells.

**Key words:** peritonitis, NKT cells, phenotype, disease outcome, postoperative period, CD3<sup>-</sup>CD56<sup>+</sup>.

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## ОСОБЕННОСТИ ФЕНОТИПА NKT-КЛЕТОК В ЗАВИСИМОСТИ ОТ ИСХОДА РАСПРОСТРАНЕННОГО ГНОЙНОГО ПЕРИТОНИТА

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**Резюме.** Целью исследования было изучение особенностей фенотипа NKT-клеток у больных распространенным гнойным перитонитом (РГП) в динамике послеоперационного периода в зависимости от исхода заболевания. Обследовано 52 пациента с острыми хирургическими заболеваниями и травмами органов брюшной полости, осложнившимися РГП, и 68 здоровых людей в качестве лиц контрольной группы. Забор крови у больных производили перед операцией (дооперационный период), а также на 7-е, 14-е и 21-е сутки послеоперационного периода. В зависимости от исхода заболевания в послеоперационном периоде, все больные РГП были разделены на две группы: больные с благоприятным исходом заболевания (n = 34), пациенты с неблагоприятным исходом (n = 18). Исследование фенотипа NKT-лимфоцитов крови проводили методом проточной цитометрии с использованием прямой иммунофлуоресценции цельной периферической крови с моноклональными антителами. У обследованных пациентов с РГП независимо от исхода заболевания в дооперационном периоде понижено относительное и абсолютное содержание NKT-клеток, причем в обеих группах больных процентное количество клеток восстанавливается сразу после операции. В то же время абсолютный уровень NKT-клеток нормализуется только у больных с благоприятным исходом РГП и только к 21-м суткам после операции. К концу периода обследования у больных с благоприятным исходом РГП в периферической крови нормализуется содержание зрелых NKT-лимфоцитов и значительно снижается количество цитотоксических клеток, что, по-видимому, определяется их миграцией в зону воспаления. Только у больных с благоприятным исходом РГП пониженный уровень неклассических (экспрессирующих CD8-маркер) зрелых и цитокин-продуцирующих NKT-клеток в дооперационном периоде нормализуется до контрольных значений к концу периода послеоперационного обследования. В то же время у пациентов с неблагоприятным исходом заболевания содержание данных субпопуляций NKT-клеток к 21-м суткам послеоперационного лечения понижено. У больных с благоприятным исходом заболевания выявляется высокий уровень зрелых и цитотоксических CD11b<sup>+</sup>NKT-клеток уже в дооперационном периоде, тогда как при неблагоприятном исходе РГП повышенное содержание цитотоксических CD11b<sup>+</sup>NKT-клеток обнаружено только к 21-м суткам после операции. Содержание NKT-клеток с экспрессией активационных маркеров (CD28 и CD57), сниженное у больных в дооперационном периоде, при благоприятном исходе нормализуется сразу после операции, тогда как при неблагоприятном исходе — ближе к концу послеоперационного обследования. Установленные особенности фенотипа NKT-клеток у больных с неблагоприятным исходом РГП характеризуют нарушения в соотношении субпопуляционного состава и механизмах функционирования данной фракции клеток, что определяет необходимость разработки иммунотерапевтических методов, направленных на стимуляцию иммунорегуляторной активности NKT-клеток.

**Ключевые слова:** перитонит, NKT-клетки, фенотип, исход заболевания, послеоперационный период, CD3<sup>+</sup>CD56<sup>+</sup>.

## Introduction

Disseminated purulent peritonitis (DPP) remains one of the unsolved problems of modern abdominal surgery due to high morbidity and mortality. The disease is a complication of a number of surgical diseases or abdominal injuries (acute appendicitis, perforated gastric and duodenal ulcer, acute gangrenous cholecystitis, pancreatic necrosis, perforation of hollow organs, their damage during trauma, etc.) in the vast majority of cases [22, 27, 28]. Mortality in DPP is about 20–30%, reaching the highest figures in the development of multiple organ failure and septic shock, the prevention and relief of which are key in the treatment of peritonitis [33, 34].

It has been proven that the course of the infectious process in the abdominal cavity, and the nature

and characteristics of the development of purulent postoperative complications, are determined not only by the severity of the underlying disease, and the adequacy of the surgical intervention performed and the completeness of the intensive care, but also by the functional state of the immune system [3, 19, 24]. Yang et al. (2022) reported that patients with spontaneous bacterial peritonitis had IL-13 overexpression in their ascites and a reduced functional activity of CD8<sup>+</sup> T cells [41]. There is evidence that an increased level of macrophage mannose receptor (CD206) expression on peritoneal macrophages was associated with an increased risk of an adverse outcome of peritonitis [38]. Previously, we found that the number of “naïve” B lymphocytes and B2 cells non-expressing and expressing CD23 in patients with an unfavorable outcome of DPP was higher

than in patients with a favorable outcome of this disease [2]. Violations of the mechanisms of the respiratory burst of neutrophils as well as a decrease in their phagocytic activity, and the level of TNF $\alpha$  synthesis in patients with DPP, were previously identified and presented in a number of publications [5, 21].

Natural killer T cells (NKT) are defined as a heterogeneous subset of T lymphocytes with a CD3<sup>+</sup>CD16/56<sup>+</sup> phenotype, i.e. they combine the phenotypic characteristics of T and NK cells [11, 40]. Accordingly, the functional activity of NKT cells is realized in various mechanisms of the immune response, realizing the relationship between natural resistance and adaptive immunity. This lymphocyte subset is involved in the mechanisms of antiviral and antiparasitic protection, and also secreted different immunoregulatory cytokines in the site of inflammation [1, 10, 12]. In addition, NKT cells are able to stimulate and inhibit antitumor immune responses [9, 39].

Semi-invariant  $\alpha\beta$ -TCR expressed by NKT cells could recognize  $\alpha$ -glycuronylceramides (one of the main components of gram-negative bacteria cell wall) that led to the formation of a complex of antimicrobial functions including those mediated by the induction of CD40L and pronounced stimulation of Th1 and Th2 lymphocytes [10, 16]. Therefore, NKT cells are also involved in immune-inflammatory processes development. For instance, Nilsson et al. demonstrated that NKT cell cytokine profile switching regulated liver sterile inflammation [31]. It was shown that NKT cells made a significant contribution to mucosal immunity regulation by intestinal homeostasis controlling and participating in the development of inflammatory diseases of the abdominal cavity [11].

Thus, the aim of our investigation was to study the characteristics of NKT cell phenotype in patients with DPP in the dynamics of the postoperative period in depending on the outcome of the disease.

## Materials and methods

*Study participants.* Fifty-two patients with acute surgical diseases and injuries of the abdominal organs complicated by DPP aged 25–65 years (the mean age of the patients was 49.6 years) who were treated at the Krasnoyarsk regional purulent-septic center at the Regional Clinical Hospital were examined. Exclusion criteria from the study were the presence of acute destructive pancreatitis (pancreatic necrosis), total mesenteric thrombosis, oncological diseases and tuberculosis. The volume of surgical intervention and the number of sanations were determined by the attending physician depending on the patient's condition. Blood sampling was performed before the surgery (preoperative period) as well as on the 7th, 14th and 21st days of the postoperative period. All patients were divided into two groups depending on the outcome of peritonitis in the postoperative period: group 1 — patients with a favorable outcome

of the disease (n = 34); and group 2 — patients with an unfavorable outcome (n = 18). Sixty-eight healthy people were examined as a control group.

All studies were performed with the informed consent of the patients and in accordance with the Helsinki Declaration of the World Association “Ethical Principles of Scientific Medical Research Involving Humans” as amended in 2013 and “Rules Of Clinical Practice In The Russian Federation” approved by the Order of the Russian Ministry of Health (19.06.2003, No. 266).

*Flow cytometry.* Study of the phenotype of NKT cells was performed by flow cytometry using direct immunofluorescence of whole peripheral blood with monoclonal antibodies (Beckman Coulter, USA). The preparation of blood samples and the adjustment of the flow cytometer were carried out in accordance with the recommendations presented in the article by Khaidukov et al. [7]. The distribution of antibodies along the fluorescence channels was carried out in accordance with the principles of panel formation for multicolor cytofluorometric studies [4]. Immunophenotyping of cells was performed by staining 200  $\mu$ l of whole EDTA-stabilized blood with the following combination of fluorochrome-conjugated monoclonal antibodies: anti-CD3 Alexa Fluor 700 (clone UCHT1, isotype — Mouse IgG1), anti-CD8 Allophycocyanin (clone B9.11, isotype — Mouse IgG1), anti-CD11b Phycoerythrin-Texas Red-X (clone J33, isotype — Mouse IgG1), anti-CD16 Phycoerythrin-Cyanin 7 (clone 3G8, isotype — Mouse IgG1), anti-CD28 Phycoerythrin (clone CD28.2, isotype — Mouse IgG1), anti-CD45 Alexa Fluor 750 (clone J33, isotype — Mouse IgG1), anti-CD56 Phycoerythrin-Cyanine 5.5 (clone N901, isotype — Mouse IgG1), and anti-CD57 Fluorescein Isothiocyanate (clone NC1, isotype — Mouse IgM).

Incubation of blood samples with antibodies was carried out for 15 min at room temperature and in the dark. Lysis of erythrocytes was carried out for 15 min using 2 ml of VersaLyse Lysing Solution (Beckman Coulter, Inc., USA) with the addition of 50  $\mu$ l of IOTest 3 Fixative Solution (Beckman Coulter, Inc., USA). Stained cells were analyzed on a Navios flow cytometer (Beckman Coulter, Inc., USA) of the Krasnoyarsk Regional Center of Research Equipment of Federal Research Center “Krasnoyarsk Science Center SB RAS”. At least 50000 lymphocytes were analyzed for each blood sample. The obtained data were analyzed using the Kaluza software package (Beckman Coulter, Inc., USA).

*Statistical analysis.* The results were presented using the median (Me) and interquartile range as 25th (Q<sub>1</sub>) and 75th (Q<sub>3</sub>) percentiles. The significance of differences between the indicators of independent samples was assessed using the non-parametric Mann–Whitney test (Mann–Whitney U test). The significance of differences in indicators in the dynamics of treatment was determined

**Table 1. Content of blood NKT cells in patients with a favorable DPP outcome in the dynamics of the postoperative period (Me, Q<sub>1</sub>–Q<sub>3</sub>)**

Parameters	Control n = 68	Preoperative period n = 34	7 days after surgery n = 34	14 days after surgery n = 34	21 days after surgery n = 34
CD3 <sup>+</sup> CD16 <sup>+</sup> 56 <sup>+</sup> , %	3.51 1.98–6.90	1.90 1.19–3.29 p <sub>1</sub> = 0.011	3.31 2.19–7.12	2.84 2.31–4.45	3.50 3.13–5.51
CD3 <sup>+</sup> CD16 <sup>+</sup> 56 <sup>+</sup> , × 10 <sup>9</sup> /l	0.07 0.04–0.19	0.02 0.007–0.03 p <sub>1</sub> = 0.007	0.03 0.02–0.04 p <sub>1</sub> = 0.039	0.03 0.01–0.05 p <sub>1</sub> = 0.039	0.08 0.05–0.14 p <sub>2</sub> = 0.043
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> , %	0.73 0.43–1.25	0.37 0.23–0.69 p <sub>1</sub> = 0.029	0.70 0.37–1.00	0.39 0.19–0.69 p <sub>1</sub> = 0.035	0.67 0.43–1.01
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>-</sup> , %	0.87 0.56–1.06	0.85 0.60–1.80	1.15 0.92–1.55	0.70 0.51–1.25	0.39 0.28–0.49 p <sub>1</sub> = 0.023 p <sub>2</sub> = 0.021 p <sub>3</sub> = 0.014
CD3 <sup>+</sup> CD16 <sup>-</sup> CD56 <sup>+</sup> , %	1.93 1.29–2.67	0.59 0.35–1.60 p <sub>1</sub> = 0.004	1.50 0.70–2.91 p <sub>2</sub> = 0.045	1.80 0.50–2.41 p <sub>2</sub> = 0.048	2.29 1.93–2.90 p <sub>2</sub> < 0.001

**Note.** p<sub>1</sub> — statistically significant differences versus controls; p<sub>2</sub> — statistically significant differences versus patients with DPP before surgery; p<sub>3</sub> — statistically significant differences versus 7 days after surgery patients.

by the Wilcoxon test (Wilcoxon matched pairs test). Friedman's rank analysis of variance (Friedman ANOVA by Ranks) was also used to assess changes in the studied parameters in the dynamics of postoperative treatment. Statistical analysis was carried out using the Statistica 8.0 software package (StatSoft Inc., USA, 2007).

## Results

We noticed that the absolute and relative numbers of NKT cell were decreased in patients in the preoperative period with a favorable outcome of DPP if compared to healthy controls, and this was mainly due to cells with CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> and CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> phenotypes (Table 1). The absolute number

of NKT lymphocytes in patients with a favorable outcome of DPP remained reduced in compared to control values on the 7th day after surgery. An increase in CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> cells levels if compared with the initial time point was also found, and this content remained until the end of the observed period.

Moreover, the consistent increase of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> cells frequency was also confirmed by the results of Friedman ANOVA test:  $\chi^2 = 13.08$ ,  $p = 0.004$ . The absolute number of NKT cells in patients with a favorable outcome of DPP remained on the 14th day of postoperative treatment, but with a decrease in the percentage of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells vs. control group. Patients with a favorable outcome of peritonitis showed a normalization of the absolute number of circulating NKT cells and a reduced percentages

**Table 2. Subsets of NKT cells with CD8 expression in patients with a favorable DPP outcome in the dynamics of the postoperative period (Me, Q<sub>1</sub>–Q<sub>3</sub>)**

Parameters	Control n = 68	Preoperative period n = 34	7 days after surgery n = 34	14 days after surgery n = 34	21 days after surgery n = 34
CD3 <sup>+</sup> CD8 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> , %	0.55 0.33–0.87	0.24 0.14–0.43 p <sub>1</sub> = 0.018	0.60 0.31–0.75	0.42 0.21–0.70	0.63 0.38–1.02 p <sub>2</sub> = 0.037
CD3 <sup>+</sup> CD8 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>-</sup> , %	0.18 0.12–0.22	0.05 0.03–0.11 p <sub>1</sub> < 0.001	0.13 0.11–0.19	0.04 0.02–0.06 p <sub>1</sub> < 0.001 p <sub>3</sub> = 0.046	0.06 0.03–0.07 p <sub>1</sub> < 0.001
CD3 <sup>+</sup> CD8 <sup>+</sup> CD16 <sup>-</sup> CD56 <sup>+</sup> , %	1.60 1.14–2.22	0.60 0.36–1.25 p <sub>1</sub> = 0.005	0.75 0.34–1.49 p <sub>1</sub> = 0.040	1.30 0.30–1.72	1.60 1.35–2.10 p <sub>2</sub> = 0.043

**Note.** p<sub>1</sub> — statistically significant differences versus controls; p<sub>2</sub> — statistically significant differences versus patients with DPP before surgery; p<sub>3</sub> — statistically significant differences versus 7 days after surgery patients.

**Table 3. Content of NKT cells expressing activation and adhesion markers in patients with a favorable DPP outcome in the dynamics of the postoperative period (Me, Q<sub>1</sub>–Q<sub>3</sub>)**

Parameters	Control n = 68	Preoperative period n = 34	7 days after surgery n = 34	14 days after surgery n = 34	21 days after surgery n = 34
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> CD11b <sup>+</sup> , %	0.012 0.003–0.041	0.043 0.017–0.074 p <sub>1</sub> = 0.035	0.059 0.028–0.114 p <sub>1</sub> = 0.030	0.031 0.014–0.049 p <sub>3</sub> = 0.041	0.030 0.005–0.110
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>-</sup> CD11b <sup>+</sup> , %	0.022 0.011–0.029	0.043 0.022–0.059 p <sub>1</sub> = 0.047	0.059 0.040–0.310 p <sub>1</sub> = 0.023	0.039 0.021–0.113	0.061 0.022–0.153 p <sub>1</sub> = 0.045
CD3 <sup>+</sup> CD16 <sup>-</sup> CD56 <sup>-</sup> CD11b <sup>+</sup> , %	0.132 0.089–0.789	0.369 0.249–0.609	0.509 0.439–0.989	0.389 0.219–0.690	0.680 0.556–1.170 p <sub>1</sub> = 0.041
CD3 <sup>+</sup> CD16/56 <sup>+</sup> CD28 <sup>+</sup> , %	1.50 0.83–3.20	0.89 0.49–1.59 p <sub>1</sub> = 0.044	1.46 0.58–3.21 p <sub>2</sub> = 0.047	0.90 0.60–2.70	1.00 0.54–4.43
CD3 <sup>+</sup> CD16/56 <sup>+</sup> CD57 <sup>+</sup> , %	1.32 0.22–2.32	0.96 0.66–1.69 p <sub>1</sub> = 0.045	1.20 0.70–2.18	1.34 0.71–1.72	1.40 1.13–1.83

**Note.** p<sub>1</sub> — statistically significant differences versus controls; p<sub>2</sub> — statistically significant differences versus patients with DPP before surgery; p<sub>3</sub> — statistically significant differences versus 7 days after surgery patients.

of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup> cells if compared to control values by the end of the observed period.

We found that the level of CD8-expressing NKT cells was altered in patients with a favorable outcome of DPP (Table 2). Thus, the percentage of peripheral blood CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> cells was reduced in this group of patients even in the preoperative period if compared to healthy controls. The relative number of NKT cells with CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> phenotype in patients

with a favorable outcome of the disease was increased vs. control ranges on the 7th day after surgery, and it reached its maximum by the end of the observed period. The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup> cells in patients of this group increased if compared to control values on the 7th day after surgery, but it decreased again at 14th day point and remained at the initial level until the end of the observed period. The frequency of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> NKT cells was reduced on the 7th day post-surgery, their number was increased vs. control values on the

**Table 4. Content of blood NKT cells in patients with an unfavorable DPP outcome in the dynamics of the postoperative period (Me, Q<sub>1</sub>–Q<sub>3</sub>)**

Parameters	Control n = 68	Preoperative period n = 18	7 days after surgery n = 18	14 days after surgery n = 18	21 days after surgery n = 18
CD3 <sup>+</sup> CD16/56 <sup>+</sup> , %	3.51 1.98–6.90	2.09 1.36–2.69 p <sub>1</sub> = 0.025	3.47 1.34–5.53	3.89 0.67–5.70	2.30 1.43–4.88
CD3 <sup>+</sup> CD16/56 <sup>+</sup> , × 10 <sup>9</sup> /l	0.07 0.04–0.19	0.02 0.01–0.03 p <sub>1</sub> < 0.001	0.02 0.01–0.04 p <sub>1</sub> = 0.004	0.02 0.01–0.05 p <sub>1</sub> = 0.021	0.03 0.01–0.06 p <sub>1</sub> = 0.045
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> , %	0.73 0.43–1.25	0.60 0.15–1.30	0.85 0.76–1.79	0.48 0.41–0.95	0.39 0.25–0.53 p <sub>1</sub> = 0.043 p <sub>3</sub> = 0.038
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>-</sup> , %	0.87 0.56–1.06	0.75 0.37–1.40	1.50 1.28–3.45 p <sub>1</sub> < 0.001 p <sub>2</sub> = 0.029	1.37 1.00–2.15 p <sub>1</sub> = 0.034	0.65 0.34–1.10
CD3 <sup>+</sup> CD16 <sup>-</sup> CD56 <sup>+</sup> , %	1.93 1.29–2.67	0.69 0.25–1.42 p <sub>1</sub> = 0.010	1.19 0.93–1.32 p <sub>1</sub> = 0.041	2.25 1.30–3.65 p <sub>2</sub> = 0.044	1.14 0.82–1.37 p <sub>1</sub> = 0.040 p <sub>4</sub> = 0.047

**Note.** p<sub>1</sub> — statistically significant differences versus controls; p<sub>2</sub> — statistically significant differences versus patients with DPP before surgery; p<sub>3</sub> — statistically significant differences versus 7 days after surgery patients; p<sub>4</sub> — statistically significant differences versus 14 days after surgery patients.

**Table 5. Subsets of NKT cells with CD8 expression in patients with an unfavorable DPP outcome in the dynamics of the postoperative period (Me, Q<sub>1</sub>–Q<sub>3</sub>)**

Parameters	Control n = 68	Preoperative period n = 18	7 days after surgery n = 18	14 days after surgery n = 18	21 days after surgery n = 18
CD3 <sup>+</sup> CD8 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> , %	0.55 0.33–0.87	0.56 0.14–1.30	0.84 0.75–1.79 p <sub>1</sub> = 0.043	0.39 0.20–0.89 p <sub>3</sub> = 0.040	0.29 0.10–0.39 p <sub>1</sub> = 0.040 p <sub>3</sub> = 0.008
CD3 <sup>+</sup> CD8 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>-</sup> , %	0.18 0.12–0.22	0.10 0.06–0.90	0.60 0.14–1.28 p <sub>1</sub> = 0.044	0.70 0.32–1.08 p <sub>1</sub> < 0.001	0.13 0.05–0.49 p <sub>4</sub> = 0.027
CD3 <sup>+</sup> CD8 <sup>-</sup> CD16 <sup>-</sup> CD56 <sup>+</sup> , %	1.60 1.14–2.22	0.19 0.07–0.87 p <sub>1</sub> < 0.001	0.79 0.30–2.09	0.83 0.49–1.85 p <sub>2</sub> = 0.047	0.64 0.19–0.71 p <sub>1</sub> < 0.001

**Note.** p<sub>1</sub> — statistically significant differences versus controls; p<sub>2</sub> — statistically significant differences versus patients with DPP before surgery; p<sub>3</sub> — statistically significant differences versus 7 days after surgery patients; p<sub>4</sub> — statistically significant differences versus 14 days after surgery patients.

14th day of treatment, and reached its maximum by the end of the observed period. The sequence of changes in the content of NKT cells with this phenotype was also confirmed using the Friedman ANOVA test ( $\chi^2 = 9.60$ ,  $p = 0.022$ ).

Next, we investigated the expression of cell adhesion molecules and activation markers by peripheral blood NKT cells (Table 3). We found increased levels of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>CD11b<sup>+</sup> and CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup>CD11b<sup>+</sup> NKT cells in blood samples from DPP patients with a favorable outcome in compared to controls, while the frequencies of CD28<sup>-</sup> and CD57<sup>-</sup> expressing NKT cells were decreased (Table 3). Moreover, CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>CD11b<sup>+</sup> cells in patients of this group were decreased if compared to control values from the 14th day, while the number of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup>CD11b<sup>+</sup> cells remained elevated by end of our observed period. The number of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup>CD11b<sup>+</sup> NKT cells in the

current patients group increased significantly vs. healthy controls only at the end of the observation period, that was also confirmed by the Friedman ANOVA test ( $\chi^2 = 12.60$ ,  $p = 0.006$ ). The percentage of NKT cells expressing CD28 and CD57 increased from the beginning of postoperative treatment and remained at the control levels until the end of the examination period.

The percentage of NKT cells in the blood of patients with DPP with an unfavorable outcome of the disease was reduced vs. control levels before the surgery, while their relative number restored after the surgery (Table 4). However, the absolute number of NKT cells in patients of this group was reduced in preoperative and postoperative periods if compared to control group. The percentage of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> NKT cells in patients with an unfavorable outcome of DPP corresponded to the control values in the preoperative period and within 14 days of subsequent

**Table 6. Content of NKT cells expressing activation and adhesion markers in patients with an unfavorable DPP outcome in the dynamics of the postoperative period (Me, Q<sub>1</sub>–Q<sub>3</sub>)**

Parameters	Control n = 68	Preoperative period n = 18	7 days after surgery n = 18	14 days after surgery n = 18	21 days after surgery n = 18
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> CD11b <sup>+</sup> , %	0.012 0.003–0.041	0.033 0.006–0.174	0.017 0.006–0.029	0.045 0.019–0.095	0.006 0.004–0.029
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>-</sup> CD11b <sup>+</sup> , %	0.022 0.011–0.029	0.017 0.006–0.104	0.007 0.003–0.008 p <sub>1</sub> < 0.001	0.010 0.004–0.025	0.074 0.069–0.084 p <sub>1,2,3</sub> < 0.001 p <sub>4</sub> = 0.014
CD3 <sup>+</sup> CD16 <sup>-</sup> CD56 <sup>+</sup> CD11b <sup>+</sup> , %	0.132 0.089–0.789	1.079 0.623–1.205 p <sub>1</sub> = 0.024	0.812 0.225–2.550 p <sub>1</sub> = 0.037	0.640 0.210–1.374 p <sub>1</sub> = 0.046	0.993 0.337–2.260 p <sub>1</sub> = 0.018
CD3 <sup>+</sup> CD16 <sup>+/56<sup>+</sup></sup> CD28 <sup>+</sup> , %	1.50 0.83–3.20	0.34 0.16–1.14 p <sub>1</sub> = 0.006	0.56 0.41–1.29 p <sub>1</sub> = 0.016	1.40 0.30–2.71	1.90 1.00–2.35 p <sub>2</sub> = 0.045
CD3 <sup>+</sup> CD16 <sup>+/56<sup>+</sup></sup> CD57 <sup>+</sup> , %	1.32 0.22–2.32	0.59 0.04–0.85 p <sub>1</sub> = 0.011	0.20 0.03–1.00 p <sub>1</sub> = 0.031	0.49 0.04–1.09 p <sub>1</sub> = 0.038	1.00 0.20–1.11

**Note.** p<sub>1</sub> — statistically significant differences versus controls; p<sub>2</sub> — statistically significant differences versus patients with DPP before surgery; p<sub>3</sub> — statistically significant differences versus 7 days after surgery patients; p<sub>4</sub> — statistically significant differences versus 14 days after surgery patients.

treatment, but it decreased by the end of the observed period. The level of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup> cells in patients of this group also corresponded to the control values in the preoperative period, while it significantly increased by the 7th and 14th days of observation. Moreover, it returned to initial ranges by the 21st day of postoperative treatment. At the same time, the percentage of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> NKT cells in patients with an unfavorable outcome of DPP was reduced in the preoperative period, but it reached the control values on the 14th day of postoperative treatment, and then decreased by the end observed period if compared to healthy controls.

The relative numbers of peripheral blood CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>-</sup> cells in patients with an unfavorable outcome of DPP in the preoperative period corresponded to the control values (Table 5). The frequencies of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells were increased by the 7th day after the surgery, but it significantly decreased by the end of the observed period if compared to controls. The level of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>-</sup> cells was increased if compared to control values on the 7th and 14th days of postoperative treatment, but their number decreased to the initial range by the end of the observed period. The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> cells in patients with an unfavorable outcome of the disease was reduced in the preoperative period, it increased to control values on the 7th and 14th days of treatment, but, finally, it was decreased by the end of the observed period if compared to controls.

The percentage of circulating CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>CD11b<sup>+</sup> cells in patients with an unfavorable outcome of DPP in the pre- and postoperative period corresponded to the control range, while the level of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup>CD11b<sup>+</sup> cells during the entire examination period exceeded the control values (Table 6). The levels of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup>CD11b<sup>+</sup> NKT cells in patients of this group in the preoperative period showed no differences with control values. However, the level of this cell subset decreased on the 7th day after the surgery, but then increased by the end of the observed period if compared to healthy controls and initial ranges.

The relative numbers of CD28-expressing NKT cells in patients with an unfavorable outcome of the disease were reduced compared to control values in the preoperative period and on the 7th day of the postoperative period, but they reached the control values by the end of the observed period significantly exceeding the initial level. Similarly, CD57-positive NKT cells in this group of patients were reduced in the preoperative period and during the first 14 days of postoperative treatment, but they increased to control values by the end of the observed period.

Differences in NKT cells content were found between patients with favorable and unfavorable outcomes of DPP (Tables 1, 4). Thus, the percentages

of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells were increased in patients with an unfavorable outcome of the disease on the 7th day after surgery ( $p = 0.045$ ), the level of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>-</sup> cells was increased on the 14th day ( $p = 0.014$ ), and the relative content of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> cells were reduced on the 21st day after surgery ( $p < 0.001$ ) if compared to patients with a favorable outcome of DPP. Additionally, the frequencies of circulating CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells were increased in the case of an unfavorable outcome of DPP during the preoperative period ( $p = 0.025$ ), and the level of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> NKT cells was reduced if compared ( $p = 0.043$ ) to patients with a favorable outcome of the disease (Tables 2, 5).

At the same time, an increased level of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells in patients with an unfavorable outcome persisted on the 7th day after surgery ( $p = 0.048$ ), while a decrease in this NKT cell subset ( $p = 0.008$ ) was observed at 21st day of postoperative treatment if compared to patients with a favorable outcome of DPP. The percentage of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup> cells in patients with an unfavorable outcome also remained lower at the end of the observed period vs. patients with a favorable outcome of DPP ( $p < 0.001$ ). The relative numbers of CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD56<sup>-</sup> cells in the blood of patients with an unfavorable outcome of DPP on the 7th and 14th days after surgery were also increased ( $p = 0.017$  and  $p < 0.001$ , respectively).

Finally, the percentages of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>CD11b<sup>+</sup> cells in patients with an unfavorable outcome of DPP on the 7th and 21st days of postoperative treatment were reduced vs. patients with a favorable outcome ( $p = 0.003$  and  $p = 0.044$ , respectively) (Tables 3, 6). Similarly, the levels of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>-</sup>CD11b<sup>+</sup> NKT cells were reduced on the 7th and 14th days of postoperative treatment vs. patients with an unfavorable outcome ( $p < 0.001$  and  $p = 0.002$ , respectively). An increase in relative numbers of CD3<sup>+</sup>CD16<sup>-</sup>CD56<sup>+</sup>CD11b<sup>+</sup> cells in patients with an unfavorable outcome vs. patients with a favorable outcome were observed only in the preoperative period ( $p = 0.002$ ). In addition, CD28<sup>+</sup> NKT cells were reduced in patients with an unfavorable outcome of DPP in the preoperative period and on the 7th day after surgery ( $p = 0.020$  and  $p = 0.028$ , respectively), and the numbers of CD57<sup>+</sup> NKT cells were reduced during the entire observation period ( $p = 0.043$ ,  $p = 0.007$ ,  $p = 0.046$  and  $p = 0.039$ , respectively) relative to the indicators found in patients with a favorable course of this infectious and inflammatory disease.

## Discussion

The functional activity of NKT cells is realized through effector (perforin/granzyme and/or FasL-mediated) mechanisms and regulatory (cytokine production) providing the relationship between innate and adaptive immunity [6, 12, 40]. The subset

composition of NKT cells is determined by CD16 and CD56 receptor expression. The CD16 is a low affinity immunoglobulin G receptor (Fc $\gamma$ RIII) that is non-covalently bound to the CD3 $\zeta$  molecule on the NKT cell membrane [17, 18]. CD56 (NCAM, Leu-19, NKH-1) is an immunoglobulin superfamily adhesion molecule that takes part in intercellular interaction [8, 23]. Mature NKT cells express both markers. Cells that exhibit CD16<sup>+</sup>CD56<sup>-</sup> phenotype are defined as cytotoxic cells, while NKT cells with CD16<sup>-</sup>CD56<sup>+</sup> phenotype are defined as cytokine-producing cells [15, 35].

In general, the relative and absolute numbers of NKT cells in the peripheral blood of patients with DPP were reduced in the preoperative period, regardless of the outcome of the disease. Moreover, if their percentage was restored already on the 7th day after the surgery then the reduced absolute level of this fraction of lymphocytes remained in the postoperative period. Only patients with a favorable outcome of the disease by the end of the observed period (on the 21st day after surgery) had increased percentages of NKT cells similar to control ranges. A feature of the dynamics of the number of NKT cells in the blood in patients with a favorable outcome of DPP was that the reduced level of cells was associated with a low content of mature and cytokine-producing NKT cells in the preoperative period, while their number was restored to control values by the end of the observed period and the number of cytotoxic NKT cells decreased. Patients with an unfavorable outcome of DPP in the preoperative period had a low level of NKT cells which was determined by a reduced content of cytokine-producing cells. The low level of cytokine-producing and mature NKT cells was also observed in patients of this group on the 21st day of postoperative treatment.

NKT cells expressing the CD8 marker are part of type II NKT cells (non-classical, non-invariant) [1, 32]. This cell fraction recognizes a wider range of antigenic molecules (compared to type I NKT cells), synthesizes cytokines that induce differentiation of Th1- and Th2-lymphocytes but also implement immunosuppressive functions [1, 14, 36]. In particular, type II NKT cells can stimulate the functional activity of myeloid suppressor cells, able to kill antigen-presenting dendritic cells and to inhibit the functional activity of cytotoxic CD8<sup>+</sup> T cells through the induction of TGF- $\beta$  expression [29, 30].

The content of mature, cytotoxic and cytokine-producing fractions of NKT cells with CD8 expression was reduced in the blood of patients with DPP with a favorable outcome of the disease in the preoperative period compared with control values. However, the level of mature and cytokine-producing CD8<sup>+</sup>NKT cells recovered to the control range by the end of the observed period (on the 21st day after the surgery). At the same time, a decrease in the content of only cytokine-producing CD8<sup>+</sup> NKT cells was

found in the examined patients with an unfavorable outcome of DPP in the preoperative period relative to the control range and values detected with a favorable outcome; the level of mature CD8<sup>+</sup> NKT cells even exceeded that detected in case of a favorable outcome of the disease. However, the number of all studied fractions of CD8<sup>+</sup>NKT cells was significantly reduced compared to control values by the 21st day of postoperative treatment.

The CD11b receptor is a type I glycoprotein defined as a subunit of the  $\alpha$ M integrin and forms the Mac-1 integrin in complex with the CD18 molecule (CD11b/CD18) [25, 26]. Expression of this marker on the membrane of NKT cells increases the level of effector and migratory activity. An increased numbers of CD11b-expressing mature NKT cells were found in patients with a favorable outcome of DPP in the preoperative period, their content returned to normal by day 21, while the content of CD11b<sup>+</sup> cytotoxic NKT cells in the pre- and postoperative period remained elevated. In addition, the level of cytokine-producing CD11b<sup>+</sup> NKT cells in individuals of this group increased towards the end of the observed period. The patients with an unfavorable outcome of the disease had a lower content of mature CD11b<sup>+</sup> NKT cells by the 21st day of the postoperative period compared with the control values and the level of these cells with a favorable outcome of peritonitis. In addition, the content of cytotoxic NKT cells significantly increased in patients with an unfavorable outcome only at the end of the observed period, while the level of cytokine-producing CD11b<sup>+</sup> NKT cells was increased throughout the entire period of the study.

Also, the content of NKT cells expressing activation markers CD28 and CD57 was studied in patients with DPP in depending on the outcome of the disease. The CD28 antigen (Tp44) belongs to the immunoglobulin superfamily and is involved in the enhancement of T-cell receptor signals, which determines its role in the regulation of adaptive immunity [13, 20]. Blockade of CD28 on the membrane of NKT cells completely suppressed cytokine production [37]. CD57 receptor (Leu-7, HNK-1, NK-1) was defined as an oligosaccharide with sulfated glucuronic acid residues which is expressed on membrane proteins, lipids, and proteoglycans, and its expression level is associated with the accumulation of perforin and granzyme B in the cytolytic killer granules cells [14, 32].

The frequencies of CD28<sup>+</sup> and CD57<sup>+</sup> NKT cells in the preoperative period was reduced relative to the control values in patients with a favorable outcome of DPP in the preoperative period. However, their number returned to normal immediately after the surgery and remained at the level of the control range until the end of the observed period. At the same time, patients with an unfavorable outcome of peritonitis had low levels of CD28<sup>+</sup> and CD57<sup>+</sup> NKT cells in the preoperative period which increased to control values only by the end of the observed period.



In general, it can be concluded that the systemic inflammatory response in DPP patients with an unfavorable outcome of the disease in the postoperative period was characterized by a violation of the ratio of the subset composition of NKT cells with a low level of non-classical NKT cells by the end of the observed period (day 21 after surgery) and a pronounced change in the cell content expressing adhesion and activation markers.

## Conclusion

Thus, significant differences in the phenotype of peripheral blood NKT cells were found between patients with different outcome of DPP. The low relative and absolute levels of NKT cells were observed all patients with DPP regardless of the disease outcome in the preoperative period. At the same time, the absolute level of NKT cells returned to normal values only in patients with a favorable outcome of DPP post 21 days after the surgery. The content of mature NKT lymphocytes was normalized in the peripheral blood of patients with a favorable outcome of DPP by the end of the examination period. The number of cytotoxic cells in the blood of these patients significantly decreased by the 21st day of the examination which is apparently determined by their migration to the area of inflammation. At the same time, patients of this group had the level of cytokine-producing cells at the level of the control range during the entire postoperative period.

Conversely, the level of mature and cytokine-producing NKT cells was reduced in the blood of patients with an unfavorable outcome of DPP by the 21st day of the postoperative period. A reduced level of non-classical (expressing the CD8 marker) mature and

cytokine-producing NKT cells was detected only in patients with a favorable outcome of DPP in the preoperative period which returned to normal by the end of the postoperative period. At the same time, patients with an unfavorable outcome of the disease had a reduced number of NKT cells of these subsets by the 21st day of postoperative treatment. It can be assumed that a high level of systemic inflammatory response in the postoperative period in patients of this category was associated with a lack of regulatory processes in the immune system including a low level of non-classical NKT cells.

In addition, it was found that a high level of NKT cells (compared to control values) expressing the CD11b receptor was observed in patients with DPP during the entire period of the study. However, only patients with a favorable outcome of the disease had a high level of mature and cytotoxic CD11b<sup>+</sup> NKT cells already in the preoperative period, while an increased content of cytotoxic CD11b<sup>+</sup> NKT cells was found in patients with an unfavorable outcome of peritonitis only by the 21st day after surgery. The content of NKT cells expressing activation markers (CD28, CD57) was reduced in patients in the preoperative period; it returned to normal with a favorable outcome immediately after surgery while patients with an unfavorable outcome had a recovery of these cell fractions towards the end of the postoperative examination. The established features of the phenotype of NKT cells in patients with an unfavorable outcome of DPP characterize disturbances in the ratio of the subset composition and the mechanisms of functioning of this cell fraction. This determines the need to develop immunotherapeutic methods aimed at stimulating the immunoregulatory activity of NKT cells.

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