

Natural killer and natural killer T-like cells in splenectomised patients

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Abstract

Introduction: Natural killer cells (NK cells) present a unique ability to recognize and kill cells infected with a variety of pathogens, regardless of prior immunization. Natural killer T-like cells (NKT-like cells) are increased in clinical conditions associated with chronic activation of the immune system. Splenectomised patients are susceptible to infections, especially those caused by encapsulated bacteria. While the knowledge about humoral immunity disturbances in asplenic persons is well established, the abnormalities of cellular response remain still under investigation.

Aim of the study was to estimate the impact of splenectomy on postoperative quantitative changes of NK cells (CD3⁻/CD16⁺CD56⁺) and NKT-like cells (CD3⁺/CD16⁺CD56⁺) as two important lymphocyte subsets involved in host defence against various pathogens.

Material and methods: Assessment of NK and NKT-like cells was performed by the flow cytometry in a group of 100 splenectomised patients and 20 healthy volunteers.

Results: Patients with secondary asplenia displayed a decreased ratio of NK cells (CD3⁻/CD16⁺CD56⁺) ($p = 0.012$) and a higher ratio of NKT-like cells (CD3⁺/CD16⁺CD56⁺) ($p = 0.001$) in comparison to controls. Patients who underwent elective splenectomy presented a higher amount of NK cells (CD3⁻/CD16⁺CD56⁺) than those with post-traumatic spleen removal ($p = 0.035$). The time since surgery negatively correlated with NK cells counts (CD3⁻/CD16⁺CD56⁺). In those patients who noticed increased susceptibility to infections, NK cells number (CD3⁻/CD16⁺CD56⁺) was lower ($p = 0.007$) than in those patients who did not observe any change in prevalence of infections. Moreover, those patients who noticed an increased infection rate had higher frequencies of NKT-like cells (CD3⁺/CD16⁺CD56⁺) comparing to those who did not observe any change in the prevalence of infections ($p = 0.006$).

Conclusions: This study provides a further piece of information on the complex immune disturbances in splenectomised individuals leading to the increased risk of bacterial infections. Results obtained suggest that monitoring of NK and NKT-like cells number may provide useful information for determining asplenia-related immunosuppression.

Key words: splenectomy, NK cells, NKT-like cells, immunity, infections.

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Introduction

Spleen is the largest lymphatic organ in the human body. Functionally it plays a fundamental role in bacterial clearance either by antibody response or macrophage bacteri-

dal capacity [1, 2]. Spleen is critical in clearing opsonised encapsulated bacteria such as pneumococci, meningococci, *Haemophilus influenzae* and *Escherichia coli* [3]. Its specific role is related to marginal zone macrophages, which are able to detect and capture encapsulated bacteria, and

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marginal zone B cells, which respond to capsule polysaccharide antigens by differentiating into IgM-producing memory B cells or antigen presenting cells [4]. The spleen is crucial in regulating immune homeostasis. It combines both innate and adaptive immunity [5]. After splenectomy, mechanisms that play a fundamental role in bacterial clearance are impaired, what results in an increased risk of life-threatening infections [6, 7]. Due to the high risk of fulminant infections occurring in this group, it is essential to take preventive actions [8].

Natural killer (NK) cells are defined as large granular lymphocytes (LGL) with CD3⁻/CD16⁺CD56⁺ phenotype [9], and constitute approximately 4-15% of blood lymphocytes [10]. Natural killer cells are not a homogeneous cell population. In humans, they can be divided into two major subtypes: CD56^{bright} and CD56^{dim}, based on the expression of the surface markers (CD16 and CD56) [11-13]. The unique ability of NK lymphocytes to recognize and kill cells infected with a variety of pathogens, regardless of prior immunization, make them first-line host defence against viruses or other intracellular pathogens [14]. They also participate in tumour immunosurveillance [15, 16] and autoimmunity [17]. It was recently discovered that NK cells have also the capacity for memory-like immune response [18]. Natural killer cells are able to mediate direct cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) against infected or neoplastic cells [19]. They act through exocytosis of various proteins including perforin, granzymes [20], FasL [21] which mediate target-cell killing and production of IFN- γ and other immunoregulatory cytokines [22]. By production of cytokines and chemokines, NK cells regulate the functions of other immune cells, such as dendritic cells (DCs) [23], neutrophils [24], macrophages and monocytes [25]. Moreover NK cell-derived cytokines activate innate immune responses and subsequently influence the development of adaptive immune response [26-28]. The adverse effects of uncontrolled or inappropriate NK cell responses result in different pathological conditions such as allograft rejection and various autoimmune diseases [29]. Complete NK-cell deficiencies in humans result in overwhelming fatal infections in childhood [30].

Natural killer T (NKT) cells represent a small but distinctive population of T lymphocytes which constitute approximately 0.02-0.2% of circulating T cells in humans [31]. Natural killer T cells are prevalent in the liver (about 4% of human hepatic T cells,) bone marrow and thymus [32]. They display properties of both T cells and NK cells through expression of an invariant $\alpha\beta$ T-cell receptor (TCR) ($V\alpha 14/J\alpha 281$ in mice and $V\alpha 24/J\alpha Q$ in humans), and NK cells surface marker NK1.1 (CD161) [33, 34]. Natural killer T-like cells can share other features with NK cells as well, e.g. expression of cytotoxic molecules [35]. In contrast to conventional T-lymphocytes, NKT cells do not respond to antigens presented by a classical major histocompatibility complex (MHC) but recognize a wide range of glycolipid

antigens presented by the MHC I-like molecule CD1d [36]. The best known example of such antigen is α -galactosylceramide (α -GalCer) [37]. Activation via CD1d initiates rapid production of a large number of both Th1 and Th2 immunoregulatory cytokines including IL-4, IL-10 and IFN- γ , activation of several immune cells (NK cells, T, B lymphocytes, dendritic cells) and increase in the cytolytic activity of NKT cells [38]. Due to the above abilities, NKT cells are called a "bridge" between innate and adaptive immunity [39]. Natural killer T cells are involved in a variety of immunological processes. They induce a host immune response against infectious pathogens, promote tumour immunosurveillance or immunosuppression and play a regulatory role in autoimmunity and allergic diseases [37, 40-45]. Natural killer T cells do not represent a homogeneous population. Three types of NKT cells have been described [46, 47].

CD1d-restricted NKT cells are important for host defence against various microbial pathogens. They contribute bacterial clearance through two different pathways. Direct recognition of bacterial lipids may occur via TCR [48]. In absence of microbial glycolipids, NKT cells are stimulated by IL-12, produced by dendritic cells and macrophages previously activated by pathogens [49]. Natural killer T cells activation may be responsible for septic shock reaction [50]. Furthermore, NKT cell subpopulations might play opposite roles due to functional differences. Type I and type II NKT cells display cross-regulation abilities, forming a new immunoregulatory axis [42, 51].

Splenectomy severely affects immunity. While the knowledge about humoral immunity disturbances in splenectomised individuals is well established, the abnormalities of cellular response remain still under investigation. The main aim of the present study was to estimate the impact of splenectomy on postoperative quantitative changes of NK and NKT-like cells as two important lymphocyte subsets involved in host defence against various pathogens.

Material and methods

Patients and controls

A study group of 100 subjects (51 women and 49 men), with an average age of 34.28 ± 22.87 years (median: 36, min. 18, max. 69 years), was recruited at the Department of Clinical Immunology and Immunotherapy of the Medical University of Lublin between October 2010 and December 2012. Fifty-six patients (56%) were splenectomised because of a blunt abdominal trauma with spleen injury. In 28 persons (28%), the reason of splenectomy was hereditary spherocytosis and 16 patients (16%) had had immune thrombocytopenic purpura (ITP). The average time since the splenectomy was 8.83 ± 9.72 years. Seventy-four persons (74%) reported more frequent infections after

splenectomy and 26 patients (26%) did not observe any change in prevalence of upper respiratory tract infections.

Control samples of peripheral blood (PB) were obtained from 20 healthy volunteers (8 women and 12 men) aged from 19 to 73 years (median age: 40.5 years, mean: 44.82 ±31.12 years). Nobody of the splenectomised subjects in the study group and controls complained of ailments characteristic of the current infection. None of them was taking immunosuppressive or immunomodulative treatment within the last 12 months. The Local Ethical Committee at the Medical University of Lublin approved the research and patients gave their prior written consent.

Assessment of NK (CD3-/CD16+CD56+) and NKT-like (CD3+/CD16+CD56+) cells

Peripheral blood in an amount of 1 ml was taken to tubes with an EDTA anticoagulant. Percentages of NK and NKT-like cells were evaluated with flow cytometry using monoclonal antibodies (MoAbs) anti-CD3 FITC/CD16+CD56 PE/CD45 PerCP (BD Biosciences), which allowed for simultaneous assessment of T (CD3+) lymphocytes and NK (CD16+CD56+) cells. During analysis, the CD3+/CD16+CD56+ population was also determined. A standard, whole-blood assay with erythrocyte cell lysis was used to prepare the PB samples. The percentage of positive cells was measured from a cut-off set using isotype matched non-specific control antibody. Three-colour immunofluorescence analyses were performed using a FACS Calibur flow cytometer (Becton Dickinson) equipped with 488 nm argon laser. A minimum of 10 000 events was acquired and analyzed using CellQuest Software. The percentage and absolute counts of cells expressing surface markers were analyzed. Dot plots, illustrating the analysis method for the

identification of NK and NKT-like cells are shown in Figure 1.

Statistical analysis

Differences between two groups were assessed using the Mann-Whitney *U* test. Spearman rank test was used to assess correlations between the variables. Statistica 9.0 PL software was used for all statistical procedures. Differences were considered as statistically significant when the *p* value was less than 0.05.

Results

The immunophenotypic analysis revealed differences between the study and control groups in total lymphocytes as well as particular numbers of lymphocyte subsets. The total lymphocyte count, percentages and absolute counts of T lymphocytes, NK cells and NKT-like cells in the study and control groups are presented in Table 1.

Patients with lymphocyte levels in the normal range [1.5-4 × 10³/mm³] represented 84% of the study group, lymphocytosis occurred in 10% of cases, lymphocytopenia in 6% of cases. The T lymphocytes (CD3+) level as compared to the normal range [60-75%] was elevated in 23% of individuals after splenectomy and normal in 67% of cases. In both groups, the level of NK cells (CD3-/CD16+CD56+) exceeded the level of NKT-like cells (CD3+/CD16+CD56+). Splenectomised patients had a lower amount of NK cells (CD3-/CD16+CD56+) and a higher amount of NKT-like cells (CD3+/CD16+CD56+). Spleen removal was followed by changes in the ratio of NK and NKT-like cell subsets. Patients with secondary asplenia displayed a decreased ratio of NK cells (CD3-/CD16+CD56+)

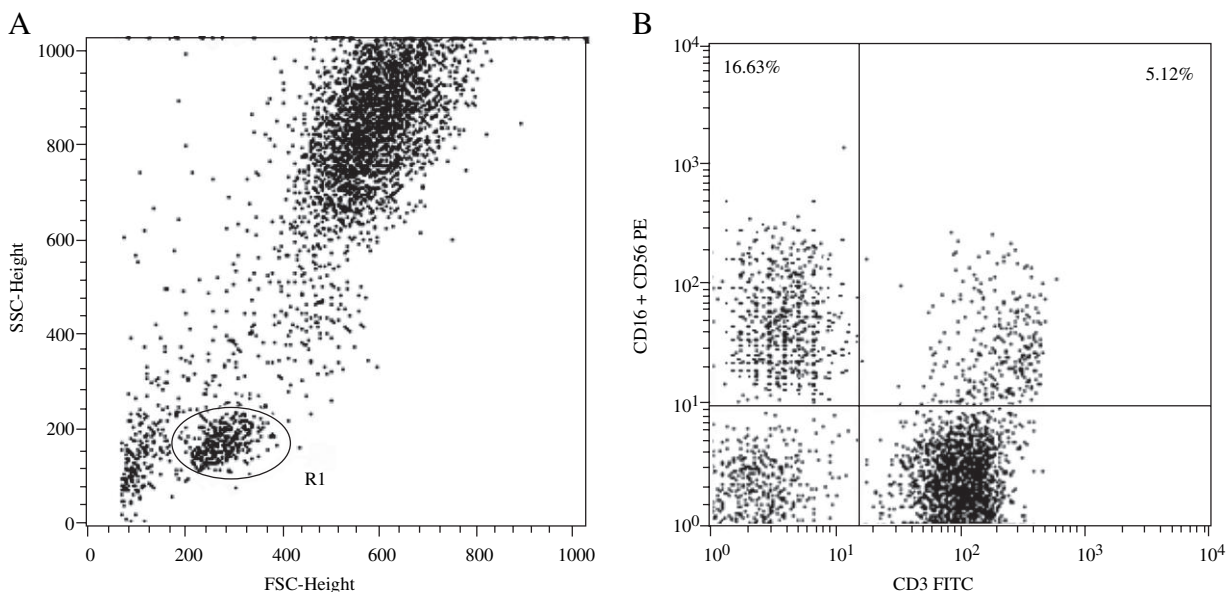


Fig. 1. Dot-plots showing the analysis method for the identification of NK and NKT-like cells in a splenectomised patient

Table 1. Total lymphocyte count, percentages and absolute counts of T lymphocytes, NK cells and NKT-like cells in the study and control groups

	Study group (n = 100)	Control group (n = 20)
Total lymphocytes count [10 ³ /mm ³]	2.66 ± 0.87	2.45 ± 0.6
T lymphocytes (CD3+) [%]	65.98 ± 8.14	65.75 ± 13.25
T lymphocytes (CD3+) [10 ³ /mm ³]	1.74 ± 0.59	1.61 ± 0.52
NK cells (CD3-/CD16+CD56+) [%]	15.68 ± 8.03	20.23 ± 10.35
NK cells (CD3-/CD16+CD56+) [10 ³ /mm ³]	0.42 ± 0.27	0.5 ± 0.3
NKT-like cells (CD3+/CD16+CD56+) [%]	4.42 ± 3.08	2.09 ± 1.03
NKT-like cells (CD3+/CD16+CD56+) [10 ³ /mm ³]	0.12 ± 0.12	0.05 ± 0.03

(*p* = 0.012) and a higher ratio of NKT-like cells (CD3+/CD16+CD56+) (*p* = 0.001) in comparison to controls (Table 1).

We revealed a positive correlation between T lymphocytes (CD3+) and NK cells (CD3-/CD16+CD56+), as well as NKT-like cells (CD3+/CD16+CD56+) number (*r* = 0.426, *p* < 0.0001, and *r* = 0.414, *p* < 0.0001, respectively) (Fig. 2 and Fig. 3).

Furthermore, there was a negative correlation between the frequencies of NK cells (CD3-/CD16+CD56+) and NKT-like cells (CD3+/CD16+CD56+) (*r* = -0.232, *p* = 0.013) (Fig. 4).

Patients who underwent elective splenectomy presented a higher amount of NK cells (CD3-/CD16+CD56+) than those with post-traumatic spleen removal (*p* = 0.035) (Fig. 5).

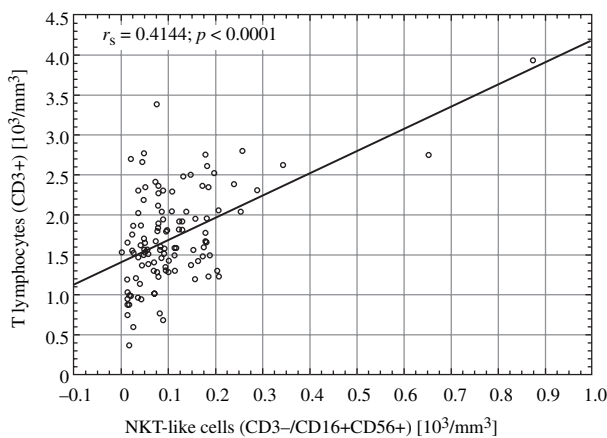


Fig. 3. Spearman correlation between total T lymphocytes count and NKT-like cells count (*r* = 0.414, *p* < 0.0001)

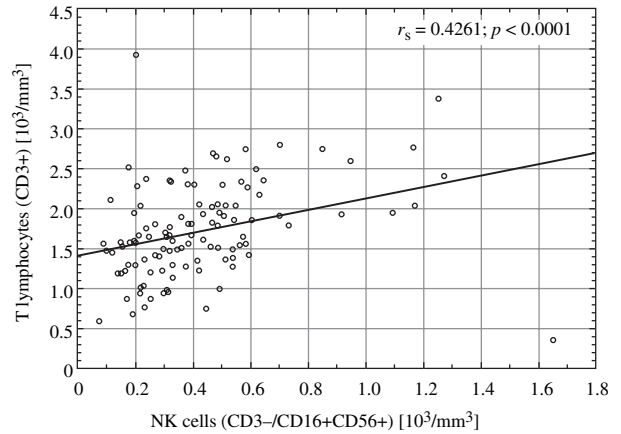


Fig. 2. Spearman correlation between total T lymphocytes count and NK cells count (*r* = 0.426, *p* < 0.0001)

The time since surgery negatively correlated with NK cells counts (CD3-/CD16+CD56+) (Fig. 6).

In those patients who noticed increased susceptibility to infections, the number of NK cells (CD3-/CD16+CD56+) was lower (*p* = 0.007) than in those patients who did not observe any change in prevalence of infections (Fig. 7).

Moreover, those patients who noticed an increased infection rate had higher frequencies of NKT-like cells (CD3-/CD16+CD56+) comparing to those who did not observe any change in the prevalence of infections (*p* = 0.006) (Fig. 8).

Discussion

Acquired asplenia following splenectomy due to post-traumatic spleen rupture or as a treatment of certain hematologic diseases is a common abnormality. It is well estab-

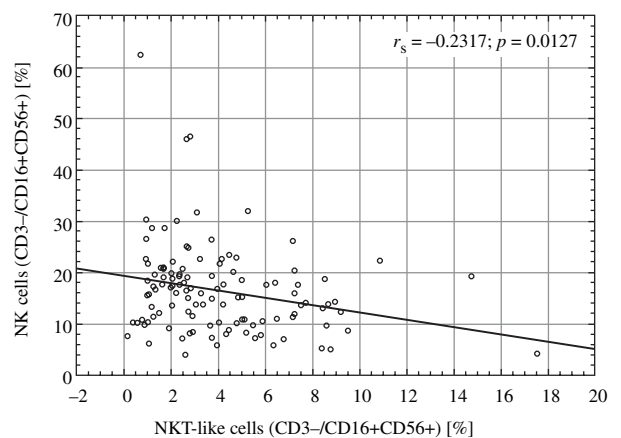


Fig. 4. Spearman correlation between the frequencies of NK cells (CD3-/CD16+CD56+) and NKT-like cells (CD3+/CD16+CD56+) (*r* = -0.232, *p* = 0.013)

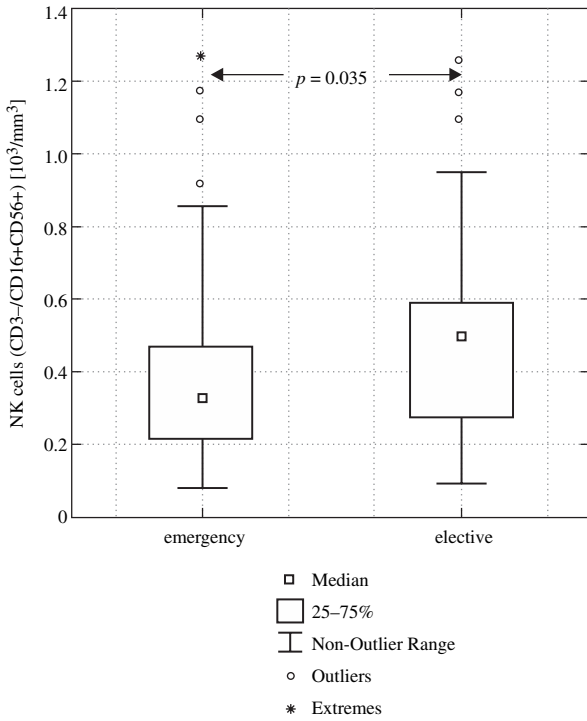


Fig. 5. Absolute counts of NK cells in the two groups of asplenic patients: those who underwent emergency splenectomy and those who underwent elective splenectomy ($p = 0.035$)

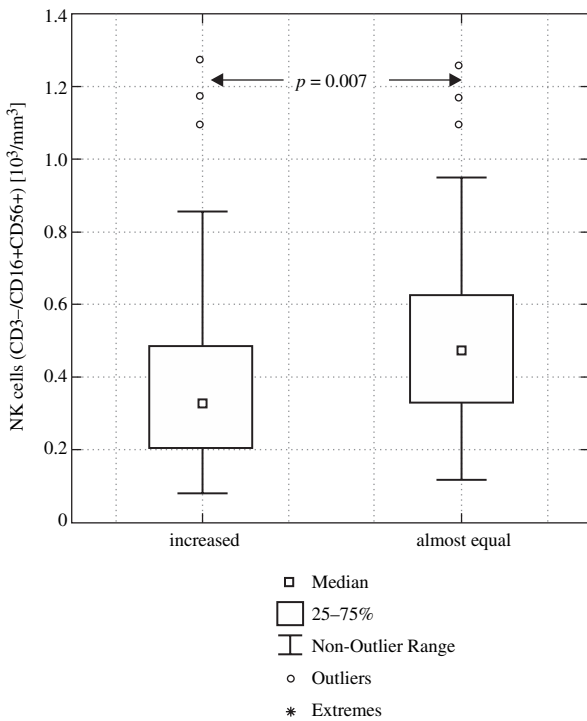


Fig. 7. Absolute counts of NK cells in the two groups of asplenic patients: those who noticed an increase in infections and those who did not observe any change in the prevalence of infections ($p = 0.007$)

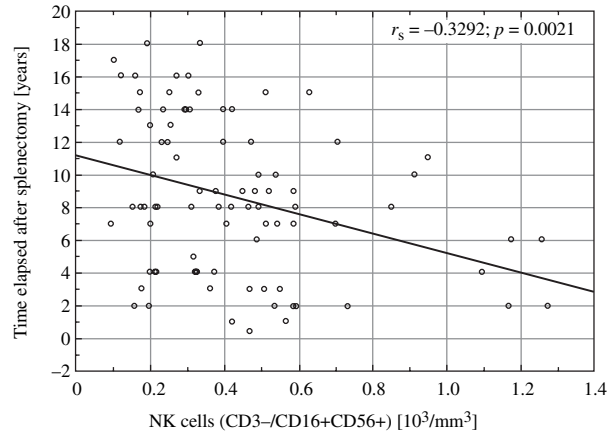


Fig. 6. A Spearman correlation between NK cells count and the time elapsed after splenectomy ($r = -0.329$, $p = -0.0021$)

lished that spleen removal has an influence on the total lymphocyte level as well as particular numbers of lymphocyte subsets [52-54]. According to our study, the total lymphocyte count and T lymphocyte subsets count after a splenectomy were insignificantly higher comparing to the control group whereas several other publications revealed a significant increase in total peripheral lymphocytes and T-lymphocytes in splenectomised patients [55, 56].

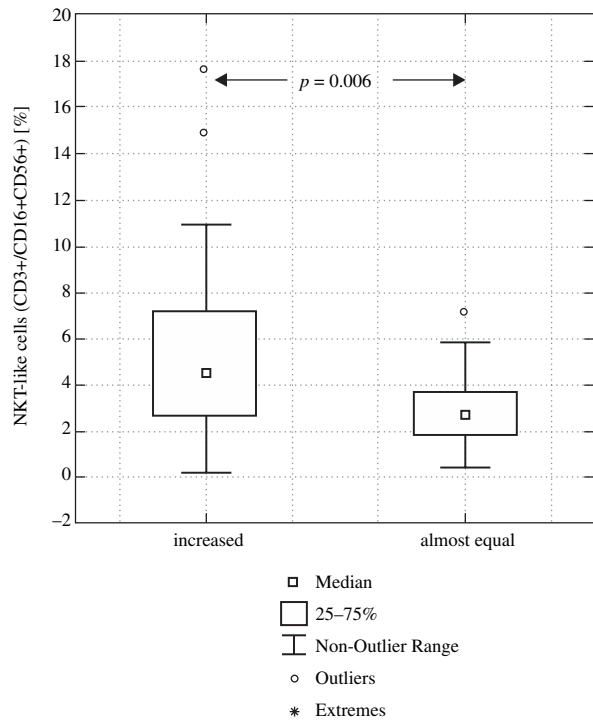


Fig. 8. Frequencies of NKT-like cells in the two groups of asplenic patients: those who noticed an increase in infections and those who did not observe any change in the prevalence of infections ($p = 0.006$)

The lymphocytosis pattern may be different considering a reason of splenectomy. Post-traumatic splenectomy patients presented an increase in the absolute numbers of CD8+ and NK cells and, to a lesser extent, of NKT cells [57]. Asplenia due to autoimmune thrombocytopenic purpura (ATP) was connected only with persistent NK cell expansion [58]. Our data showed a higher amount of NK cells in patients who underwent elective splenectomy than those with post-traumatic spleen removal. However, in contrast to other authors, our results demonstrated a lower amount of NK cells and a higher amount of NKT-like cells than in the study group. Moreover, we observed that the ratio of NK and NKT-like cells was inversely proportional.

Several studies with a more detailed lymphocyte subsets analysis showed a substantial increase in the number of CD4+ and CD8+ lymphocytes but reduction in the CD4/CD8 ratio in splenectomised patients [52-54, 59, 60]. There is clinical evidence that a decrease in CD4+ T cells ratio was caused by a selective and long-term decrease in the percentage of CD4+CD45RA+ lymphocytes [61]. There is a difference between clinical studies and animal model research. In humans, post-splenectomy T cells subset redistribution lasted significantly longer [62, 63].

Our study revealed a relation between NK cells level and time elapsed after a splenectomy, while Winkelmeyer *et al.* did not notice any differences in immunological parameters in two groups that had undergone splenectomy 7 months to 5 years or 6 to 14 years earlier [64].

The mechanisms that play a fundamental role in bacterial clearance after a splenectomy are impaired, which results in an extended risk of infections. In accordance with that common observation, we noticed an increased frequency of infections following splenectomy. Natural killer T-like cells are important regulators of immunity against infectious diseases. They control bacterial clearance involving both innate and adaptive immunity [42]. On the other hand, they may be responsible for septic shock reaction through production of cytokines [50]. We described an elevated number of NKT-like cells in patients with a higher infection rate. Natural killer cells have a major role in defence against a broad spectrum of pathogens as well. A persistent increase in the absolute number and cytotoxic activity of NK cells which occurs after a splenectomy might probably compensate partially for the lack of phagocytic activity [65]. However, according to our findings, NK cells number in the study group was decreased, what was surprising, and related to a higher infection rate.

Conclusions

In conclusion, this research confirmed previous findings that total counts of lymphocytes and T lymphocyte subsets after a splenectomy were higher, but observed elevations were statistically insignificant. A lower post-splenec-

tomy amount of NK cells is an unexpected result, considering other studies. An inversely proportional correlation between the ratio of NK and NKT-like cells was noticed. Increase in the infection rate corresponded with an elevated NKT-like cells number and a decreased NK cells count. In comparison to the preliminary research, this study provides a further piece of information on the complex immune disturbances in splenectomised individuals leading to the increased risk of bacterial infections. Results obtained suggest that monitoring of the NK and NKT-like cells number may provide useful information for determining asplenia-related immunosuppression. Those outcomes require further investigation.

The authors declare no conflict of interests.

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