Research Article

Journal of Innate Immunity

J Innate Immun 2017;9:598–608
DOI: 10.1159/000479382

Received: April 21, 2017
Accepted after revision: July 10, 2017
Published online: October 4, 2017

Innate Immunity Protein Tag7 Induces 3 Distinct Populations of Cytotoxic Cells That Use Different Mechanisms to Exhibit Their Antitumor Activity on Human Leukocyte Antigen-Deficient Cancer Cells

Tatiana N. Sharapova Olga K. Ivanova Natalia V. Soshnikova Elena A. Romanova Lidia P. Sashchenko Denis V. Yashin

Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russian Federation

Keywords
Natural killer cells · Cytotoxicity · Monocytes · FasL · Apoptosis · Necroptosis · Tag7 · NKG2D · TREM-1

Abstract
The search for new immune response mechanisms capable of controlling immune-evasive tumor cells devoid of the MHC antigen is a challenging task for immunologists. In this study, we found that the treatment of human peripheral blood lymphocytes with the innate immunity protein Tag7 (PGRP-S, PGLYRP1) induces differentiation of the populations of NK (natural killer) cells and CD8+ and CD4+ T lymphocytes that are cytotoxic for human leukocyte antigen-negative tumor cells. These populations employ different mechanisms of tumor cell lysis (based on the release of granzymes in the case of NK cells and on the FasL-Fas interaction in the case of CD8+ and CD4+ T lymphocytes) and induce different death pathways (apoptosis or necroptosis) in tumor cells. An analysis of genes activated in leukocyte populations after Tag7 treatment and experiments with specific inhibitors have shown that the TREM-1 receptor expressed on the monocyte cell surface is essential for activation of cytotoxic activity. Overall, the results of this study provide evidence for a novel role of the Tag7 protein in the immune response.

Introduction

Analysis of the mechanisms of activation of the immune response is a major field of study in modern immunology. In particular, it involves the search for new proteins that can induce an immune response against immune-evasive tumor cells, including those completely devoid of the major histocompatibility complex (MHC) and, hence, invulnerable to classical cytotoxic lymphocytes [1, 2]. Of special interest in this respect are cytokines as proteins that cause the proliferation and differentiation of lymphocytes.

The best-studied cytokines are TNF-α, INF-γ, and IL-2. One of the first cytokines to be discovered was TNF-α [3]. The biologically active form of TNF-α is a trimer, and

E-Mail karger@karger.com
www.karger.com/jin

Dr. Denis V. Yashin
Institute of Gene Biology, Russian Academy of Sciences
Vavilova 34/5
RU–119334 Moscow (Russian Federation)
E-Mail yashin_co@mail.ru

it has 2 cell surface receptors, TNFR1 and TNFR2, that transduce different signals. TNFR1, the main receptor for this cytokine, contains the death domain and can transduce not only the signals triggering cell death by apoptosis or necroptosis [4], but also the signal stimulating cell proliferation [5]. The TNFR2 receptor lacks the death domain and mainly transduces cell activation signals [6].

The CD8+ effector T lymphocytes produce cytokine INF-γ, which can block virus replication and has a strong antitumor effect [7]. The cytokine IL-2 deserves special attention. It was as early as 1982 that Steven A. Rosenberg and colleagues showed that human peripheral blood lymphocytes incubated with IL-2 give rise to lymphokine-activated killer (LAK) cells that are cytotoxic to malignant cells [8]. It has also been found that CD8+NKG2D+ T lymphocytes treated with IL-2 or IL-15 can transform into LAK cells with NK (natural killer)-like activity [9, 10]. We have recently shown that human peripheral blood mononuclear cells (PBMCs) incubated with IL-2 for 6 days proved to generate subpopulations of CD4+ and CD8+ T lymphocytes capable of lysing human leukocyte antigen (HLA)-negative human erythroblastoma K562 cells. In particular, the CD4+ T lymphocyte subpopulation killed the K562 cells that carried surface-bound Hsp70 protein. These lymphocytes were found to express the Tag7 protein that formed a complex with Hsp70 [11].

The innate immunity protein Tag7 is also known as peptidoglycan recognition protein (PGLYRP1). Its structure is highly conserved from insects to mammals. The gene encoding this protein in mice was identified in our laboratory [12], and its homologs (PGRP-S) were subsequently found in insects [13]. In *Drosophila,* PGRP-S is a key component of immune defense, which binds bacterial peptidoglycan and activates the Toll and IMD pathways of the immune system [14]. The mammalian Tag7 protein has a direct antibacterial activity [15].

We have found that Tag7 also has other functions in the immune defense. In addition to the aforementioned recognition and lysis of Hsp70+ K562 cells, Tag7 in complex with the Mts1 protein can attract NK cells and lymphocytes to the focus of infection [16]. Moreover, it interacts with Hsp70 to form the cytotoxic Tag7-Hsp70 complex capable of killing tumor cells [17], which binds to the known TNFR1 death receptor expressed on their surface [18]. We have shown that it is the Tag7 protein that is responsible for the interaction of this complex with the receptor [19]. Taking into account that TNFR1 is a multifunctional receptor that can trigger not only the apoptotic and necroptotic pathways but also cell proliferation and differentiation, we assumed that Tag7 may be involved in lymphocyte activation as well as in the mechanisms of cell death.

The purpose of this study was to determine: (i) whether Tag7 protein can induce cytotoxic activity in PBMCs, (ii) what cellular subpopulations exhibit cytotoxic activity, and (iii) what cytotoxic mechanism are used to kill cancer cells.

Materials and Methods

Cell Culture and Sorting

Human erythroblastoma K562 cells and human lymphoblastoma MOLT-4 cells were cultured in RPMI-1640. Human cervical carcinoma HeLa cells and murine L929 fibroblasts were cultured in DMEM, with 2 mM L-glutamine and 10% fetal calf serum in both media (all from Invitrogen, Carlsbad, CA, USA). Human PBMCs were isolated from the total leukocyte pool of healthy donors by Ficoll-Hypaque density gradient centrifugation, as described previously [20], and cultured at a density of 4×10^6 cells/mL in RPMI-1640 (see above) with 10^{-9} M of Tag7 for 6 days. In some experiments, the medium was supplemented with the LP17 peptide (LQVTDSGLYRCVIYHPP, 0.1 mg/mL, as shown by Qian et al. [21]) 1 h before adding Tag7. Cell sorting and isolation of monocytes were performed using standard magnetic bead kits (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's protocol. The results were regularly tested by flow cytometry. The purity of cell fractions in all tests was no less than 80%.

Proteins and Antibodies

The cDNAs encoding recombinant human Tag7 (GenBank accession No. NM_005091) were subcloned in pQE-31 and expressed in *E. coli* M15 (pREP4; Qiagen, Hilden, Germany). Tag7 was purified as described previously [22]. Limulus assays (Limulus amebocyte lysis chromogenic endpoint assay; Cambrex) of recombinant Tag7 did not detect LPS above the detection limit of 1 endotoxin unit per microgram of protein. In some of the experiments we used Tag7 purified from the human lymphocytes. For this purpose, we gently lysed human lymphocytes via freeze thawing, then the cytosolic fraction was collected in the presence of protease inhibitors, and loaded onto the anti-Tag7 conjugated BrCN-Sepharose. The purity of the eluted protein was assessed by SDS-PAGE. Polyclonal antibodies to the N-terminus of Fas (N-18), C-terminus of FasL (C-178), NKG2D, and MicA were from Sigma-Aldrich (St. Louis, MO, USA); CD4-FITC, CD4-PE, CD8-FITC, CD8-TC, CD3-PE, CD3-TC, CD16-FITC, CD56-PE, NKG2D-FITC, MicA-PE, FasL-FITC, FasL-PE, and Fas-PE antibodies were from Caltag Medsystems (Buckingham, UK).

Cytotoxicity Assays

The K562 cells cultured in 96-well plates (3×10^4 cells per well) were mixed with lymphocytes added at a 20:1 ratio and incubated at 37°C for 3–24 h. The inhibition test was conducted with polyclonal antibodies (anti-NKG2D, anti-MicA, anti-Fas, anti-FasL, and anti-granzyme B) at a concentration of 20 μg/mL. This concentration was selected based on the dose-dependency curve (see online suppl. Fig. S1; see www.karger.com/doi/10.1159/000479382 for all online suppl. material). Dead cells were detected with a Cytotox

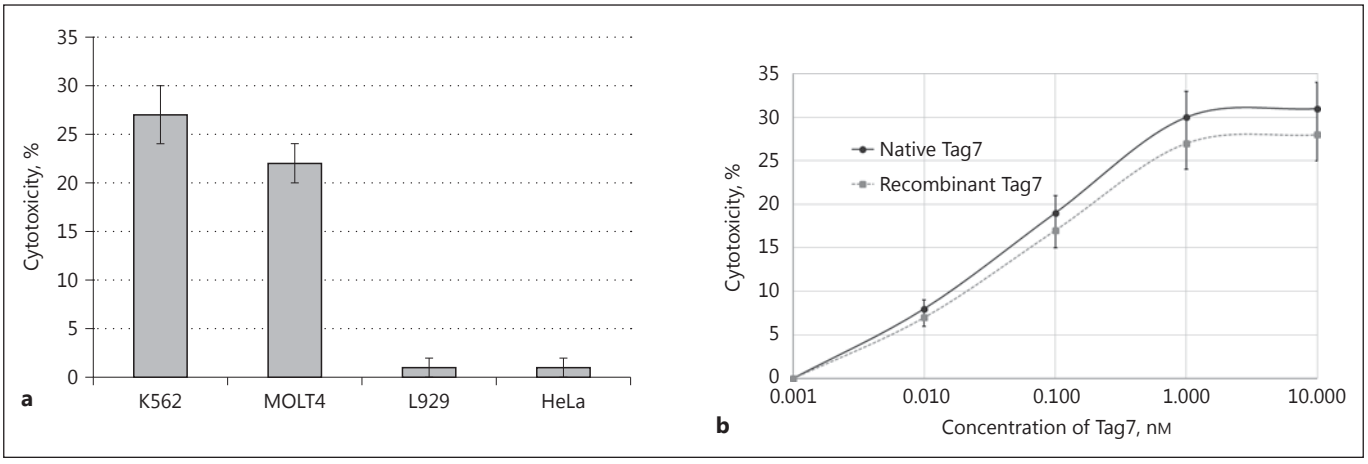

Fig. 1. Tag7 activates lymphocytes that acquire cytotoxic activity against K562 and MOLT-4 cells. **a** PBMCs treated with Tag7 (10^{-9} M) for 6 days were incubated with K562, MOLT-4, L929, and HeLa cells for 3 h, and the cytotoxicity was evaluated. **b** Cytotoxic activity of human lymphocytes against K562 cells on day 6 of activation by different concentrations purified from human lymphocytes Tag7 (native Tag7) or recombinant Tag7 protein (rTag7). Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (Student *t* test).

96 Assay kit (Promega, Madison, WI, USA). The death rate of control cells (lymphocytes or target cancer cells) did not exceed 3%.

In the enzyme inhibition assays, the cells were initially treated for 1 h with the caspase 3 inhibitor Ac-DEVD-CHO, caspase 8 inhibitor Ac-IETD-CHO (50 μM each), or RIP1 kinase inhibitor necrostatin 1 (5 mM; all from Sigma-Aldrich), and then the lymphocytes were added.

Flow Cytometry

The cells were fixed with 1% paraformaldehyde (Sigma-Aldrich) and stained with appropriate antibodies at room temperature. The samples (at least 10^4 cells each) were analyzed with an Epics Elite flow cytometer (Coulter, Marseille, France) in the logarithmic channel of fluorescence. The data were processed with EXPO32 software (Applied Cytometry Systems, Sheffield, UK). Cells were gated in forward and side scatter to remove cell debris.

Microarray Analysis

The mRNA was extracted from control PBMCs cultured in RPMI-1640 or from PBMCs incubated for 3 or 24 h with 10^{-9} M of Tag7. Two samples from each time point were used for analysis with the HumanHT-12 expression microarray kit (Illumina Inc., San Diego, CA, USA). The resulting data were filtered with a threshold detection value of $p = 0.05$ and then normalized by the quantile normalization method. The sample-to-reference ratio was used to identify genes induced or reduced more than 2-fold (at $p \leq 0.05$), which were classified as significantly changed genes.

RNAi Knockdown, Transfection, and Stable shMicA K562 Cell Line Generation

The pGPV RNAi system (Eurogen) was used to downregulate hMicA expression in the K562 cells. Two fragments of hMicA (5′-GCAGAAGATGTCCTGGGAAA-3′ and 5′-CTATGTCCGTTGTTGTAAGA-3′) were cloned as hairpins in pGPV and used for the transfection with Lipofectamine® LTX Reagent with PLUS™ Reagent (Thermo Fisher Scientific). The control cells were transfected with pGVP vector expressing a "scrambled" shRNA sequence. A heterogeneous stable K562 cell line was generated with puromycin (1 μ/mL), and then the individual clones with the most dramatic decrease in the MicA mRNA were selected. Downregulation of the MicA mRNA was tested with the primer pairs: forward, 5′-AAGACCAAGACACACTATCACGC and reverse, 5′-GGTGTCGTGGCTCAAAGATAC. The level of the ENY2 mRNA was used as a reference (primers 5′-GCAGATGAGAGCAGCGATTAACC-3′ and 5′-GGAGTGATTTCAGCCACCAAGTC-3′). Primers for RT-PCR were as follows: TNF-α: forward, 5′-TCTCTCTAATCAGCCCTCTGGC and reverse, 5′-GGTTATCTCTCAGCTCCACGCC; IL-6: forward, 5′-GGAACAAGCCAGAGCTGTGC and reverse, 5′-TGCCGAAGAGCCCTCAGG; IL-1β: forward, 5′-GTACGATCACTGAACTGCACGC and reverse, 5′-CACGCAGGACAGGTACAGATTC.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). All data were calculated from at least 3 independent biological replicates. Differences from the control were tested for statistical significance with MathCad software (PTC, Cambridge, MA, USA) using the Student *t* test for experiments on cell treatment with a single agent and 2-way ANOVA for experiments on cell treatment with 2 or more agents (see individual figure legends).

Results

Tag7 Induces Cytotoxic Activity against K562 and MOLT-4 Tumor Cells in Human Lymphocytes

PBMCs isolated from the blood of healthy donors were incubated with Tag7 (10^{-9} M) for 6 days and then tested

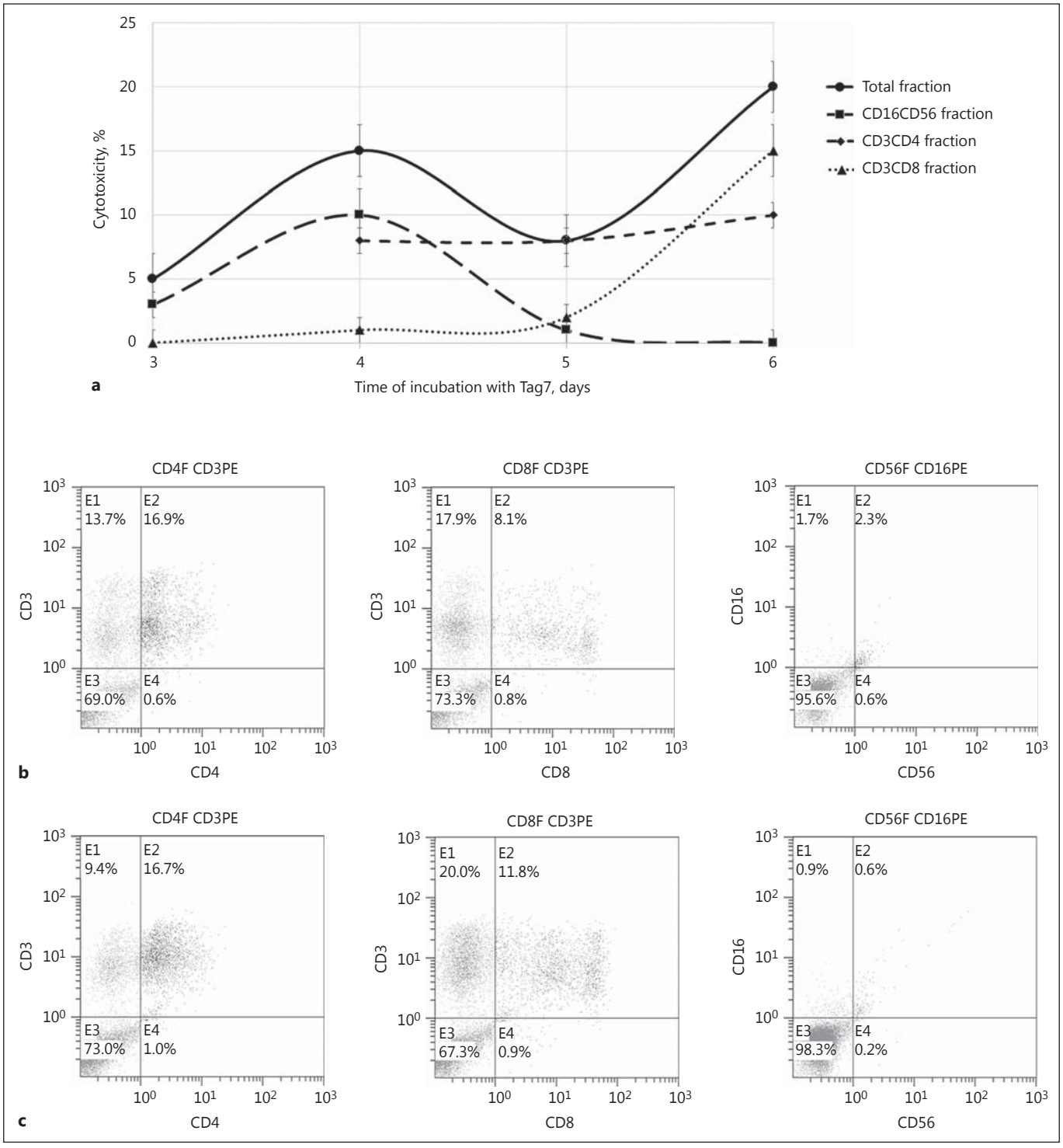

Fig. 2. Incubation with Tag7 results in activation of CD16+CD56+ lymphocytes (NK cells) on day 4 and of CD8+CD3+ lymphocytes on day 6, while CD3+CD4+ cells are active in both cases. **a** Dynamics of cytotoxic activity of Tag7-activated lymphocytes and their subpopulations against K562 cells. PBMC or CD3+CD8+, CD3+CD4+, and CD16+CD56+ lymphocyte subpopulations isolated from the Tag7-treated (10^{-9} M) PBMC pool by negative magnetic separation on days 3–6 were incubated with target cells for 3 h. Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (Student t test). Flow cytometry diagrams of the PBMC pool incubated with Tag7 for 4 (**b**) or 6 (**c**) days and stained with antibodies to CD4-FITC, CD8-PE, and CD3-TC (left and middle panels), or CD16-FITC and CD56-PE (right panels).

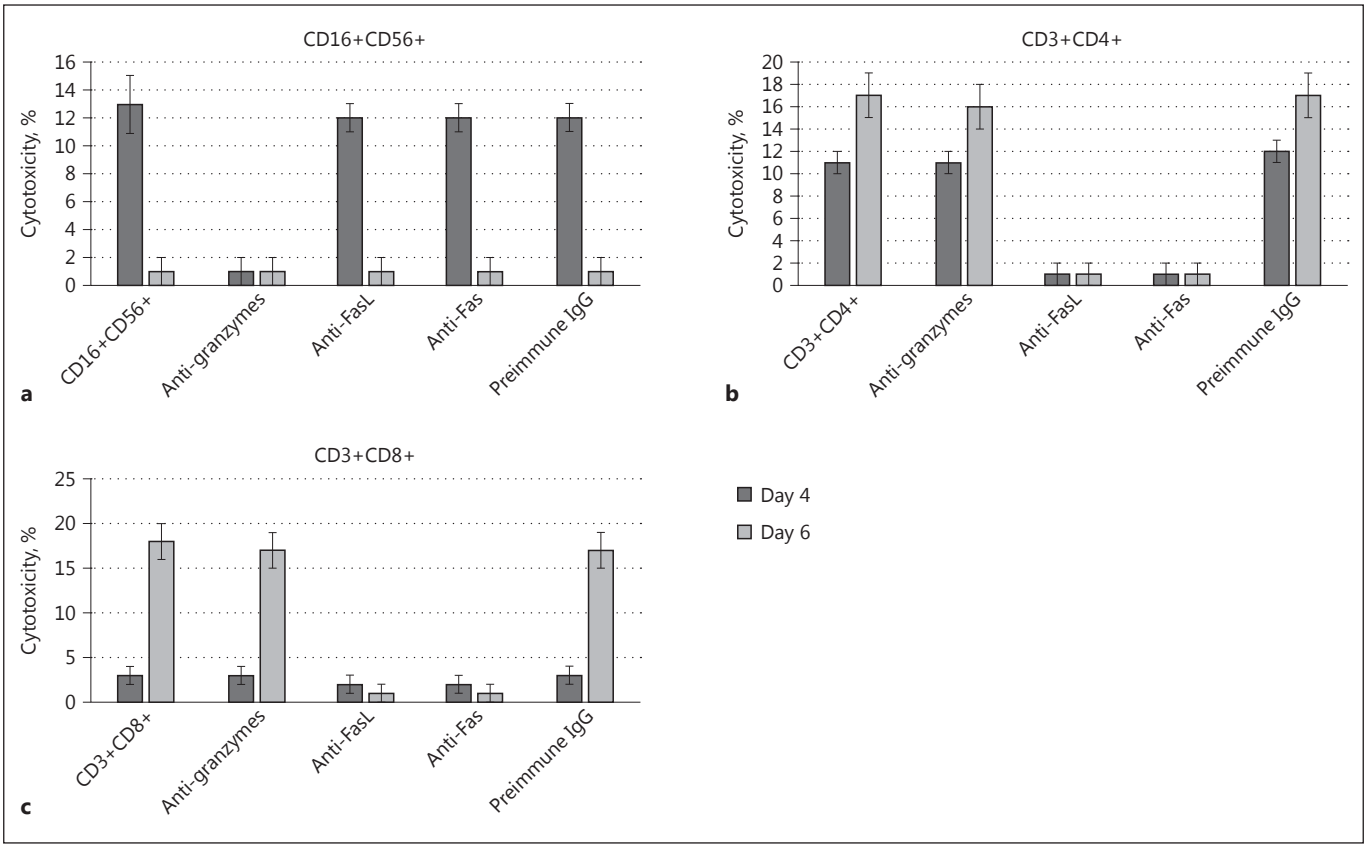

Fig. 3. Target cell lysis by CD16+CD56+ lymphocytes is based on the release of granzymes, while that by CD3+CD4+ and CD3+CD8+ lymphocytes is based on the Fas-FasL interaction. Cytotoxicity of the CD16+CD56+ (**a**), CD3+CD4+ (**b**), and CD3+CD8+ (**c**) lymphocyte subpopulations isolated from the PBMC pool, treated with Tag7 for 4 (dark gray) or 6 days (light gray) and incubated with K562 cells in the presence of antibodies to granzymes, Fas, FasL (all 20 μg/mL) or preimmune serum, added to lymphocytes or target cells 1 h before the cytotoxicity assay. Differences from the control in all cases are significant at $p < 0.05$ (2-way ANOVA). Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (2-way ANOVA).

for cytotoxic activity against the tumor cell lines K562, Molt4, HeLa, and L929. As shown in Figure 1a, Tag7-activated lymphocytes killed K562 and MOLT-4 cells, but were not cytotoxic for HeLa and L929 cells. Such a specificity for target cells is characteristic of NK cells and cells with NK-like activity. Subsequent experiments were performed with the K562 culture, devoid of HLA antigen, since these target cells proved to be most sensitive to activated lymphocytes. An optimal concentration of the Tag7 protein was determined experimentally after analysis of the cytotoxic activity curve of PBMCs incubated with different concentrations of Tag7 protein. For comparison, the activation of PBMCs was performed either with the recombinant Tag7 protein or with Tag7 purified from the human lymphocytes (see Materials and Methods). Since the activation curves were identical, this allowed us to use the recombinant Tag7 for the subsequent experiments (Fig. 1b). No traces of cytotoxic activity were observed after incubation without Tag7 protein. As a positive control, we used incubation with IL-2 protein. As we have shown previously, in this case cytotoxic activity is observed [11] (see online suppl. Fig. S2).

Incubation with Tag7 Results in Activation of NK Cells and CD4+ T Lymphocytes on Day 4 and of CD8+ and CD4+ T Lymphocytes on Day 6

To evaluate the time dependence of Tag7-induced lymphocyte cytotoxicity, PBMCs incubated with this protein were tested for cytotoxic activity at 1-day intervals (Fig. 2a). This activity reached a peak on day 4, decreased on day 5, and then reached a second peak on day 6 (Fig. 2a).

Using flow cytometry, we estimated the composition of the population of lymphocytes activated on days 4 and 6 (Fig. 2b, c). The results showed that the PBMC culture on day 4 contained CD16+CD56+ NK cells along with CD3+CD4+ and CD3+CD8+ lymphocytes. The same subpopulations of T lymphocytes were revealed on day 6, while NK cells proved to be almost absent.

To estimate the contribution of each subpopulation to the overall cytotoxicity, fractions of NK cells, CD8+ lymphocytes, and CD4+ lymphocytes were isolated by negative magnetic separation from the heterogeneous population of Tag7-activated cells on days 4 and 6, and tested for cytotoxic activity against K562 cells. This activity on day 4 was observed for NK cells and CD4+ lymphocytes, and on day 6 for both CD4+ and CD8+ lymphocytes (Fig. 2a).

These data suggest that the observed cytotoxicity of Tag7-treated lymphocytes was accounted for by 3 cell subpopulations: NK cells were activated on day 4 and then disappeared, CD8+ lymphocytes were activated on day 6, while CD4+ lymphocytes showed cytotoxic activity at both these time points.

NK Cells Use Granzymes to Kill Target Cells, While CD4+ and CD8+ Lymphocytes Kill Them via the FasL-Fas Interaction

To analyze the mechanisms of tumor cell lysis by different lymphocyte subpopulations, we used antibodies to known cytotoxic agents that may be involved in the transduction of the programmed cell death signal. Target cells or lymphocytes were preincubated with these antibodies before coincubation of the cells, and then cytotoxicity was evaluated (Fig. 3a–c). Antibodies to granzyme B completely suppressed the cytotoxic activity of NK cells but had no effect on that of CD8+ and CD4+ lymphocytes. In turn, antibodies to FasL and Fas prevented target cell death under the effect of CD8+ and CD4+ lymphocytes. Thus, different lymphocyte subpopulations proved to use different mechanisms for inducing programmed cell death pathways in the target tumor cells.

NKG2D and MicA Are Necessary for Tumor Cell Lysis by CD8+CD3+ Lymphocytes via the FasL-Fas Interaction

It is known that NK cells can kill tumor cells devoid of the HLA antigen, but this is not characteristic of typical CD8+ lymphocytes. We supposed that the cytotoxic effect observed in our experiments was caused by specialized subpopulations of these lymphocytes.

As shown previously, CD8+NKG2D+ lymphocytes treated by IL-2 or IL-15 can transform into LAK cells with NK-like activity [9, 10], which carry the NK cell activating receptor NKG2D. This receptor interacts with MicA, an unconventional MHC molecule expressed on the surface of HLA-negative tumor cells. Using flow cytometry and specific antibody staining, we showed that K562 expressed MicA, while CD3+CD8+ lymphocytes expressed both FasL and NKG2D on their surface (Fig. 4a, b). We then analyzed the effect of antibodies to MicA and NKG2D on the cytotoxicity of the CD3+CD8+ lymphocyte subpopulation. The results showed that either anti-NKG2D or anti-MicA antibodies blocked the cytotoxic activity of this subpopulation, while treatment with preimmune IgG was completely ineffective (Fig. 4c).

To verify these results, we knocked down MicA expression in the K562 cells and subsequently treated the cells with the CD3+CD8+ lymphocyte subpopulation. We produced 2 stable knockdown lines and selected clones in which the level of the unprocessed MicA transcript was only 20–30% of the control cells (quantitative PCR data; online suppl. Fig. S3). These clones were not susceptible to the cytotoxic effect of the CD3+CD8+ lymphocyte subpopulation. Therefore, MicA knockdown completely blocked the cytotoxic effect of activated lymphocytes (Fig. 4c). The control clone was killed by the CD3+CD8+ lymphocyte subpopulation (Fig. 4c). Our data suggest that MicA is essential for cancer cell recognition and killing by CD3+CD8+ lymphocytes. Thus, the active lymphocyte subpopulations differ in the mechanisms by which they recognize and kill HLA-negative tumor cells: the NK cells are known to recognize them through the NKG2D-activating receptor [10] and kill by releasing granzymes; the CD8+ lymphocytes also use this receptor to recognize target cells but kill them by a FasL-mediated mechanism; the same FasL-Fas mechanism is used by the CD4+ lymphocytes to kill target cells.

Cytotoxic Lymphocytes Induce Apoptosis and Necroptosis in Target Cells via the FasL-Fas Interaction

To gain a deeper insight into the mechanisms of tumor cell lysis by lymphocytes, we analyzed the dependence of their cytotoxic activity on the time of incubation with K562 cells (online suppl. Fig. S4). This dependence proved to be nonlinear: the cytotoxic activity peaked after 3 h, dropped after 6 h, and then reached the second peak after 24 h. Such a pattern of the activity curve suggested that lymphocytes induced different mechanisms of programmed death in the target cells, with consequent differences in its dynamics.

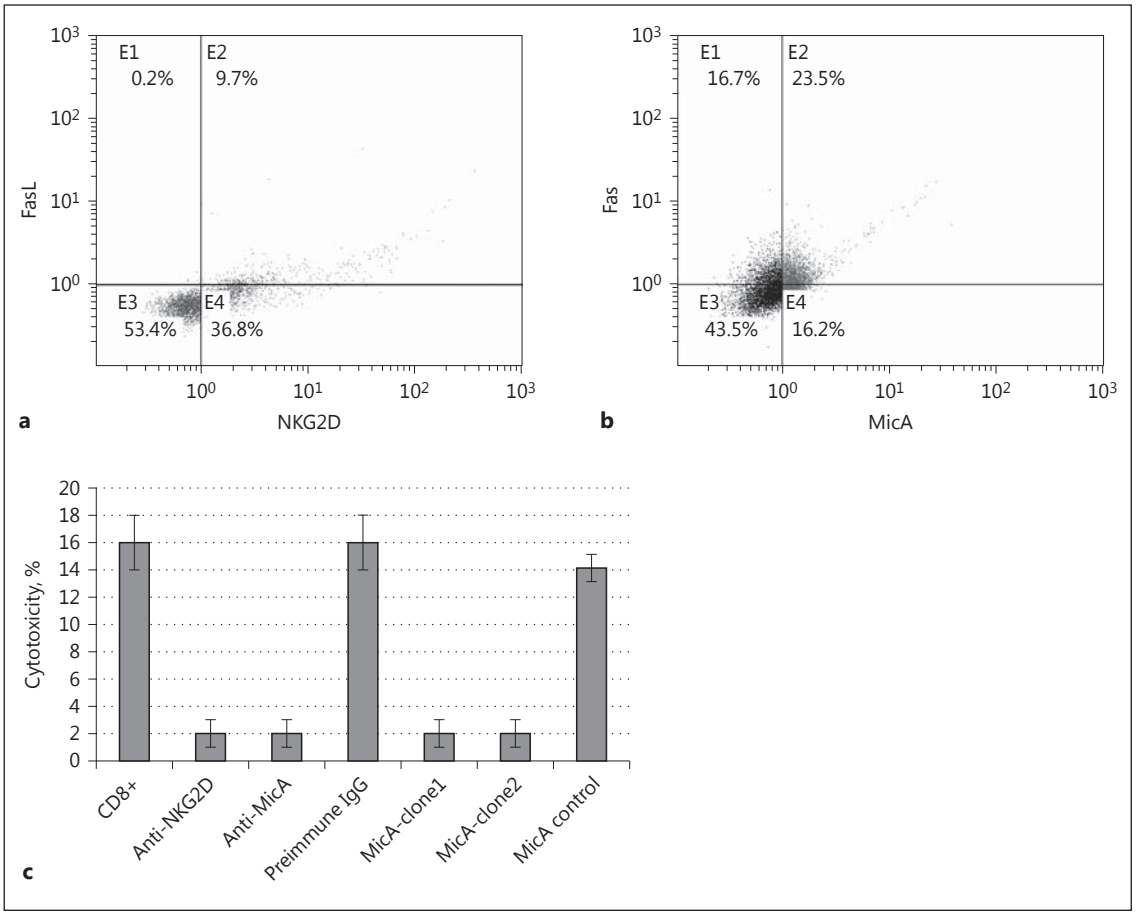

Fig. 4. NKG2D expressed on the surface of CD8+CD3+ lymphocytes binds to MicA on the surface of K562 cells and causes their lysis. **a** Flow cytometry of the CD8+ subpopulation isolated from the PBMC pool on day 6 of its incubation with Tag7 and stained with NKG2D-FITC and FasL-PE. **b** Flow cytometry of K562 cells stained with Fas-FITC and MicA-PE. **c** Cytotoxicity of the CD8+CD3+ subpopulation isolated on day 6 of PBMC treatment with Tag7 and incubated with K562 cells or their MicA downregulated clones in the presence of antibodies to NKG2D and MicA. The antibodies (20 μg/mL) were added to lymphocytes and target cells 1 h before the cytotoxicity assay. Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (2-way ANOVA).

To identify the type of programmed cell death induced by activated lymphocytes, their cytotoxicity was evaluated in the presence of inhibitors of the key molecules involved in the apoptotic and necroptotic signaling pathways (Fig. 5). The inhibitors of proapoptotic caspases 3 and 8 proved to completely block target cell death at the 3-h time point but had no effect at the 24-h time point; conversely, the antinecroptosis inhibitor necrostatin-1 blocked cell death at the 24-h time point, but was ineffective at the 3-h time point. These results provide evidence that lymphocytes incubated with target tumor cells induce their death by apoptosis after 3 h and by necroptosis after 24 h.

Treatment with Tag7 Leads to Activation of Monocytes and Consequent Increases in the Expression of Cytokines IL-1b, IL-6, and TNF-α

In order to understand the processes occurring in human peripheral blood cells under the effect of Tag7, we analyzed the pattern of gene activation in PBMCs treated with this protein. For this purpose, PBMCs from the same pool were incubated in RPMI-1640 with 10% FBS (control) or in the same medium containing 10^{-9} M Tag7 for 3 and 24 h, and the total RNA extracted from these cells was used for global gene expression profiling with the Illumina HumanHT-12 microarray. The results of this experiment are shown in the online Supplementary Table S1. We have devoted special attention to several genes involved in the

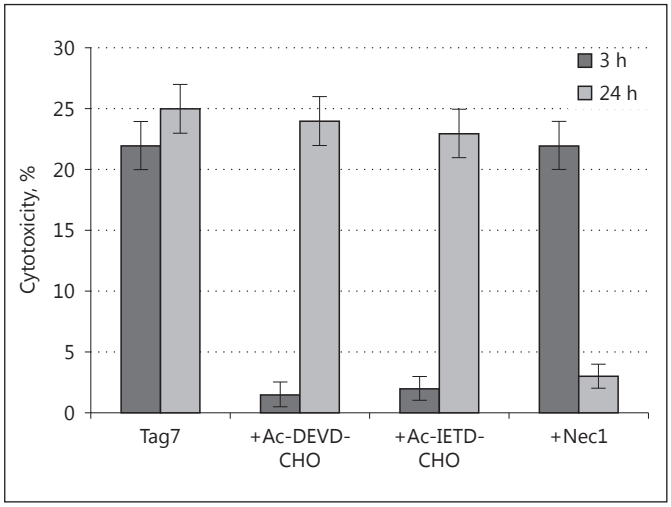

Fig. 5. Lymphocytes incubated with K562 cells induce their death by apoptosis after 3 h and by necroptosis after 24 h. Cytotoxicity of lymphocytes treated with Tag7 for 6 days and incubated with K562 cells in the presence of caspase 3 inhibitor Ac-DEVD-CHO, caspase 8 inhibitor Ac-IETD-CHO, or RIP1 kinase inhibitor necrostatin 1 (Nec1) for 3 and 24 h. The inhibitors were added to lymphocytes and target cells 1 h before the cytotoxicity assay. Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (2-way ANOVA).

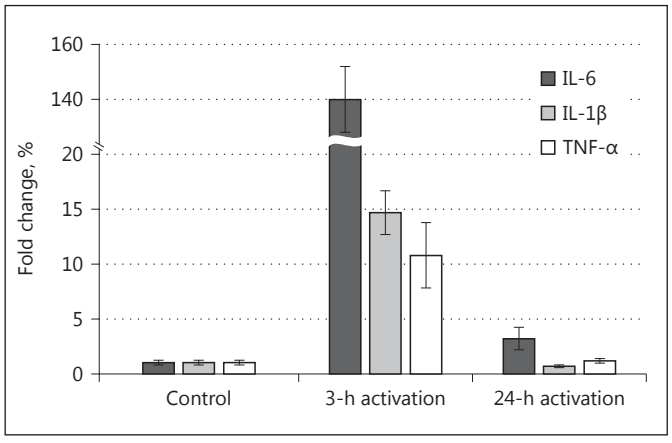

Fig. 6. Incubation of monocytes with Tag7 leads to activation of IL-1β, IL-6, and TNF-α genes. Transcription levels of IL6, IL-1β, and TNF-α upon incubation of an isolated monocytes fraction with Tag7 for 3 or 24 h and in control cells as measured by qPCR. The level of the ENY2 mRNA was used as a reference. Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (2-way ANOVA).

immune response. In particular, this concerned genes coding for the cytokines released by monocytes at the initial stages of the immune response (IL-1β, increases 3.8 times after 24 h, IL-6, increases 3.5 times after 3h, and TNF-α, increases 1.5 times after 24 h). To further confirm these data, we performed additional RT-PCR analyses of the mRNA activation of these genes in the isolated monocytes, which were treated for 3 or 24 h with Tag7 (Fig. 6). It can be seen that the levels of expression of these genes, IL-1β, IL-6, and TNF-α, are strongly increased. As a control, we tested the monocyte-deprived PBMC population for the levels of expression of these genes, and found no increase (see online suppl. Fig. S5). These data confirm that the cytokine genes expressed by monocytes at the initial phase of the immune response are activated significantly upon incubation with the Tag7 protein.

To test for the involvement of monocytes in the induction of PBMC antitumor activity, they were removed from the PBMC pool, and the remaining cells were treated with Tag7 for 6 days. No cytotoxicity was observed in this variant (Fig. 7). We then treated previously isolated monocytes with Tag7 for 24 h, added them to the monocyte-free PBMC pool, and incubated this cell mixture for 6 days without Tag7. As a result, lymphocytes acquired cytotoxic activity against K562 cells (Fig. 7). This is evidence that monocytes are indeed required for lymphocyte activation by Tag7.

According to published data, the genes coding for the aforementioned cytokines in monocytes can be induced upon activation of TREM-1, triggering receptors expressed on myeloid cells [23]. Furthermore, it has been shown by Read et al. [24] that Tag7 (PGLYRP1) in the complex with bacterial peptidoglycan or in a multimeric form is capable of binding to TREM-1 during immune responses triggered by bacteria. These authors also demonstrated the direct binding of these 2 proteins. Hence, we hypothesized that it is the interaction of Tag7 with TREM-1 on the surface of monocytes that triggers the development of lymphocyte subpopulations with antitumor activity. To test this hypothesis, PBMCs were treated with Tag7 in the presence of the LP17 peptide, an inhibitor of TREM-1. This peptide is used to disrupt the signal transduction via the TREM-1 receptor [21]. As expected, this peptide completely blocked lymphocyte activation (Fig. 7).

Discussion

The results of this study show that treatment of PBMCs with the Tag7 protein stimulates the development of lymphocyte subpopulations with antitumor activity. This is due to the ability of Tag7 to interact with the TREM-1

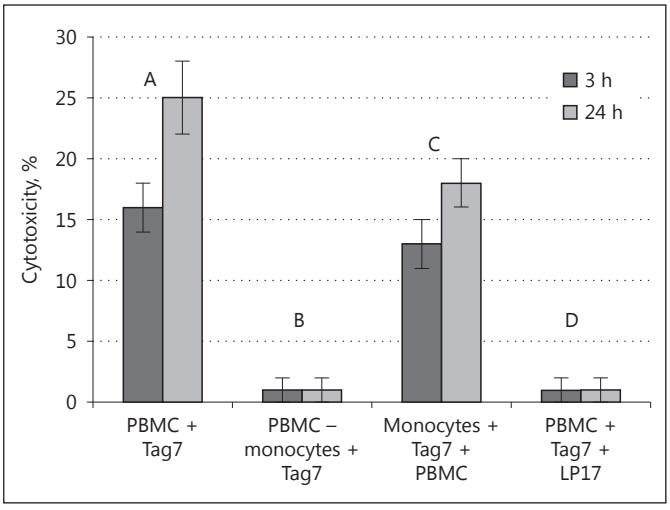

Fig. 7. TREM-1 receptor on the monocyte surface is required for the induction of cytotoxicity under the effect of Tag7. Tests for cytotoxicity of lymphocytes from the PBMC pool after different variants of treatment: A, the PBMC pool was treated with Tag7 for 6 days; B, the PBMC pool was treated with Tag7 for 6 days after the removal of monocytes; C, isolated monocyte fraction was treated with Tag7 for 24 h, added to the monocyte-free cell pool, and the resulting mixture was incubated without Tag7 for 6 days; D, the PBMC pool was treated with Tag7 for 6 days in the presence of peptide LP17, a TREM-1 inhibitor, added 1 h prior to treatment. Data are presented as the mean ± SD of 3 independent experiments. Differences from the control in all cases are significant at $p < 0.05$ (2-way ANOVA).

receptor on monocytes, with the consequent emergence of NK cells and T lymphocytes with cytotoxic activity against target tumor cells devoid of MHC.

TREM-1 belongs to the immunoglobulin superfamily, is expressed on macrophages and neutrophils, and can be activated in response to various proinflammatory stimuli, such as PAMPs [25, 26]. This receptor is probably implicated in monocyte activation and anti-inflammatory immune responses [27]. Its silencing markedly reduces activation of the key cytokine genes under the effect of LPS [28]. Read et al. [24] showed that Tag7 is able to interact with the TREM-1 receptor, inducing the secretion of proinflammatory cytokines. Here, we have shown an activation of the proinflammatory cytokine genes in response to the Tag7 stimulation of TREM-1. We have also shown here that TREM-1 may be involved in lymphocyte activation and the consequent development of cytotoxic subpopulations capable of killing MHC-negative tumor cells.

The data presented above contribute to the list of known activities in which Tag7 may be involved. As shown in our previous studies, this protein not only has antibacterial properties, but can exert a direct antitumor effect, acting in complex with heat shock protein Hsp70. Being expressed on the surface of CD3+CD4+ lymphocytes, Tag7 is involved in the recognition of target tumor cells devoid of MHC [11]. It also forms a complex with the Mts1 protein, which causes the chemotactic migration of lymphocytes [16]. Here we have shown that Tag7 has one more function – it is capable of activating human peripheral blood lymphocytes.

The treatment of a peripheral lymphocyte pool with Tag7 results in the generation of subpopulations of active NK cells capable of killing HLA-negative tumor cells and of CD8+ and CD4+ T lymphocytes, which also cause the lysis of tumor cells but by a different mechanism, via FasL-Fas interaction. Such cytotoxic lymphocytes are also generated upon activation of peripheral blood leukocytes by the IL-2 cytokine, as we have shown previously [11]. Treatment with either Tag7 or IL-2 leads to activation of unconventional lymphocyte subpopulations. The CD4+ subpopulation kills tumor cells using the FasL-Fas mechanism, and this function is novel for this subset of cells. For the CD3+CD4+ fraction treated by IL-2, we have shown previously that the CD4+ subpopulation kills tumor cells using the FasL-Fas mechanism and the membrane-bound Tag7 protein that recognizes the Hsp70 stress molecule on the tumor cell surface [11]. Since Hsp70 is not expressed on the surface of normal cells, it can be regarded as a danger signal, with Tag7 (which recognizes this signal) serving as a danger receptor similar to NK cell-activating receptors and PAMP-recognizing receptors. The CD8+CD3+ cytotoxic subpopulation is also unconventional: these lymphocytes use the NK cell-activating receptor NKG2D to recognize HLA-negative tumor cells. A noteworthy fact is that the 3 subpopulations of effector cells differ in the dynamics of activation. The NK subpopulation is activated by day 4 of Tag7 treatment, and then these cells disappear within a very short time. Active CD8+ lymphocytes appear later, by day 6, making no significant contribution to the overall cytotoxicity prior to that. In contrast, CD4+ lymphocytes remain active for a long time, and their cytotoxic effect is manifested both on day 4 and on day 6.

The cytotoxic processes induced in target cells by FasL+ lymphocytes are also not ordinary. Affecting K562 tumor cells via the FasL-Fas interaction, they trigger 2 different pathways of programmed cell death. As a result, 1 K562 subpopulation dies by apoptosis (with the involvement of caspase cascade), and the other dies by necroptosis (with the involvement of RIP1 kinase). It was shown

previously that soluble FasL can induce not only apoptosis but also RIP1-dependent cell death [29]. We have recently shown that lymphocytes activated via IL-2 can induce both apoptosis and necroptosis via FasL-Fas interaction [30]. In our work, we have found that FasL from the lymphocytes surface can also trigger RIP1-dependent necroptosis in target cells. This property can contribute to the enhanced antitumor activity of generated cytotoxic lymphocytes by countering apoptosis resistance of evasive tumor cells.

It should also be noted that, as follows from the results of this study, the innate immunity protein Tag7 interacting with the innate immunity receptor TREM-1 on the surface of monocytes (involved in the innate immunity response) can stimulate processes that lead to the induction of acquired immunity cells with antitumor activity. Thus, this protein appears to be one more connecting link between the 2 branches of the immune response. We have shown that the lymphocyte activation either by the recombinant Tag7 protein or by the Tag7 isolated from the human lymphocytes is equivalent. Taking into account that Tag7 is present in cells of the human immune system, and can be secreted by them [15], it could be suggested that this protein may be involved in the immune response regulation.

Acknowledgement

This work was supported by RSF grant 15-14-00031.

Disclosure Statement

The authors declare no competing financial interests.

References

1 Bubeník J: MHC class I down-regulation: tumour escape from immune surveillance? Int J Oncol 2004;25:487–491.
2 Seliger B: Strategies of tumor immune evasion. BioDrugs 2005;19:347–354.
3 Kolb WP, Granger GA: Lymphocyte in vitro cytotoxicity: characterization of human lymphotoxin. Proc Natl Acad Sci USA 1968;61:1250–1255.
4 Christofferson DE, Yuan J: Necroptosis as an alternative form of programmed cell death. Curr Opin Cell Biol 2010;22:263–268.
5 Wertz IE: TNFR1-activated NF-κB signal transduction: regulation by the ubiquitin/proteasome system. Curr Opin Chem Biol 2014;23:71–77.
6 Cabal-Hierro L, Lazo PS: Signal transduction by tumor necrosis factor receptors. Cell Signal 2012;24:1297–1305.
7 Young HA, Bream JH: IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications. Curr Top Microbiol Immunol 2007;316:97–117.
8 Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA: Lymphokine-activated killer cell phenomenon: lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 1982;155:1823–1841.
9 Gamero AM, Ussery D, Reintgen DS, Puleo CA, Djeu JY: Interleukin 15 induction of lymphokine-activated killer cell function against autologous tumor cells in melanoma patient lymphocytes by a CD18-dependent, perforin-related mechanism. Cancer Res 1995;55:4988–4994.
10 Meresse B, Chen Z, Ciszewski C, Tretiakova M, Bhagat G, Krausz TN, Raulet DH, Lanier LL, Groh V, Spies T, Ebert EC, Green PH, Jabri B: Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity 2004;21:357–366.
11 Sashchenko LP, Dukhanina EA, Shatalov YV, Yashin DV, Lukyanova TI, Kabanova OD, Romanova EA, Khaidukov SV, Galkin AV, Gnuchev NV, Georgiev GP: Cytotoxic T lymphocytes carrying a pattern recognition protein Tag7 can detect evasive, HLA-negative but Hsp70-exposing tumor cells, thereby ensuring FasL/Fas-mediated contact killing. Blood 2007;110:1997–2004.
12 Kiselev SL, Kustikova OS, Korobko EV, Prokhortchouk EB, Kabishev AA, Lukanidin EM, Georgiev GP: Molecular cloning and characterization of the mouse *tag7* gene encoding a novel cytokine. J Biol Chem 1998;273:18633–18639.
13 Kang D, Liu G, Lundström A, Gelius E, Steiner H: A peptidoglycan recognition protein in innate immunity conserved from insects to humans. Proc Natl Acad Sci USA 1998;95:10078–10082.
14 Michel T, Reichhart JM, Hoffmann JA, Royet J: *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 2001;414:756–759.
15 Liu C, Gelius E, Liu G, Steiner H, Dziarski R: Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth. J Biol Chem 2000;275:24490–24499.
16 Dukhanina EA, Yashin DV, Galkin AV, Sashchenko LP: Unexpected deeds of familiar proteins: interplay of Hsp70, PGRP-S/Tag7 and S100A4/Mts1 in host versus cancer combat. Cell Cycle 2010;9:676–682.
17 Yashin DV, Ivanova OK, Soshnikova NV, Sheludchenkov AA, Romanova EA, Dukhanina EA, Tonevitsky AG, Gnuchev NV, Gabibov AG, Georgiev GP, Sashchenko LP: Tag7 (PGLYRP1) in complex with Hsp70 induces alternative cytotoxic processes in tumor cells via TNFR1 receptor. J Biol Chem 2015;290:21724–21731.
18 Sashchenko LP, Dukhanina EA, Yashin DV, Shatalov YV, Romanova EA, Korobko EV, Demin AV, Lukyanova TI, Kabanova OD, Khaidukov SV, Kiselev SL, Gabibov AG, Gnuchev NV, Georgiev GP: Peptidoglycan recognition protein tag7 forms a cytotoxic complex with heat shock protein 70 in solution and in lymphocytes. J Biol Chem 2004;279:2117–2124.
19 Yashin DV, Romanova EA, Ivanova OK, Sashchenko LP: The Tag7-Hsp70 cytotoxic complex induces tumor cell necroptosis via permeabilisation of lysosomes and mitochondria. Biochimie 2016;123:32–36.
20 Sashchenko LP, Gnuchev NV, Lukjanova TI, Redchenko IV, Kabanova OD, Lukanidin EM, Blishchenko EY, Satpaev DK, Khaidukov SV, Chertov OYu: Time-dependent changes of LAK cell phenotypes correlate with the secretion of different cytotoxic proteins. Immunol Lett 1993;37:153–157.
21 Qian L, Weng XW, Chen W, Sun CH, Wu J: TREM-1 as a potential therapeutic target in neonatal sepsis. Int J Clin Exp Med 2014;7:1650–1658.

22 Cavaillon JM: Monocyte TREM-1 membrane expression in non-infectious inflammation. Crit Care 2009;13:152.
23 Guan R, Wang Q, Sundberg EJ, Mariuzza RA: Crystal structure of human peptidoglycan recognition protein S (PGRP-S) at 1.70 Å resolution. J Mol Biol 2005;347:683–691.
24 Read CB, Kuijper JL, Hjorth SA, Heipel MD, Tang X, Fleetwood AJ, Dantzler JL, Grell SN, Kastrup J, Wang C, Brandt CS, Hansen AJ, Wagtmann NR, Xu W, Stennicke VW: Cutting edge: identification of neutrophil PGLYRP1 as a ligand for TREM-1. J Immunol 2015;194:1417–1421.
25 Bleharski JR, Kiessler V, Buonsanti C, Sieling PA, Stenger S, Colonna M, Modlin RL: A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. J Immunol 2003;170:3812–3818.
26 Radsak MP, Salih HR, Rammensee HG, Schild H: Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. J Immunol 2004;172:4956–4963.
27 Gibot S, Kolopp-Sarda MN, Béné MC, Bollaert PE, Lozniewski A, Mory F, Levy B, Faure GC: A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. J Exp Med 2004;200:1419–1426.
28 Cannon JP, O'Driscoll M, Litman GW: Specific lipid recognition is a general feature of CD300 and TREM molecules. Immunogenetics 2012;64:39–47.
29 Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J: Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489–495.
30 Ivanova OK, Sharapova TN, Romanova EA, Sashchenko LP, Yashin DV: CD3+ CD8+ NKG2D+ T lymphocytes induce apoptosis and necroptosis in HLA-negative cells via FasL-Fas interaction. J Cell Biochem 2017;118:3359–3366.