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In Vitro Studies May be Useful in Donor Selection and Evaluating the Effectiveness of CD8⁺ T-cell Reprogramming: Experience of a Pilot Study†

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Abstract: Survival and cytotoxicity of reprogrammed human CD8⁺ T-cells (hrT-cells) were evaluated in a culture of cancer stem cells (CSCs) isolated from a patient with small cell lung cancer (SCLC). T-cells were isolated from the blood of healthy volunteers and patients with lung diseases. Reprogramming with MEK and PD-1 inhibitors increased the survival and cytotoxicity of allogeneic T-cells *in vitro*. The positive effect of reprogramming is more pronounced in patients with lung diseases that in healthy donors. Autologous hrT-cells showed high effective in eliminating CSCs. Thus, *in vitro* studies are significant in selection of a potential cell donor and evaluating the effectiveness of their reprogramming.

Keywords: reprogrammed; autologous and allogeneic CD8⁺ T-cells; healthy donors; SCLC; COPD; asthma; cancer stem cells; *in vitro* study

1. Introduction

Small cell lung cancer (SCLC) is the most aggressive form of lung cancer. The 5-year survival rate is about 5%. The treatment of patients with SCLC underwent significant transformation by the introduction of immune checkpoint inhibitors. However, significant progress has still not been made [1]. At the time of diagnosis, more than 60% of patients have distant metastases [2, 3], a significant part of metastases is found in the brain, liver, and bones [3]. The presence of distant metastases and their number determine the patient's life expectancy and prognosis [4]. Circulating tumor cells (CTCs) are detected before the appearance of other signs of lung cancer, including precancerous conditions [5]. CTCs and cancer stem cells (CSCs) play a leading role in SCLC metastasis and can be used as diagnostic and prognostic markers [3, 6, 7], potential therapeutic targets [8].

CAR T-cells therapy has proven for the treatment of malignant blood disorders. Meanwhile, the creation of CAR T-cells for the treatment of solid tumors, including SCLC, is difficult due to the variability of tumor cells and the problem with the access of modified cells to solid tumor cells [9]. In addition, when developing cell therapy approaches, special attention should be paid to cell material. For example, the blood of healthy donors or umbilical cord blood is a source of T-cells [10]. However, allogeneic cell transplantation is associated with graft-versus-host disease. Autologous cells can avoid negative consequences. However, patients' T-cells are often dysfunctional. After chemotherapy, T-cells are more differentiated and show lower proliferative activity *in*

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vivo and *ex vivo* [10]. Antigen specific stimulation leads to rapid depletion of such cells. Previously, we developed an approach to reprogramming mouse T-cells which increase their survival and target CSCs, and thus increase the effectiveness of LLC cell therapy [8]. MEK and PD-1 inhibitors and targeted training of CSCs were used for the reprogramming.

In the present study, using *in vitro* allogeneic and autologous cell therapy model, we reprogrammed $CD8^+$ T-cells isolated from the blood of healthy volunteers (non-smoker and smoker) and patients with chronic lung diseases (chronic obstructive pulmonary diseases (COPD), SCLC, asthma in different combination) and assessed their survival and cytotoxic activity relative to targeted CSCs of a patient with SCLC and COPD.

2. Materials and Methods

2.1. Design of Investigation

The study included non-smoking (*V1*) and smoking (*V2*) volunteers, patient with COPD (*P1*), COPD and asthma (*P2*), SCLC and COPD (*P3*). The characteristics of the subjects are presented in the table 1. The study included four stages. At the first stage, mononuclear cells and then $CD8^+$ T-cells were isolated from the venous blood samples of the subjects. At the second stage, CSCs were isolated from the peripheral blood mononuclear cell fraction of patient *P3* and a culture of CSCs was obtained. At the third stage, $CD8^+$ T-cells of the subjects were reprogrammed using the MEK inhibitor and the PD-1 blocker nivolumab. CSCs from patient *P3* were used for T-cells "training". At the fourth stage, apoptosis and cytotoxicity of hrT-cells were assessed in culture of CSCs isolated from a patient *P3*.

Table 1. Patient characteristics.

Characteristic	Volunteer G. *V1*	Volunteer K1. *V2*	Patient R. *P1*	Patient K2. *P2*	Patient A. *P3*
age (years)	35	35	58	63	56
sex	male	male	male	male	male
smoke	non-smoker	smoker	smoker	non-smoker	smoker
COPD	without COPD	without COPD	with COPD	with COPD	with COPD
asthma	without asthma	without asthma	without asthma	with asthma	without asthma
SCLC	without SCLC	without SCLC	without SCLC	without SCLC	with SCLC

COPD – chronic obstructive pulmonary diseases, **SCLC –** small cell lung cancer.

2.2. Isolation of Human $CD8^+$ T-cells

$CD8^+$ T-cells were isolated from blood mononuclear cells using Lympholyte-H (CEDARLANE, Netherlands, Cedarlane Laboratories) and the EasySepTM Human $CD8^+$ T Cell Isolation Kit for magnetic separation.

2.3. Reprogramming of Human $CD8^+$ T-cells

Reprogramming of human $CD8^+$ T-cells was carried out as described previously [8]. For "training" T-cells used CSCs patient *P3* (with COPD and SCLC). After reprogramming, the expression of CCR7 hrT-cells was assessed [11].

2.4. Cultivation and Detection of CSCs Obtained from Blood of Patient P3

Cultivation of CSCs and evaluation of spheroid formation were carried out according to the protocols described previously [8]. For confirmation of the presence of

specific markers on tumor spheroids and single cells, cell staining with monoclonal antibodies (CD117 BB515, CD87 BV421, CD274 BV421, Axl BV480, EGF Receptor Alexa Fluor® 647, all Becton Dickinson, San Jose, CA, USA) was performed. The Cytation 5 instrument was used to obtain images of cells. Followed image analysis was performed using Gen5™ data-analysis software (BioTek, Instruments, Friedrichshall, Germany).

2.5. Detection of the Cytotoxicity and Apoptosis of Reprogrammed Human CD8⁺ T-cells in Vitro

Apoptosis and cytotoxicity of CD8⁺ T-cells were studied as described earlier [11]. The Cytation 5 instrument was used to obtain images of cells. For cell analysis using Gen5™ data-analysis software (BioTek, Instruments, Friedrichshall, Germany).

2.6. Statistical Analysis

Statistical analysis was processed by methods of variational statistics using the SPSS Statistics 12.0 software as described earlier [8].

3. Results

3.1. Tumor Cells Isolated from the Blood of Patient P3 Form Spheroids in Vitro which Included Cells Expressing CD87, CD117, CD274, EGF, and Axl

In a culture of the adherent fraction of mononuclear cells isolated from the blood of patient *P3*, we found spheroids (Figure 1a). Based on the Cytation5 images, a spheroid was defined as a three-dimensional cellular structure. The total number of spheroids was 74 per 200,000 cells. Spheroids were divided into three classes by cellularity: class 1 includes spheroids with the number of cells n=10-19; class 2 – n=20-29; class 3 – n=30-39 (Figure 1b).

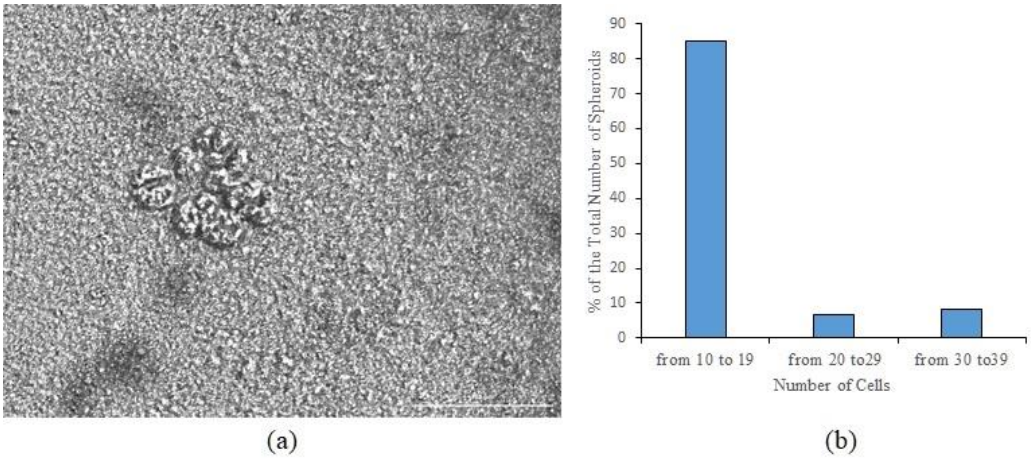

Figure 1. Detection of spheroids in culture of the adherent fraction of mononuclear cells isolated from the blood of patient *P3*. **(a)** Representative images of spheroids in culture of the adherent fraction of mononuclear cells after 14 days of culture. Images were obtained using the Cytation 5 Multi-Mode Reader. Native preparations. All scale bars are 100 μm. **(b)** Spheroids' differentiation depending on the number of cells.

100% staining of cells in spheroids were stained with dyes in various combinations. The following combinations were used: CFSE/Hoechst/EGF, CD117/CD87/EGF, CD117/Axl/EGF, CD117/CD274(PD-L1)/EGF. Cells stained with 7AAD were not found in the structure of spheroids.

3.2. Apoptosis of hrT-cells and hnT-cells of the Subjects in the CSCs Culture Isolated from the Blood of Patient P3

In primary CSCs culture isolated from the blood of patient *P3* (COPD with SCLC), apoptosis of hrT-cells volunteers *V1* (healthy volunteer) and *V2* (smoker volunteer), patients *P1* (smoker with COPD), *P2* (COPD with asthma) and *P3* was studied in comparison with the corresponding naive human $CD8^+$ T-cells (hnT-cells). The maximum number of apoptotic cells was observed in the culture of hrT-cells of the volunteer *V1*. The level of apoptosis of hnT-cells of the volunteer *V2* was higher than in *V1* cell culture. However, hrT-cells of volunteer *V2* were more resistant to apoptosis than *V1* cells.

Apoptosis of hrT-cells isolated from the patient *P1* was significantly reduced at ratios of 0.25:1.0, 5.0:1.0, and 10.0:1.0. The most pronounced decrease in apoptosis was observed in hrT-cells isolated from the blood of the patient *P3*. A decrease in apoptosis in this group was noted at a ratio of 1.0:1.0. At ratios of 2.5:1.0, 5.0:1.0, and 10.0:1.0, the number of apoptotic cells decreased more. Apoptosis of hrT-cells obtained from patient *P2* did not change significantly (Figure 2).

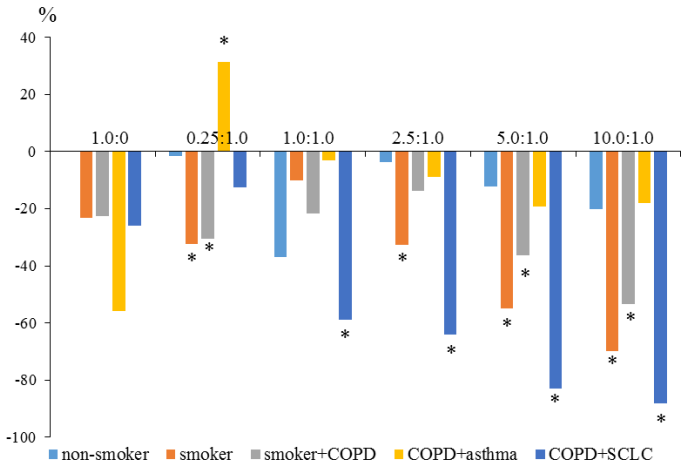

Figure 2. Apoptosis of reprogrammed T-cells of volunteers *V1* (non-smoker volunteer) and *V2* (smoker volunteer), patients *P1* (smoker with COPD), *P2* (COPD with asthma), and *P3* (COPD with SCLC), in a culture of CSCs isolated from the blood of patient *P3* compared to naive T-cells (apoptosis of naive T-cell was taken as 100%). * – for comparison with naive T-cells ($p < 0.05$).

3.3. Cytotoxic Activity of hrT-cells and hnT-cells of the Subjects in the Culture of CSCs Isolated from the Patient P3

In the primary CSCs culture isolated from the blood of patient *P3*, the cytotoxic activity of hnT-cells and hrT-cells of the subjects was studied. In all groups, the cytotoxicity of hrT-cells increased with increasing the concentration of T-cells in culture (compared to hnT-cells). Cytotoxicity reached the maximum at a T-cells:CSCs ratio of 10.0:1.0. At the same time, a significant increase in the cytotoxicity of hrT-cells in volunteer *V2*, patients *P1*, and *P2* was observed at a ratio of 1.0:1.0. Cytotoxicity of hrT-cells in patients *P1*, *P2* and *P3* increased more significantly relative to hnT-cells in comparison with volunteers *V1* and *V2* (Figure 3).

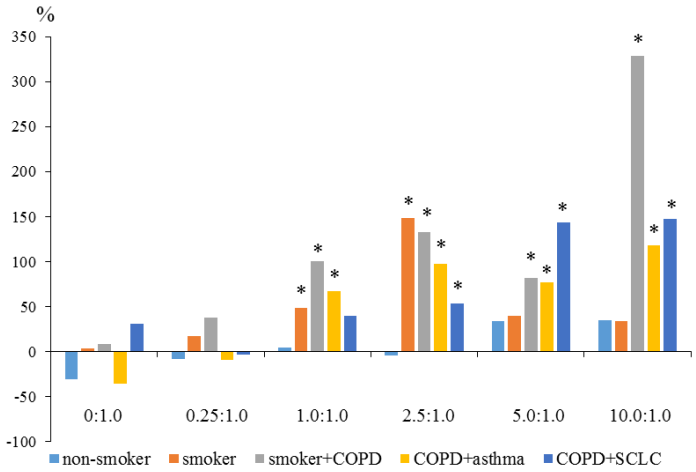

Figure 3. Cytotoxic activity of reprogrammed T-cells (hrT-cells) of volunteers *V1* (non-smoker volunteer) and *V2* (smoker volunteer), patients *P1* (smoker with COPD), *P2* (COPD with asthma), and *P3* (COPD with SCLC), in a culture of CSCs isolated from the blood of patient *P3* to naive T-cells (cytotoxic of naive T-cell was taken as 100%). * – for comparison with naive T-cells ($p < 0.05$).

Discussion

Cell therapy with modified immune cells is a promising approach for the treatment of SCLC. An unresolved issue of this approach to therapy is the choice of the optimal cell donor whose modified cells could eliminate the given target of CSCs. This issue can be partially resolved by assessing the cells *in vitro*. In the present pilot study, we evaluated the activity of allogeneic (from volunteers *V1* and *V2*, patients *P1* and *P2*) and autologous (from patient *P3*) hrT-cells on a culture of CSCs isolated from the blood of patient *P3* (COPD+SCLC). In a culture of CSCs, hrT-cells from volunteers *V1*, *V2* and patients *P1*, *P2*, and *P3* showed significantly greater cytotoxicity and less apoptosis than corresponding nrT-cells. The most pronounced increase in cytotoxicity was observed in hrT-cells patients with lung disease than in volunteers. In addition, the number of apoptotic hrT-cells in a culture obtained from patients with pulmonary diseases was less. The exception was T-cells isolated from the blood of a patient with COPD and asthma. Reprogramming did not have a significant effect on the change in this indicator.

Thus, T-cells reprogramming using the MEK inhibitor, the PD-1 blocker, and personalized "training" with CSCs are effective for allogeneic T-cells of volunteers and patients with lung diseases. We demonstrated the efficacy of autologous hrT-cells in SCLC *in vitro*. The use of autologous cells can significantly reduce the risk of negative immune reactions.

We understand that the study has limitations: the sample of patients was limited, and no follow-up study was conducted. We are currently recruiting additional patients through this project. Further studies will confirm the effectiveness of cellular reprogramming and provide additional data required for the improvement of the personalization of cell therapy.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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