γδT-КЛЕТКИ У ПАЦИЕНТОВ С ОПУХОЛЯМИ ПОЛОСТИ НОСА И ОКОЛОНОСОВЫХ ПАЗУХ

Нижегородова Д.Б.[1, 2], Колядич Ж.В.[3], Морозова Н.А.[3], Зафранская М.М.[1, 2]

[1] Белорусская медицинская академия последипломного образования, г. Минск, Республика Беларусь
[2] Международный государственный экологический институт имени А.Д. Сахарова Белорусского государственного университета, г. Минск, Республика Беларусь
[3] Республиканский научно-практический центр онкологии и медицинской радиологии имени Н.Н. Александрова, Минский район, а/г Лесной, Республика Беларусь

Резюме. Иммунологические факторы могут играть важную роль в качестве предикторов или прогностических биомаркеров при онкопатологических процессах. В последнее время в качестве перспективных эффекторных клеток для иммунотерапии злокачественных новообразований рассматривается популяция неклассических γδT-лимфоцитов, сочетающих свойства врожденного и приобретенного иммунитета. В данной работе представлена структурно-функциональная характеристика субпопуляций γδT-лимфоцитов, вовлекающихся в формирование противоопухолевого иммунитета у пациентов со злокачественными и доброкачественными опухолями полости носа и околоносовых пазух. Цель исследования – оценить субпопуляционный состав и функциональные особенности γδT-клеток у пациентов с новообразованиями полости носа и околоносовых пазух для характеристики механизмов клеточного иммунитета при опухоль-ассоциированном патологическом процессе.

Материалом исследования явилась периферическая венозная кровь 21 пациента (13 мужчин и 8 женщин, средний возраст 63,0 (56,0-69,0) лет) с новообразованиями полости носа и околоносовых пазух и 10 условно здоровых доноров. Фенотип лимфоидных клеток и внутриклеточную продукцию цитокинов оценивали с использованием моноклональных антител и метода проточной цитометрии, внеклеточную продукцию цитокинов исследовали в супернатантах культур методом иммуноферментного анализа.

Установлено увеличение общего количества γδT-клеток у пациентов с плоскоклеточным раком и изменение соотношения $V\delta2^+/V\delta1^+$T-клеток в периферической крови как у пациентов со злокачественными, так и доброкачественными опухолями полости носа и околоносовых пазух по сравнению со здоровыми донорами. В обеих исследуемых группах пациентов выявлено повышение уровня фосфоантиген-индуцируемой активации γδT-клеток в сочетании со снижением индексов стимуляции и различным цитокиновым профилем: у пациентов с плоскоклеточным раком отмечалось увеличение внутриклеточной продукции IFNγ в γδT-клетках, в то время как у пациентов с инвертированной папилломой перераспределение субпопуляций γδT-лимфоцитов связано с преимущественной продукцией IL-17. При этом процент γδT-лимфоцитов, синтезирующих IFNγ, коррелировал с его концен-

Адрес для переписки:

Нижегородова Дарья Борисовна
Белорусская медицинская академия последипломного образования
220013, Республика Беларусь, г. Минск,
ул. П. Бровки 3, корп. 3.
Тел.: +375 296089994.
E-mail: nzh@tut.by

Address for correspondence:

Darya B. Nizheharodava
Belarusian Medical Academy of Postgraduate Education
3 P. Brovki St, Bldg 3
Minsk
220013 Belarus
Phone: +375 296089994.
E-mail: nzh@tut.by

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трацией в супернатантах клеточных культур у пациентов со злокачественными новообразованиями полости носа и околоносовых пазух ($R = 0,61$; $p < 0,05$).

Полученные данные свидетельствуют о вовлечении γδT-лимфоцитов в патогенез злокачественных и доброкачественных опухолей и могут являться фундаментальной основой для дальнейшего определения возможных предикторов опухоль-ассоциированной воспалительной реакции и малигнизации.

Ключевые слова: опухоли полости носа, опухоли околоносовых пазух, лимфоидные клетки, γδT-лимфоциты, IFNγ, IL-17, изопентенил пирофосфат

γδT CELLS IN PATIENTS WITH TUMORS OF THE NASAL CAVITY AND PARANASAL SINUSES

Nizheharodava D.B.[a, b], Kolyadich J.V.[c], Marozava N.A.[c], Zafranskaya M.M.[a, b]

[a] *Belarusian Medical Academy of Postgraduate Education, Minsk, Republic of Belarus*
[b] *International Sakharov Environmental Institute of Belarusian State University, Minsk, Republic of Belarus*
[c] *N. Alexandrov National Cancer Centre of Belarus, Minsk District, Republic of Belarus*

Abstract. The immunological factors can play an important role as predictive and prognostic biomarkers in oncopathology. Recently, non-conventional innate-like γδT-lymphocytes have received a lot of attention as a promising effector cell population for cancer immunotherapy. This study describes structural and functional subpopulations of γδT lymphocytes involved in antitumor immunity in patients with malignant and benign tumors of the nasal cavity and paranasal sinuses. The aim of the study was to estimate γδT cell subsets composition and functions in patients with neoplasms of nasal cavity and paranasal sinuses in order to characterize cellular immunity in tumor-associated pathological process.

The peripheral venous blood was obtained from 21 patients (13 men and 8 women, average age of 63.0 (56.0-69.0) y. o.) with neoplasms of nasal cavity and paranasal sinuses, and 10 healthy donors. Lymphoid cells phenotype and production of intracellular cytokines were investigated using monoclonal antibodies and flow cytometry, production of extracellular cytokines was measured using enzyme-linked immunosorbent assay kits.

The increase of total γδT cells number in patients with squamous cell carcinoma accompanied by changes in $Vδ2^+/Vδ1^+$T cells ratio in peripheral blood of both patients' groups with malignant and benign nasal cavity and paranasal sinuses tumors were revealed as compared to healthy donors. The upregulated γδT cell response to phosphoantigen induction in combination with reduced indices of stimulations were shown in the both patients groups but cytokine profile was different, i.e., the elevated IFNγ production has been determined in patients with squamous cell carcinoma. However, in patients with inverted papilloma, redistribution of γδT cell subsets has been associated with IL-17-producing γδT cells. Moreover, the percent of $IFNγ^+$γδT lymphocytes did correlate with IFNγ concentration in cell culture supernatants of patients with malignant nasal cavity and paranasal sinuses neoplasms ($R = 0.61$; $p < 0.05$).

The revealed data suggest an involvement of γδT lymphocytes in malignant and benign tumor pathogenesis and may provide a fundamental basis for further detection of possible tumor-associated inflammation and malignization predictors.

Keywords: nasal cavity, paranasal sinuses, neoplasms, lymphoid cells, γδT lymphocytes, IFNγ, IL-17, isopentenyl pyrophosphate

Introduction

Nasal cavity (NC) and paranasal sinuses (PNS) neoplasms account for approximately 5% of the upper respiratory tract tumors. About half of these tumors are benign, mainly squamous and inverted papilloma (IP), and the remaining are malignancies amounting for 1-3% of malignant tumors of all localizations, of which squamous cell carcinoma (SCC) comprises approximately 75%. More than 76% of patients have malignant tumors of NC and PNS detected at stage III-IV [18, 22].

One of the issues is the absence of biomarkers or predictors that contribute to the early detection of the malignant process and, therefore, late diagnosis of malignant NC and PNS neoplasms. According to numerous studies, neoplasm has a dual effect on the immune system: on the one hand, the local tumor microenvironment is characterized by changes in immune cells populations and immune control check-points including the interaction of

co-inhibitor receptors (cytotoxic T lymphocyte-associated antigen 4, programmed death-1, T cell immunoglobulin domain and mucin domain 3, lymphocyte activation gene 3, etc.) on lymphocytes with their ligands, on the other hand, tumor factors production induces an imbalance and leads to immunosuppression contributing to tumor cells escape from immunosurveillance [4, 7]. These molecular events determine a certain lymphoid cells subsets composition in the systemic circulation as well as local infiltration into the tumor, the functional potential of which can be characterized by cytotoxic reactions and also contribute to the immunosuppression [11]. In view of the immune system involvement in the tumor pathogenesis, it seems important to search for immunological biomarkers, which may include T lymphocytes with T cell receptors (TCR) composed of γ and δ chains (γδT lymphocytes).

Over the past decade, unconventional innate-like γδT lymphocytes have received a lot of attention as attractive effector cells for cancer immunotherapy [16, 33]. Being a minor T cell population in peripheral blood (0.5-10% of all T lymphocytes), they are abundant in the mucosa within intraepithelial lymphocytes (up to 50%) and participate in protective immunity against tumors and infectious organisms providing immunosurveillance. Their high and quick antitumor activity is characterized by non-MHC-restricted antigen recognition, cytotoxic potential, antigen-presenting function, abundant cytokine and chemokine secretion capacity [15, 28].

In recent years, there have been a number of reports about diverse roles of γδT lymphocytes in tumor immunity owing to their structural and functional heterogeneity [13, 16, 32]. Compared to antigenic receptors of αβT and B lymphocytes, the great γδT cells potential to various ligand-binding sites formation comes from the high polymorphism of γδTCR, the variable domains of which are encoded with 3 main Vδ-genes and at least 6 Vγ-genes, resulting in high γδT lymphocytes heterogeneity. Moreover, similar to αβT lymphocytes γδT cells are able to polarize into γδT1 cells (secreting interferon γ (IFNγ) and tumor necrosis factor α (TNFα)), γδT1/17 cells (secreting IFNγ and interleukin (IL) 17), γδT17 cells (secreting IL-17 only), γδT2 cells (secreting IL-4), follicular B helper $γδT_{FH}$ cells (secreting IL-4, IL-10) and regulatory fork head box $P3^+$ ($FoxP3^+$) γδTreg cells. It was demonstrated that γδT1 cells (express $CD56^+$ phenotype and are involved in cytolytic reactions) and $γδT_{FH}$ cells (enhance B lymphocyte maturation and antibody formation) have the both a direct and indirect antitumor effect, while γδT17 cells, $FoxP3^+$γδTreg cells show protumorigenic effect due to the immunosuppression [28, 32].

Thus, the determination of γδT lymphocytes specific immunological features in benign and malignant NC and PNS neoplasms is of actual interest and may be considered as a basis for detailing and systematizing new methods for diagnosis and therapy as well as developing an algorithm for their application in preventing disease complications and relapses.

In this article, the characteristic of γδT cell subsets composition and functions in patients with NC and PNS neoplasms is presented for the first time, aimed at γδT mediated cellular immunity assessment in tumor-associated pathological process.

Materials and methods

The peripheral venous blood was obtained from 21 patients with NC and PNS neoplasms (13 men and 8 women, average age of 63.0 (56.0-69.0) y. o.) hospitalized at N.N. Alexandrov National Cancer Centre of Belarus. All subjects were divided in three groups: group 1 – 10 patients with SCC; group 2 – 11 patients with IP; group 3 consisted of 10 healthy donors. Clinical and demographic characteristics of patients and healthy donors are presented in Table 1.

Peripheral blood mononuclear cells isolation and cultivation

Peripheral blood was collected in sterile heparin tubes, diluted 1:1 with physiological saline, layered onto a Histopaque-1077 density gradient (Sigma, Germany) and centrifuged for 30 min at 300 g at 4 °C. The resulting interphase ring of peripheral blood mononuclear cells (PBMC) was washed twice in physiological saline for 10 min at 300 g and 4 °C. PBMC were cultured in RPMI-1640 medium (Bio-Whittaker, USA) completed with 10% fetal calf serum (Gibco, Germany), 2 mM L-glutamine (Bio-Whittaker, USA), 1% antibiotic-antimycotic (Gibco, Germany), 100 U/mL IL-2 (Fluka, Germany) during 3 days (for further estimation of γδT cells cytokines production) and in the presence or absence of 20 μM isopentenyl pyrophosphate (IPP, Sigma, Germany) during 6 days (for further estimation of γδT cells proliferation rate) as previously described with minor modifications [20].

Flow cytometry method

Immunophenotyping of peripheral blood lymphoid cells

The populations of lymphoid cells in whole peripheral blood were determined using the next CYTO-STAT tetraCHROME monoclonal antibody panels: CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5 (Beckman Coulter, USA). γδT lymphocytes subsets were identified in whole peripheral blood using a DuraCloneIMTCRs monoclonal antibody panel: γδTCR-FITC/αβTCR-PE/HLA-DR-ECD/Vδ1TCR-PC7/CD4-APC/CD8-AF700/CD3-AF750/Vδ2TCR-PB/CD45-KrO (Beckman Coulter, USA). Monoclonal antibody reagents were added according to the manufacturer's instructions to 100μL of the venous blood specimen, and reaction mixtures were incubated at 20-25 °C for 15 min in the dark. Then red blood cells were lysed with VersaLyse solution (Beckman Coulter, USA) for 10 min. Results were analyzed on

TABLE 1. CLINICAL AND DEMOGRAPHIC CHARACTERISTICS OF PATIENTS AND HEALTHY DONORS

Groups	Diagnosis	n	Gender, male / female	Age, y. o.	Disease stage (TNM system)	Disease duration, months
Group 1	Squamous cell carcinoma	10	7/3	58.0 (56.0-71.7)	stage I (20%) stage II (20%) stage III (20%) stage IV (40%)	6.0 (4.0-10.0)
Group 2	Inverted papilloma	11	6/5	64.0 (50.5-67.5)	–	5.0 (2.0-18.0)
Group 3	Healthy donors	10	5/5	49.0 (44.0-61.0)	–	–

Note. TNM, Tumor, Node, Metastasis system; n, patients' number in a group; y. o., years old.

10000 $CD3^+$T lymphocytes or 1000 γδT lymphocytes using a 10-channel Cytoflex flow cytometer (Beckman Coulter, USA).

Intracellular cytokine detection

IFNγ and IL-17 syntheses were evaluated in 3-days PBMC cultures as previously described with minor modifications [31]. For quantitative intracellular cytokines determination, 4 ng/mL phorbol 12-myristate 13-acetate (Sigma, Germany), 1 μg/mL of ionomycin calcium salt and 10 μg/mL brefeldin A (Cayman Chemicals, USA) were added in the last 4 hours of cell culture activation. Then PBMC were stained with monoclonal antibodies to surface CD3-FITC and γδTCR-PC7 (Beckman Coulter, USA) at 20-25 °C for 15 min in the dark, fixed with 4% paraformaldehyde (Sigma, Germany), permeabilized with 2% Triton X (Sigma, Germany), and then intracellular staining was performed using monoclonal antibodies IFNγ-PE (Beckman Coulter, USA) and IL-17A-PerCP (R&D Systems, USA). Results were analyzed on 1000 γδT lymphocytes using flow cytometer.

γδT cells proliferation rate

γδT cells proliferation rate was estimated in 6-days PBMC cultures stained with monoclonal antibodies to surface CD3-FITC and γδTCR-PC7 (Beckman Coulter, USA). PBMC cultures without IPP were used as controls. Results were analyzed on 1000 γδT lymphocytes using flow cytometer. The index of stimulation (IS) was calculated as the ratio of IPP-stimulated γδT cells number to the unstimulated cultures in conventional units (c. u.).

ELISA extracellular IFN detection

IFNγ concentration was determined in the cell-free culture supernatants using commercial human ELISA kit "γ-Interferon-EIA-BEST" (Vektor-Best, Russia) according to the manufacturer's instruction.

Statistical method

Statistical data processing was performed using Statistica 8.0 (StatSoft Inc., USA). The median (Me), 25^{th} and 75^{th} percentiles were used as descriptive statistics of the studied groups. Significant differences between investigated groups were determined by nonparametric criteria: Mann–Whitney U test and Wilcoxon test; p-values < 0.05 (*) and $p < 0.01$ (**) were considered as statistically significant.

Results

Lymphoid cells population in patients with malignant and benign NC and PNS neoplasms

The absolute and relative numbers of peripheral blood lymphocytes populations in patients with NC and PNS neoplasms and donors are presented in Table 2. Patients from group 1 were characterized by changes in the following classical lymphoid cell populations: decreased $CD3^+CD4^+$T helpers ($p < 0.01$) as well as increased $CD3^+CD8^+$T lymphocytes ($p < 0.05$) have been detected as compared to healthy donors. Meanwhile, the total number of $CD3^+$T lymphocytes, $CD19^+$B cells and $CD56^+$NK cells in patients with SCC did not significantly differ from those in groups 2 and 3. By contrast, the significantly increased relative number of γδT lymphocytes was found in patients with malignant NC and PNS tumors as compared to the both patients with IP as well as to healthy donors. The quantitative parameters of peripheral blood lymphocytes subsets in patients with IP did not statistically differ from those in healthy donors (Table 2).

In view of significant changes and involvement of non-classical γδT lymphocytes in patients with NC and PNS tumors, a further analysis of γδT cells subsets and potential functions was performed.

γδT cell subsets in patients with malignant and benign NC and PNS neoplasms

Three subpopulations of γδT lymphocytes depending on the expression of the TCR δ chain were investigated in patients with NC and PNS neoplasms. The original flow cytometry dot-plots of γδT cells numbers are presented in Figure 1 and displayed a pattern of typical subsets composition in investigated groups.

As seen from the original dot-plots of $Vδ1^+$T and $Vδ2^+$T cells numbers in patient S. with a confirmed diagnosis of SCC from group 1 (Figure 1A) and in patient N. with a confirmed diagnosis of IP from group 2 (Figure 1B), $Vδ2^+/Vδ1^+$T cells ratio has been decreased as compared to healthy donor R. Like that, $Vδ1^+$T cells subset prevailed in patient S.

(47.33%, Figure 1A), and Vδ2$^+$/Vδ1$^+$T cells ratio reached 1,1, while in healthy donor R. Vδ2$^+$T cells subset dominated (94.63%, Figure 1C), and Vδ2$^+$/Vδ1$^+$T cells ratio made 23.9. Meanwhile, in patient N. with IP with decreased Vδ2$^+$T cells subset number (72.02%) as well as Vδ2$^+$/Vδ1$^+$T cells ratio (3.9), an increase of the both Vδ1$^+$T cells and Vδ1$^-$/Vδ2$^-$T cells (corresponds to Vδ3$^+$T cells) subsets was observed (Figure 1B).

The statistical analysis of γδT cells subsets number in investigated groups revealed the significantly decreased Vδ2$^+$T cells number and the increased Vδ1$^+$T cells percentage as well as the tendency to increase of Vδ1$^-$Vδ2$^-$T lymphocytes (Vδ3$^+$T cells) in peripheral blood of patients with both malignant and benign NC and PNS tumors as compared to healthy donors (Figure 1D).

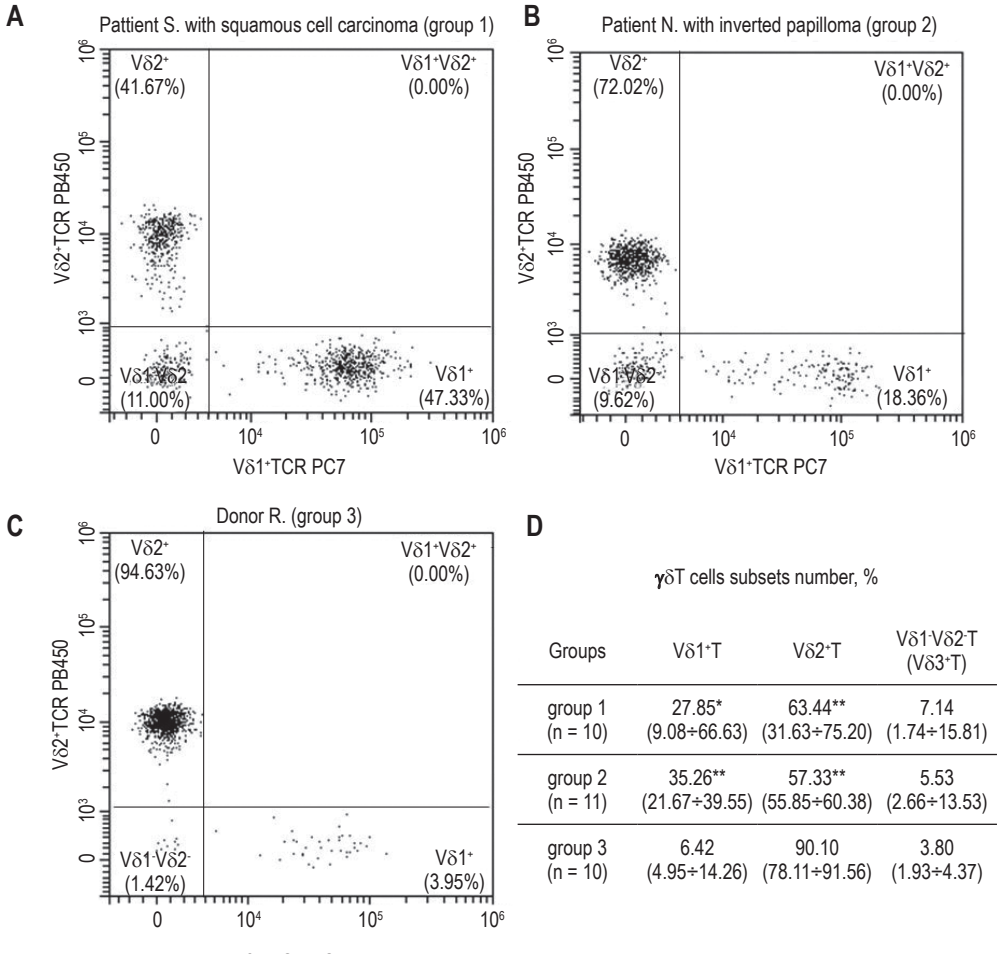
γδT cells proliferation response to IPP in patients with malignant and benign NC and PNS neoplasms

For the assessment of functional status, the γδT lymphocytes number was determined in 6-days PBMC cultures under IPP-stimulated conditions. The increased γδT cells percentage in response to phosphoantigen was shown in the both groups of patients ($p < 0.05$) as well as in healthy donors ($p < 0.01$) (Figure 2, significance is not shown). But the number of IPP-stimulated γδT cells in PBMC cultures of patients with SCC was significantly higher (21.6 (10.3-32.4) %) than in patients with IP (11.5 (3.8-19.6) %) or healthy donors (12.3 (8.9-26.6) %). Nevertheless, the indices of stimulations in the both groups of patients were significantly reduced (IS_{group1} = 1.87 (1.69-2.35) c. u. and IS_{group2} = 2.10 (1.88-3.11) c. u.) as compared to healthy donors

TABLE 2. LYMPHOID CELLS IN PERIPHERAL BLOOD OF PATIENTS WITH NC AND PNS NEOPLASMS AND HEALTHY DONORS, Me ($Q_{0.25}$-$Q_{0.75}$)

Lymphoid cells	Patients with NC and PNS neoplasms		Healthy donors	p-value
	Group 1 (SSC) n = 13	Group 2 (IP) n = 15	Group 3 n = 10	
	1	2	3	
Lymphocytes, %	30.50 (23.50-35.25)	36.00 (27.50-51.00)	36.00 (31.00-44.00)	n. s.
Lymphocytes, × 10^9/L	2.03 (1.37-2.86)	2.70 (1.88-3.53)	2.62 (2.23-3.08)	n. s.
CD3$^+$T cells, %	72.81 (68.86-79.02)	70.52 (65.21-75.33)	70.53 (68.22-76.76)	n. s.
CD3$^+$T cells, × 10^9/L	1.32 (0.94-1.89)	2.09 (1.38-2.50)	1.91 (1.67-2.17)	n. s.
CD3$^+$CD4$^+$ T cells, %	53.13 (49.34-64.83)	60.32 (54.98-62.73)	64.41 (60.30-69.71)	$p_{1-3} < 0.05$
CD3$^+$CD4$^+$ T cells, × 10^6/L	769.42 (502.57-1150.03)	1152.95 (818.77-1284.44)	1172.99 (1013.61-1363.15)	n. s.
CD3$^+$CD8$^+$ T cells, %	40.57 (26.51-44.65)	30.97 (28.14-33.83)	29.25 (25.10-33.07)	$p_{1-3} < 0.05$
CD3$^+$CD8$^+$ T cells, × 10^6/L	454.27 (378.06-646.46)	651.77 (367.62-784.22)	561.30 (418.69-714.54)	n. s.
γδTCR$^+$CD3$^+$ T cells, %	5.82 (3.76-7.24)	3.49 (2.72-6.24)	3.32 (1.93-5.17)	$p_{1-2} < 0.05$ $p_{1-3} < 0.05$
γδTCR$^+$CD3$^+$ T cells, × 10^6/L	68.01 (30.43-101.12)	72.53 (41.56-123.44)	55.81 (35.95-104.42)	n. s.
CD19$^+$B cells, %	8.35 (5.38-9.42)	10.34 (5.93-11.06)	9.03 (7.56-10.92)	n. s.
CD19$^+$B cells, × 10^6/L	168.64 (75.28-248.99)	251.34 (146.54-339.12)	222.49 (194.44-332.79)	n. s.
CD56$^+$ NK cells, %	17.57 (12.39-19.56)	18.25 (12.49-22.48)	15.25 (11.48-18.54)	n. s.
CD56$^+$ NK cells, × 10^6/L	290.96 (229.02-423.22)	419.85 (302.16-638.14)	401.56 (250.99-491.42)	n. s.

Note. SCC, squamous cell carcinoma; IP, inverted papilloma; n, patients' number in a group; p-value, statistically significant test result; n. s., not significant; CD, cluster of differentiation; TCR, T cell receptor; NK cells, natural killer cells.



Groups	Vδ1+T	Vδ2+T	Vδ1-Vδ2-T (Vδ3+T)
group 1 (n = 10)	27.85* (9.08÷66.63)	63.44** (31.63÷75.20)	7.14 (1.74÷15.81)
group 2 (n = 11)	35.26** (21.67÷39.55)	57.33** (55.85÷60.38)	5.53 (2.66÷13.53)
group 3 (n = 10)	6.42 (4.95÷14.26)	90.10 (78.11÷91.56)	3.80 (1.93÷4.37)

Figure 1. γδT cells subsets (%) in peripheral blood of patients with NC and PNS neoplasms and healthy donors

Note. (A-C) Original dot-plots from flow cytometer of typical Vδ1+T and Vδ2+T cells distribution in investigated groups (X-axis, Vδ1+T cell receptor expression; Y-axis, Vδ2+T cell receptor expression). (D) Descriptive statistics of γδT cells subsets number. TCR, T cell receptor; PB450, pacific blue dye; PC7, phycoerythin-cyanine 7 dye; *, p < 0.05; **, p < 0.01 as compared to group 3; group 1, patients with squamous cell carcinoma; group 2, patients with inverted papilloma; group 3, healthy donors.

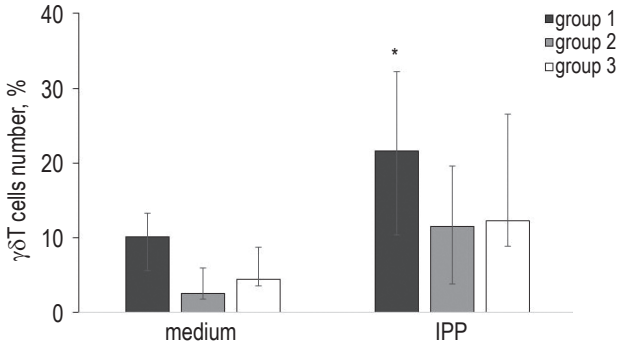


Figure 2. Proliferation rate of γδT cells (%) in stimulated (IPP) and unstimulated (medium) 6-days PBMC cultures

Note. *, p < 0.05 as compared to group 3; medium, unstimulated PBMC cultures; IPP, PBMC cultures stimulated with isopentenyl pyrophosphate; group 1, patients with squamous cell carcinoma; group 2, patients with inverted papilloma; group 3, healthy donors.

(IS_{group3} = 2.88 (2.58-3.15) c. u., respectively, p < 0.01 and p < 0.05).

γδT cells functional subsets in patients with malignant and benign NC and PNS neoplasms

One of the main characteristics of γδT lymphocytes is their inherent ability to very rapidly secrete pro-inflammatory cytokines [23]. In this regard, the functional maturity of γδT lymphocytes was characterized by their ability to produce intracellulary either IFNγ or IL-17, followed by the determination of IFNγ+γδT lymphocytes, IFNγ+IL-17+γδT lymphocytes or IL-17+γδT lymphocytes in 3-days PBMC cultures from patients with malignant and benign NC and PNS tumors and healthy donors.

The main source of IFNγ among T cells was a subpopulation of γδT lymphocytes (Figure 3) whereas CD3+T cells synthesized IFNγ at low levels (group 1: 7.8 (5.2-11.2) %; group 2: 4.3 (3.9-4.7) %; group 3: 5.2 (4.6-6.2) %). A significant increase of spontaneous intracellular IFNγ production by γδT lymphocytes was observed in group 1 as compared to the control group (Figure 3), while the significant differences

in the population of $CD3^+$T lymphocytes were not established.

Moreover, $IFN\gamma^+\gamma\delta T$ lymphocytes correlated with IFNγ concentration in supernatants of patients with malignant NC and PNS neoplasms ($R = 0.61$; $p < 0.05$), which varied from 22.7 to 1396.2 pg/mL. At the same time, the percentage of γδT lymphocytes spontaneously synthesizing the both IFNγ and IL-17 was increased in patients with malignant and benign tumors as compared to the controls ($p < 0.05$). The number of $\gamma\delta TCR^+IFN\gamma^+IL\text{-}17^+T$ cells in patients from group 2 was significantly higher than in group 1 (Figure 3). In addition, in patients with benign tumors, the number of γδT lymphocytes producing only IL-17 was higher than in group 1 or healthy donors (Figure 3).

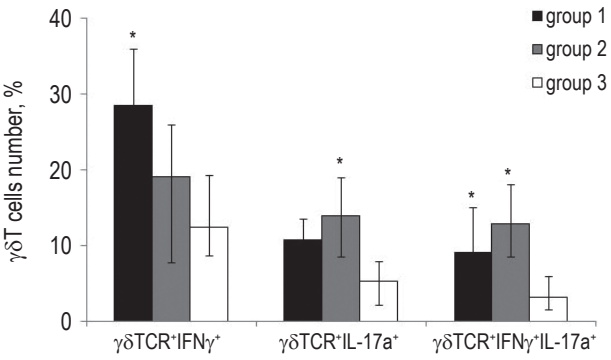


Figure 3. IFNγ and IL-17 synthesis in γδT lymphocytes (%) of patients with malignant and benign NC and PNS neoplasms

Note. *, $p < 0.05$ as compared to group 3; group 1, patients with squamous cell carcinoma; group 2, patients with inverted papilloma; group 3, healthy donors; TCR, T cell receptor; IFNγ, interferon γ; IL-17, interleukin 17.

Discussion

Recently, the immune system has been established to play a key role in the control of tumor growth and progression [14]. In view of this, the phenotype of main lymphoid cells subsets was investigated in peripheral blood of patients with malignant and benign tumors of NC and PNS. The revealed CD4/CD8 ratio decrease characterizes the redistribution of T cells towards cytotoxic profile in patients with SCC. Thus, the activation of the classical T cell immunity with a pronounced cytotoxic potential is observed in patients with malignant NC and PNS neoplasms reflecting the formation of antitumor immunity and was previously reported by many authors [8, 12, 17].

In this regard, our attention essentially focused on non-classical γδT cells, which were discovered three decades ago and still remain an enigmatic population of lymphocytes [26, 30]. γδT cells perform a wide variety of functions, but some discrete subsets have more restricted effector properties with strong evidence for functional plasticity in the periphery during immunopathological process [23, 32]. The elevation of γδT cells relative number was detected in peripheral blood of patients with SCC. So, taking into account that γδT cells structural and functional features can be used as possible biomarkers of NC and PNS neoplasms, the subsets composition, proliferative ability and cytokine profile were further investigated.

According to literature data, three subpopulations of γδT lymphocytes are distinguished depending on the expression of the TCR δ chain: a) $V\delta1^+$T cells that populate mainly gastrointestinal epithelium, skin, spleen, liver, and also are found in a small amount in peripheral blood (< 30%) recognizing lipid-presenting MHC-like molecules of the CD1 family or stress-induced molecules MICA/B, ULBP; b) $V\delta2^+$T cells that predominate in peripheral blood (> 70%) and are activated by microbial phosphoantigens ((E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate) or phosphoantigens of transformed cells (IPP); c) $V\delta3^+$T cells, which are localized in the liver and gastrointestinal epithelium, express the degranulation marker CD107α and are identified in patients with chronic viral infection (cytomegalovirus, HIV), B cell leukemia and lymphoma [28, 33].

Our results showed changes in γδT lymphocytes subsets characterized by the significantly decreased number of $V\delta2^+$T cells and the increased amount of $V\delta1^+$T cells as well as the tendency to increased $V\delta1^-V\delta2^-$T lymphocytes ($V\delta3^+$T cells) in peripheral blood of patients with both malignant and benign NC and PNS tumors as compared to healthy donors. Previously, Wu D. et al. have reported a redistribution of γδT lymphocytes subsets between peripheral blood and tissue under oncopathological conditions because of changes in the cytokine microenvironment [28]. Taking into account that $V\delta2^+$T lymphocytes mainly recognize phosphorylated antigens generated in mevalonate pathway and accumulate in tumor cells, as well as F1-ATPase expressed on the surface of tumor cells and stress-induced molecules (MICA and MICB, UL16-binding protein) [26], a decrease in $V\delta2^+$T cells number in the peripheral blood results from cells migration into tissues for their effector functions implementation.

Previous studies demonstrated that $V\delta2^+$T lymphocytes subset has a pronounced antitumor potential, can inhibit cell proliferation, angiogenesis, lymphangiogenesis and induce apoptotic death of cancer cells [13, 15]. The role of $V\delta1^+$T lymphocytes in malignant neoplasms pathogenesis is still being debated. In contrast to $V\delta2^+$T cells, $V\delta1^+$T lymphocytes exhibit a more expressed regulatory function and are involved in immunosuppression due to the following mechanisms: inhibition of dendritic cell maturation as well as activation and differentiation of αβT lymphocytes into effector cells; IL-17-mediated angiogenesis and myeloid-derived suppressor cells (MDSC) recruitment; transforming growth factor β (TGF-β) production and promotion of epithelial-mesenchymal transition [5, 16, 25, 27]. In this regard, malignant cancer cells avoid immunosurveillance,

that results in invasion and metastasis. However, some authors demonstrated that the antitumor cytolytic effect of Vδ1$^+$T lymphocytes in certain tumors is much higher than that of Vδ2$^+$T lymphocytes [29]. In turn, the role of Vδ3$^+$T lymphocytes has not been studied in oncopathology, and the data concerning Vδ3$^+$T cells cytotoxic properties are contradictory [32].

Thus, elevated Vδ1$^+$T and Vδ3$^+$T lymphocytes numbers in the peripheral blood, on the one hand, can reflect an increased activation of these subsets, and on the other hand, indicate an unfavorable microenvironment that leads to the immunosuppression and then to the tumor formation.

Moreover, γδT lymphocytes redistribution was accompanied by changes in their functional status. Thus, in patients with SCC, the proliferative potential of γδT lymphocytes in response to phosphoantigen IPP was significantly higher than in patients with IP or healthy donors. The recognition of tumor cells by γδT lymphocytes is known to occur through a host of cell surface receptors for self and non-self ligands, including TCR recognition of tumor antigen and stress ligand receptors, such as NKG2D, FCγIII (CD16), FasL, TRAIL and DNAM-1 (CD226) [6]. Vδ2$^+$T lymphocytes recognize tumor-derived phosphorylated prenyl metabolites in a TCR-dependent manner, which may accumulate intracellularly as a by-product of dysregulated tumor metabolism. IPP is one of well-studied phosphoantigen, which can accumulate in cancer cells as a result of the elevated metabolic flux through the mevalonate pathway of cholesterol biosynthesis [1, 9, 19]. Non-peptidic antigens are not presented in the context of classical MHC and are instead presented through a non-polymorphic type I transmembrane protein called butyrophilin 3A1 (BTN3A1). But the mechanism of activation of Vδ2$^+$T cells by BTN3A1-bound phosphoantigen remains controversial [19]. Non-MHC-restricted, possessing innate-like recognition kinetics Vδ2$^+$T cells are an attractive candidate for cancer immunotherapy and have been targeted in clinical settings using aminobisphosphonate drugs – potent inhibitors of the mevalonate pathway. Thereby, aminobisphosphonates not only promote direct antitumor effects but also lead to a build-up in endogenous isoprenoid metabolites resulting in activation and proliferation of type 1 cytotoxic effector γδT cells with antitumor potential to produce IFNγ, TNFα, perforin and granzymes [6]. Despite the detected high level of γδT cells proliferation rate in response to IPP in patients groups, the indices remained reduced that possibly reflects the exhaustion of γδT cells functions as a result of chronic stimulation with tumor antigens.

Together with phenotypic heterogeneity γδT lymphocytes demonstrate functional plasticity, which is determined by both the anatomical localization and the presence of an inflammatory or tolerogenic signal of the microenvironment [13]. In this regard, the cytokine profile (IFNγ or IL-17) of γδT lymphocytes in patients with NC and PNS neoplasms was further investigated. An increased spontaneous intracellular and extracellular IFNγ production respectively in γδT cells and PBMC cultures was found in patients with SCC. By secreting large amounts of IFNγ, γδT cells participate in controlling infection or tumor progression through the activation of macrophages and cytotoxic lymphocytes and provide antitumor immunosurveillance [23]. But in the both groups of patients, IL-17-producing γδT cells were also detected, which may play a pathogenic role, as their main function is extremely fast neutrophil recruitment at the site of inflammation. The established differences in IL-17-producing γδT cells in patients with malignant and benign NC and PNS neoplasms may reflect an active change in the functional potential of γδT lymphocytes from IFNγ-mediated antitumor to IL-17-mediated protumorigenic or migration of IL-17$^+$ cells in tissues. IFNγ$^+$IL-17$^+$γδT cells have also been characterized in patients with malignant and benign NC and PNS neoplasms. Although their precise physiological relevance is still to be established, IFNγ$^+$IL-17$^+$γδT cells can clearly be a distinct component of γδT cells response in scenario of tumor immunity. Sheridan et al. showed that IFNγ$^+$IL-17$^+$γδT cells lack of CD27 and become memory phenotype providing enhanced protection against recall infection [24]. Thus, IFNγ$^+$IL-17$^+$γδT cells may potentially play host-protective versus pathogenic role in a distinct microenvironment.

γδT lymphocytes are known to participate in antitumor immunosurveillance via the following mechanisms: direct cytotoxicity mediated by perforins and granzymes; FasL and TRAIL expression and elimination of Fas$^+$ and TRAIL-R$^+$ tumor cells; CD16-mediated antibody-dependent cellular cytotoxicity; the ability to present tumor antigens; rapid and early production of IFNγ and TNFα, which enhance the cells antitumor activity and inhibit tumor angiogenesis [10, 23, 32]. However, in recent years, there have been publications about the protumorigenic activity of γδT lymphocytes. In particular, IL-17$^+$γδT lymphocytes, being the main source of IL-17 in the tumor microenvironment, can contribute to angiogenesis by inducing vascular endothelial growth factor synthesis, increasing the MDSC population and MDSC-mediated depletion of CD8$^+$T lymphocytes [3], as well as tumor progression because of tumor-associated inflammation and immunosuppression, including IL-10 and TGF-β production [2, 28]. In addition, IL-1β and IL-17 secreted by IL-17$^+$γδT lymphocytes stimulate the expansion and polarization of neutrophils, which in turn acquire the ability to suppress cytotoxic CD8$^+$T lymphocytes and contribute to metastases [21].

Conclusion

The activation of the classical and non-classical T cell immunity with an expressed cytotoxic potential is observed in patients with SCC, which is typical for

antitumor immunity development. In both groups of patients with malignant and benign NC and PNS neoplasms, γδT lymphocytes demonstrate phenotypic heterogeneity characterized by the increase of $Vδ1^+T$ and $Vδ3^+T$ lymphocytes as well as by the exhaustion of γδT cells proliferation. At the same time cells functional plasticity differed between study groups: γδT lymphocytes of patients with SCC are characterized by predominantly IFNγ production that mediates antitumor immunity, while in the group with IP the prevalence of IL-17 synthesis is detected that is typical for protumorigenic microenvironment. The revealed data point the involvement of γδT lymphocytes in malignant and benign tumor pathogenesis and may provide a fundamental basis for further identification of possible tumor-associated inflammation and malignization predictors. But for the application of γδT lymphocytes parameters as biomarkers for diagnosing NC and PNS neoplasms, there is a need for their further investigation including the correlation with patients' clinical data.

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Авторы:

Нижегородова Д.Б. *— к.б.н., доцент, ведущий научный сотрудник отдела иммунологии и биомедицинских технологий научно-исследовательской лаборатории, Белорусская медицинская академия последипломного образования; доцент кафедры иммунологии, Международный государственный экологический институт имени А.Д. Сахарова Белорусского государственного университета, г. Минск, Республика Беларусь*

Колядич Ж.В. *— д.м.н., профессор, заведующая лабораторией онкопатологии центральной нервной системы с группой онкопатологии головы и шеи, Республиканский научно-практический центр онкологии и медицинской радиологии имени Н.Н. Александрова, Минский район, а/г Лесной, Республика Беларусь*

Морозова Н.А. *— аспирант, врач — онколог-хирург отделения опухолей головы и шеи, Республиканский научно-практический центр онкологии и медицинской радиологии имени Н.Н. Александрова, Минский район, а/г Лесной, Республика Беларусь*

Зафранская М.М. *— д.м.н., профессор, главный научный сотрудник отдела иммунологии и биомедицинских технологий научно-исследовательской лаборатории, Белорусская медицинская академия последипломного образования; заведующая кафедрой иммунологии, Международный государственный экологический институт имени А.Д. Сахарова Белорусского государственного университета, г. Минск, Республика Беларусь*

Authors:

Nizheharodava D.B., *PhD (Biology), Associate Professor, Leading Research Associate, Immunology and Biomedical Technologies Department of Central Research Laboratory, Belarusian Medical Academy of Postgraduate Education; Associate Professor, Immunology Department, International Sakharov Environmental Institute of Belarusian State University, Minsk, Republic of Belarus*

Kolyadich J.V., *PhD, MD (Medicine), Professor, Head, Laboratory of Oncopathology of the Central Nervous System with a Group of Oncopathology of the Head and Neck, N. Alexandrov National Cancer Centre of Belarus, Minsk District, Republic of Belarus*

Marozava N.A., *Postgraduate student, Oncologist-Surgeon, Head and Neck Tumors Department, N. Alexandrov National Cancer Centre of Belarus, Minsk District, Republic of Belarus*

Zafranskaya M.M., *PhD, MD (Medicine), Professor, Chief Research Associate, Immunology and Biomedical Technologies Department of Central Research Laboratory, Belarusian Medical Academy of Postgraduate Education; Head, Immunology Department, International Sakharov Environmental Institute of Belarusian State University, Minsk, Republic of Belarus*

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