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TUMOR INFLATING LYMPHOCYTES. PURIFICATION, EXPANDING AND CYTOTOXICITY ANALISYS ON PRIMARY TUMOR CULTURES

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Background. *Tumor Infiltrating Lymphocytes (TILs) is one of the most promising sources of autologous cytotoxic T-cells for adoptive immunotherapy, which has already shown high efficiency in the treatment of metastatic melanoma. However, the isolation of TILs from solid tumors is technically difficult. A suppressive tumor microenvironment, in particular, a high level of expression of check-point inhibitors PD-1 CTLA4, tissue hypoxia and other factors cause that T cells isolated from the tumor do not proliferate well and do not exhibit cytotoxic properties.* ***Aims.*** *In this study, we isolated TILs from surgical material obtained by resection of solid tumors (primary and metastatic adenocarcinomas of various localization, melanoma, glioblastoma), studied their population composition and developed protocols for the purification expanding, and activation of CD4+, CD8+ cytotoxic antitumor lymphocytes.* ***Methods.*** *An urgent task is the activation of TILs, turning off immunosuppressive mechanisms and increasing their antitumor cytotoxic activity. Various approaches are used for this: activation by a cocktail of cytokines and antibodies, editing the lymphocyte genome by knocking out suppressor genes or, conversely, transduction of activating genes, coincubation with feeder cells, etc. Cells were obtained from samples of resected tumors in 16 patients; in each case we obtain an autologous pair: the primary tumor culture and the TILs culture.* ***Results.*** *We could isolate viable lymphocytes in 100% of cases. Isolated TILs were successfully expanded in our specialized medium using various combinations of IL-2, IL-15, IL-21, IL-7, anti-CD3 and anti-CD28. Immunophenotyping showed that the obtained TILs are a heterogeneous mixture of CD4+, CD8+ cells containing populations of CD3+CD8+CD45+(CTL) CD3+CD4+CD45+ (T-helpers), CD4+CD25+CD127- (T-regulatory cells), CD3-CD56+CD45+ (NK-cells), CD3+CD56+CD45+ (T-NK-cells). The initial cultures of TILs were also characterized by a high level of PD1 expression, indicating their low antitumor cytotoxicity. Using different protocols of isolation, expansion, and activation, we obtained a cell preparation containing 80% of CD8+ PD-1- activated TILs in an amount sufficient for adoptive therapy (500×106 or more). An in vitro study of the cytotoxicity of obtained TILs in primary cultures of homologous tumors using RTCA Icelligence showed high cytotoxicity, providing almost 100% tumor cell death.* ***Conclusion.*** *Our developed protocol for the production and activation of TILs can be recommended for the phase I–II clinical trials of adoptive immunotherapy of recurrent, highly metastatic solid tumors.*

Keywords: *adoptive immunotherapy, solid tumors, tumor infiltrating lymphocytes, TILs, Tregs, CTL.*

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BACKGROUND

Adoptive immunotherapy — the therapeutic use of immune cells grown and activated ex vivo — is one of the modern alternative methods of anticancer therapy, which is used both together with standard therapy and instead of it, as a “therapy of despair” in multidrug-resistant tumors with multiple metastases [1–4]. One of the sources of cytotoxic cells for immunotherapy are tumor infiltrating lymphocites (TILs) [5]. In contrast to immune cells derived from blood [6], TILs are secreted from tumor tissue; therefore, it is believed that they exhibit tropism towards tumor cells [3]. Since the description of this technology in 1987 by Steven Rosenberg’s group [5], the preparation of TILs has been a complex, time-consuming and individualized laboratory procedure. It took the developers of the technology more

than 20 years to prove the effectiveness of the technology in the treatment of metastatic melanoma in clinical trials [7, 8]. Until recently, attempts to treat other solid tumors with TILs have not been successful [9–13].

An alternative technology for targeting a tumor is the use of CAR-T cells carrying a chimeric antigen receptor (CAR) that recognizes a particular target antigen on tumor cells [14]. However, CAR-T cells can recognize, as a rule, only one [15–17] or, in the case of bivalent CARs, two antigens [18]. Although trivalent CARs have been described for a number of solid tumors [19], they have not yet reached the stage of clinical trials. Unlike CAR-T cells, TILs can recognize a wide range of antigens that are unique to patient tumor cells [11]. Rosenberg's group, which implemented the introduction of TILs immunotherapy in the treatment of metastatic melanomas, and a number of other teams by 2018 showed the possibility of using this technology in the treatment of breast cancer, including the triple-negative (triple) variant [20-22]. Since 2011, one of the largest biotechnology companies IOVANCE (USA) has received a license for the TILs technology and is currently on the verge of obtaining FDA TIL approval for progressive cervical cancer and metastatic melanoma [23].

In terms of population composition, TILs are a heterogeneous mixture of subpopulations of immune cells, including T, B, NK cells, macrophages, and dendritic cells [21, 24–26]. The composition and functional state of TILs can vary greatly depending on the type of tumor, stage of the disease, therapy, method of isolation and cultivation [27]. At the same time, it is the population composition and activation status of antitumor immune cells that are crucial for the development of effective and reproducible immunotherapy using TILs [28].

The aim of this study is to obtain a panel of TILs from surgical material of solid tumors (primary foci and metastases of adenocarcinomas of various localization, melanoma, glioblastoma), to study their population composition and to develop protocols for the purification, augmentation and activation of CD4 +, CD8 + cytotoxic antitumor lymphocytes.

METHODS

Study design

The study involved 16 patients who received surgical treatment at the Federal Research Center of the Federal Medical and Biological Agency of the Russian Federation for malignant tumors of neuroepithelial origin (glioblastoma, 7 patients), lung cancer (2 patients), ovarian cancer (2 patients), colorectal cancer metastases in the liver (2 patients); melanoma metastases in the liver (2 patients); cervical cancer (1 patient).

Eligibility criteria

Grade IV neuroepithelial tumors (glioblastoma multiforme) were selected for the study; as well as malignant solid tumors of epithelial origin, corresponding to the classification of T4, N3, M1.

Conditions of conduction

Intraoperative material was taken in the operating unit of the Federal Research Center for Clinical and Biological Research of the Federal Medical and Biological Agency of Russia (Moscow), the preparation of primary cultures of tumors and TILs was performed in the laboratory of cell technologies at the Federal Research Center for Scientific and Clinical Center of the Federal Medical and Biological Agency of Russia. Immunophenotyping was performed at the National Medical Research Center for Children's Health (Moscow).

Obtaining primary tumor cells and TIL. Fragments of tumor tissue with a pathomorphologically confirmed histotype were washed with sterile phosphate buffered saline (PBS) and divided into two parts in a sterile box. The first part was mechanically crushed and enzymatically dissociated in a solution of 10% type I collagenase, dispase, and 1 mg/ml DNase in a thermal shaker for 30–60 min at 37 °C. Then 10% autologous serum was added to the preparation and centrifuged twice at 250G. The cells from the pellet were seeded on adhesive plastic in DMEM F12 growth medium (Gibco, USA) with 10% inactivated autologous serum or universal human AB serum (Innovative, USA) with the addition of an antimycotic antibiotic (Gibco). After 3-5 passages, primary tumor cultures were cryopreserved in liquid nitrogen.

The second part of the tumor was mechanically crushed to a size of 1–2 mm, transferred into 24-well plates (Costar, United States) and cultured in a specialized Immunocult medium (United States) containing 10% autologous inactivated patient serum, interleukin (interleukin, IL) 2 (Milteny biotech, Germany), 50 U/ml penicillin and 50 mg/ml streptomycin (Sigma, USA) in a CO_2 incubator at 37 °C, 5% CO_2 and 90% humidity for 7 days. To improve lymphocyte proliferation, Dynabeads™ Human T-Expander CD3/CD28 magnetic microparticles (11141D, Life Technologies, USA) were added to the medium. After 14 days, the microparticles were removed using a Dynal MPC-S universal magnet (A13346, Thermo Fisher, USA) according to the

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manufacturer's instructions, the number of cells was counted, and transferred into 25 cm2 mattresses (Eppendorf, USA). In order to activate TILs, a mixture of IL2, IL7, IL15, and IL21 was added to the medium at a dose of 10–1000 U/ml (Milteny biotech, Germany) and, in some cases, instead of microparticles anti-CD3, anti-CD28 — antibodies at a concentration of 30 μg/ml.

Obtaining autologous serum from a patient. To obtain autologous serum, blood was collected in tubes with a Vacuette Z Serum Clot Activator collection tubes (Greiner Bio One, Austria), centrifuged at 400 g for 20 min, the serum was collected and inactivated by heating in a water bath for 40 min at 56 °C. WITH.

Flow cytometry for phenotype analysis. Immunophenotyping was performed using an ACEA Novocyte three-laser flow cytometer (ACEA Bioscience, USA). In peripheral blood and in the culture of lymphocytes isolated from the tumor, the main and small populations of CD45+ lymphocytes were quantified using the following monoclonal antibodies: CD3+ (T-lymphocytes), CD3+ CD4+ (T-helpers), CD3+ CD8+ (cytotoxic T-lymphocytes), CD3-CD19+ (B-lymphocytes), CD3-CD16+/CD56+ (NK-cells), CD3+ HLA-DR+ (activated T-lymphocytes), CD4 + CD127low CD25high (regulatory T-cells-Treg), CD4 + CD25 + CD127high (activated T-helpers-Tact), CD3 + CD4 + CD161 + (Th17 lymphocytes), all antibodies produced by Beckman Coulter, Sony Biotechnology (USA) and Miltenyi Biotec (Germany). We also evaluated the expression of the depletion marker PD-1 (CD279, BioLegend, USA), as well as the population of activated T-regulatory cells CD4-CD25-aFoxP3 (Treg detection kit; Miltenyi Biotec, Germany). For staining, cells were washed with PBS (phosphate-buffered saline) with 2% bovine serum albumin, 1 mM EDTA, 0.1% sodium azide. Surface markers were stained on unfixed cells for 20 min at 4°C. To stain intracellular markers, fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin were used.

Cytotoxic analysis using RTCA Icelligence. The analysis of the cytotoxicity of TILs against primary cultures of tumor cells was performed using an RTCA Icelligence cellular analyzer (ACEA, Bioscienses Inc., USA). The principle of the method is based on continuous measurement of the impedance on the surface of the plate, the value of which correlates with the density of the monolayer. Target tumor cells were seeded in DMEM medium in the wells of an 8-well E-plate (10,000 cells/well) and cultured for 48 h until the formation of 100% confluence of the monolayer (exit of the cell index curve to a plateau). After that, 100,000 (autologous or allogeneic) TILs were added to the primary tumor cultures and cultured with target cells for 48–72 h. Wells with tumor cells without TILs (positive control) and wells with only TILs were used as controls. without target cells to exclude background impedance due to lymphocytes (negative control).

Ethical review

The study was carried out in accordance with the protocol of the 1999 Council of Europe Convention on Human Rights and Biomedicine and the 2000 Declaration of Helsinki by the World Medical Association); all patients participating in the study signed an informed consent.

Statistical analysis

When performing experiments on the analysis of cytotoxicity, the comparison of the indicators of the cell index during incubation with autologous and allogeneic tumor-infiltrating immune cells was performed using the parametric Student's t-test. The experiments were performed in triplicate, with the mean values and standard deviations of the cell index values being determined.

RESULTS

Study subject

The biomaterial was obtained from 16 patients who were undergoing surgical treatment for oncological disease at the Federal State Budgetary Institution Federal Research Center of the Federal Medical and Biological Agency of Russia. The following solid tumors were investigated: adenocarcinomas of various localization (7 samples), glioblastoma (7 samples), melanoma (2 samples).

Main study results

As a result of the work, from all 16 samples of intraoperative tumor material, in addition to the primary culture of solid tumor cells, it was possible to obtain a culture of TILs. When isolating TILs from large tumor fragments (volume 1–2 cm3), we used the Rapid Growth Protocol (REP) [8, 20], in which the yield of TILs was up to 10×10^6 cells within 24–48 h. analysis by flow cytometry on the first day and obtaining the number of cells required for therapy (5×10^8) within a week. If the biopsy specimen was less than 0.5 cm^3 (almost all glioblastoma specimens), conventional mechanical dissociation and fragment culture were used; in this case, obtaining a culture of TILs was delayed up to 2–3 weeks.

The initial cultivation of TILs derived from melanoma and adenocarcinomas was performed according to one protocol with the addition of 10–100 μg/ml IL2. On the 6–7th day of cultivation, TILs began to migrate from the tumor tissue, forming a characteristic "carpet" (Fig. 1). At this stage, it is important to assess the proliferative potential of the first released lymphocytes. The choice of the protocol for their further successful cultivation depends on this. The protocol modifications consisted in increasing the concentration of exogenous IL2 to 6000 ME/ml, replacing IL2 with IL15, the time of adding activating antibodies, and choosing feeder cells.

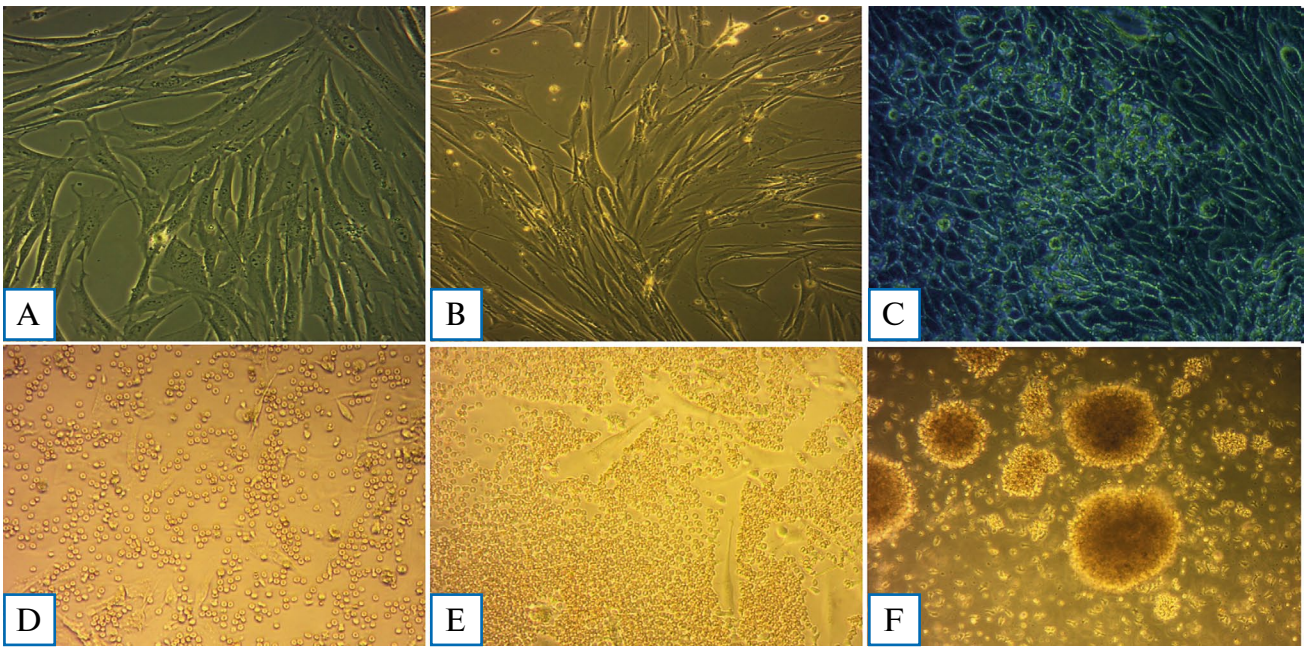
Immunophenotyping showed that the obtained TILs are a heterogeneous mixture of immune cells containing subpopulations of CD3 + CD4 +, CD3 + CD8 + T cells, CD4 + CD25 + CD127- T regulatory cells, CD3-CD56 + CD45 + NK cells, CD3-CD19 + B -cells. A major population of NK-T cells (CD3 + CD56 +) was also found in TILs derived from glioblastoma. The proportion of cytotoxic CD3 + CD8 + T cells in the culture of TILs varied depending on the histogenesis of the tumor and the stage of the disease. In TILs obtained from adenocarcinomas, at the time of isolation, the proportion of CD3 + CD8 + cells remained 16–36% (Fig. 2, A – B). The highest content of CD3 + CD8 + TILs was observed in the culture obtained from melanoma (Fig. 2, C). In TILs obtained from patients with glioblastoma, the content of cytotoxic CD + CD8 + cells was minimal and ranged from 2 to 20%.

The low initial concentration of cytotoxic T cells in TILs preparations from carcinomas and especially from glioblastomas may be one of the reasons for the low efficiency of adoptive immunotherapy for these diseases. In the process of growing TILs, we tried to increase the proportion of cytotoxic T cells and to activate them as much as possible. During activation in the presence of magnetic microparticles CD3/CD28 and/or a cocktail of IL2, IL7, IL15 and IL21, we managed to achieve an increase in CD3 + CD8 + up to 60–90% (Fig. 2, D – F).

Primary TILs, especially glioblastoma-derived lymphocytes, were characterized by the presence of a significant proportion of T-regulatory cells (Tregs) that are immunosuppressive and thus contraindicated for administration in adaptive immunotherapy. To get rid of this undesirable subpopulation in this case, we used positive selection for CD8 (MagniSort™ Human CD8 Positive Selection Kit, Thermofisher, USA). Glioblastoma preparations with very high initial Tregs content (up to 80%) were recovered by negative selection for CD25 + (Dynabeads™ CD25, ThermoFisher). As a result, the fraction of Tregs in the final preparations of TILs did not exceed 1–3% (Fig. 3, A, B).

The original cultures of TILs were also characterized by a high level of expression of the checkpoint inhibitor PD-1 (up to 90%), which indicated their low an-

Fig. 1. Light microscopy of primary cultures of tumor cells (A – B) and primary cultures of cytotoxic tumor-infiltrating lymphocytes (D – E), × 200, phase contrast



Note. The resulting tumor cells have a fibroblast-like phenotype (A, B) or a glandular phenotype (C). D, E — different stages of TILs cultivation from the initial (D) to the final (E), in which cytospheres are formed in large quantities.

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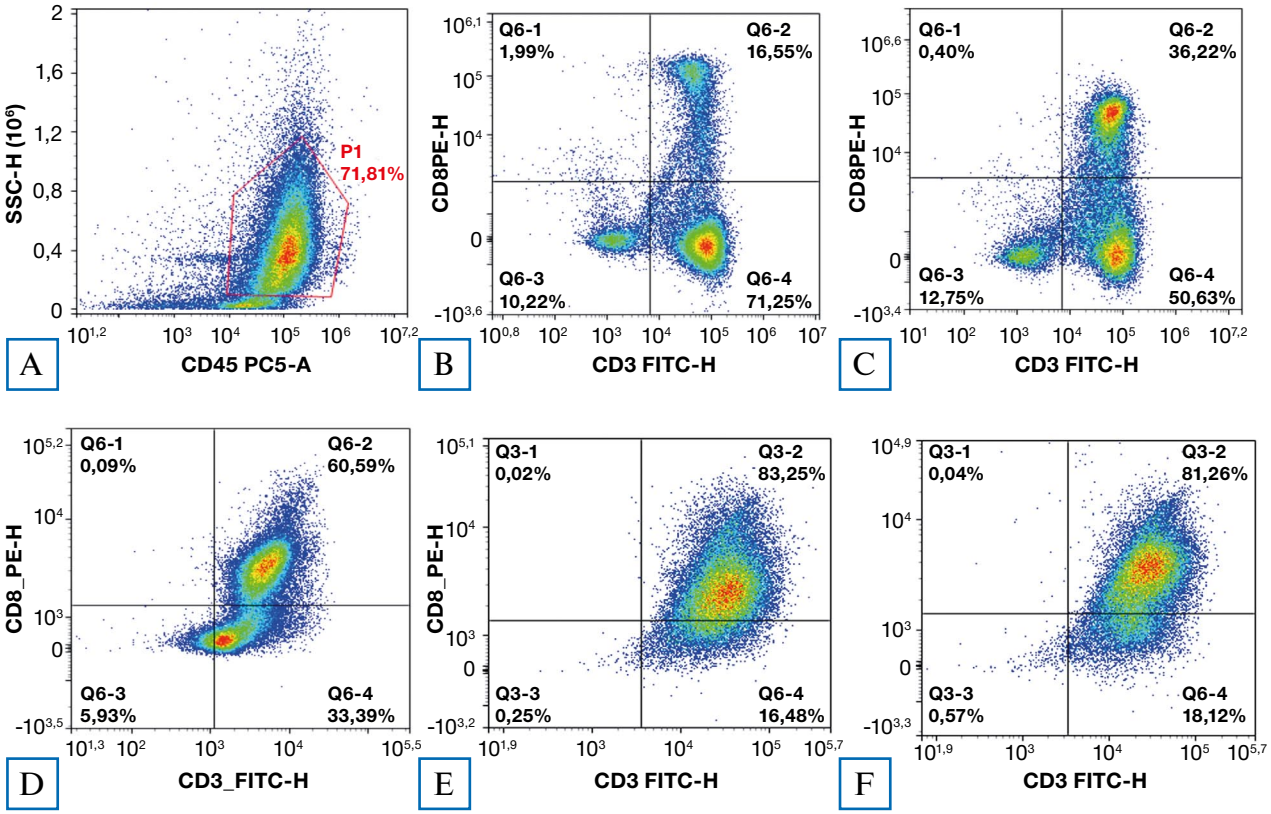
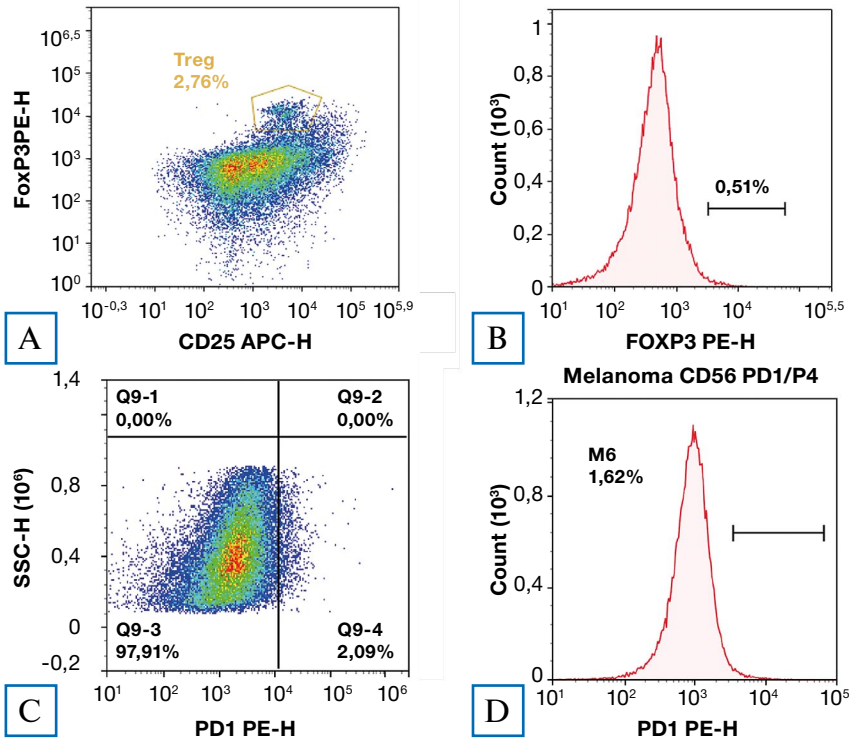
Fig. 2. Immunophenotyping of cytotoxic CD45 + CD3 + CD8 + TILs



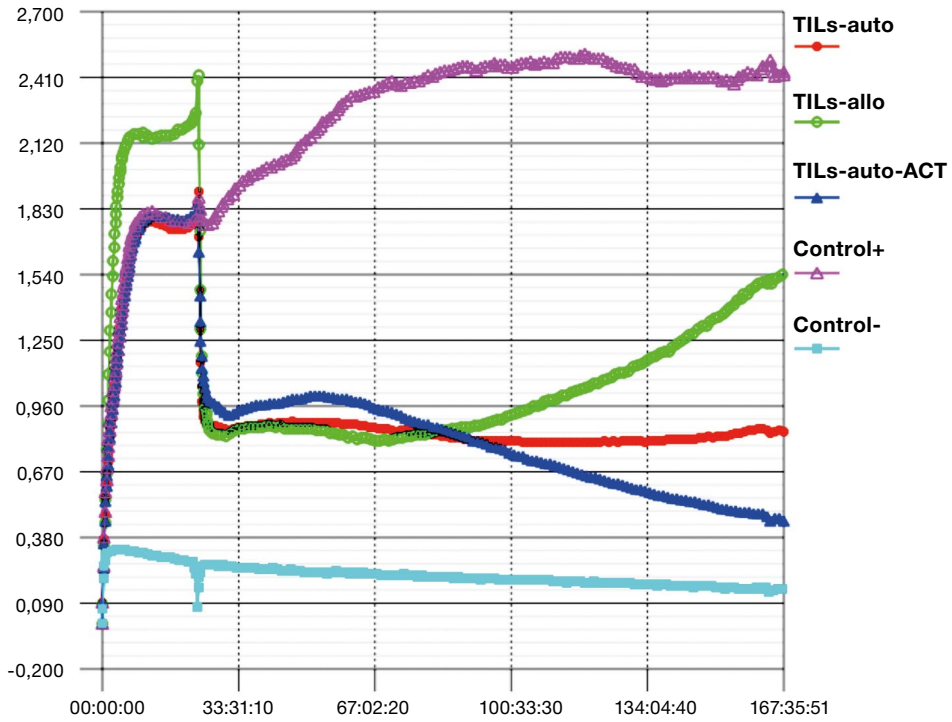
Note. Testing soon after receiving the primary culture (B, C) and after cultivation in a selective medium (D – F). A — gating of CD45 + lymphocyte population in which CD3 + CD8 + was determined; B — initial culture of TILs from adenocarcinoma; C — parental culture of TILs from melanoma. Bottom row — activated TILs from adenocarcinoma (D), melanoma (E), and glioblastoma (F). Flow cytometry using a Novocyte device (ACEA, USA).

Fig. 3. The content of T-regulatory cells and the level of expression of the marker of depletion of cytotoxic lymphocytes PD-1 in the final culture of TILs



Note. A, B — flow cytometry CD25 + FoxP3 + Tregs (their content in preparations does not exceed 3%); C, D — PD-1 expression (1.62–2.09%).

Fig. 4. Evaluation of the cytotoxicity of the obtained preparations of autologous and allogeneic TILs in relation to cultures of primary tumor cells



Note. TILs-auto — preparation of autologous TILs; TILs-allo — preparation of allogeneic TILs; TILs-auto-ACT is a preparation of autologous TILs activated by a modified technique. The abscissa shows the incubation time of TILs with a monolayer culture of tumor cells. The ordinate is the cell index. Differences between indicators in the TILs-allo, TILs-auto and TILs-auto-ACT groups are significant (p <0.05). Test using a cellular analyzer RTCA Icelligence (ACEA, USA).

titumor activity. During cultivation and activation, PD-1 + -positive depleted TILs ceased to express the gene of this protein, and by the end of the growth protocol, the content of PD-1 + cells in the culture of TILs did not exceed 2.5%, which indicated their high level of activation (Fig. 3, C, D).

The resulting preparations of TILs were investigated in in vitro experiments to assess cytotoxicity against autologous and allogeneic primary tumor cultures. It was found that activated autologous TILs exhibit the greatest antitumor cytotoxic activity (Fig. 4).

DISCUSSION

In this study, we simultaneously solved several problems: obtaining a panel of primary cultures of solid tumors, standardizing the isolation and accumulation of TILs from a biopsy specimen of the primary focus or regional/distant metastasis; immunoprofiling of freshly isolated TILs and assessment of their population composition during cultivation, as well as development of protocols for the activation of TILs obtained from various sources (adenocarcinoma, melanoma, glioblastoma). The creation of an autologous pair of a primary tumor cell culture and an actively proliferating culture of TILs is an interesting model system for studying various approaches to activation/inhibition of cytotoxicity.

The study showed that TILs obtained from different solid tumors have different initial composition: the largest number of cytotoxic cells is found in the preparation obtained from melanoma (up to 40%), the smallest - in the preparation from glioblastoma (20% or less). In the latter case, the major population of TILs, as a rule, are T-regulatory cells, the recruitment of which into the tumor enhances the already pronounced immunosuppressive tumor microenvironment and prevents the cytotoxic antitumor activity of immune cells. In addition, we found that TILs isolated from solid tumors exhibit a so-called “depleted” phenotype with increased PD-1 expression in both the CD4 + T helper population and cytotoxic CD8 + cells. Preliminary experiments with these cells have shown that they have virtually no antitumor cytotoxicity. Having analyzed a number of protocols for activating TILs, we synthesized on their basis the optimal protocol characterized by the sequential addition of CD3/CD28 magnetic microparticles or similar antibodies, as well as a cocktail of IL2, IL-7, IL15, and IL21. The cytotoxic activity of the thus obtained TILs was tested in in vitro experiments, showing the

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advantage of using autologous activated TILs over allogeneic ones.

CONCLUSION

An optimized protocol for the isolation, augmentation and activation of TILs from intraoperative biopsies of various solid tumors has been developed, which makes it possible to obtain a cell preparation containing up to 80% of CD8 + PD-1-activated TILs in an amount sufficient for adoptive therapy (5×10^8 and more). The protocol developed by us for obtaining and activating TILs can be recommended for conducting phase I – II clinical trials of adoptive immunotherapy of recurrent, actively metastatic solid tumors.

ADDITIONAL INFORMATION

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Competing interests. The authors declare no conflict of interest which should be reported.

AUTHOR CONTRIBUTIONS

Yusubalieva G.M. — research design, obtaining, primary cultures of tumor cells, obtaining, growing, activation and immunochemical analysis of TILs, writing a manuscript; Petrichuk S.V. Kuptsova D.G. — immunophenotyping of TILs using flow cytometry; Krivoshapkin A.L., Vinokurov A.G., Kalinkin A.A. — sampling and characterization of glioblastoma intraoperative material; Kedrova A.G., Ivanov Yu.V., Sanzharov A.E. Ishchenko R.V. — sampling and characteristics of intraoperative material of adenocarcinomas; Ponomarev A.V., Kim S.V., Ivanov Yu.V. — sampling and characteristics of intraoperative material of melanoma metastases; Troitsky A.V. — general research management; V.P. Baklaushev — research design, manuscript writing. All authors have read and approved the final version prior to publication.

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