Reprogrammed CD8+ T-Cells Isolated from the Spleen Increase the Number of Immune Cells with Antitumor Activity and Decrease the Amount of Cancer Stem Cells

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Abstract: We have developed an approach to reprogramming immune cells by inhibiting the MAPK/ERK pathway through MEKi and the PD-1/PD-L1 immune checkpoint signaling pathway. We hypothesized that reprogramming of spleen $CD8^+$ T-cells could also create a population of immune cells with high antitumor activity. We reprogrammed $CD8^+$ T-cells derived from the spleen of C57BL/6 mice (rs$CD8^+$T-cells). In orthotopic LLC model, cell therapy with rs$CD8^+$T-cells increased the amount of proliferating $CD8^+$ and $CD4^+$ T-cells in blood and lung tissue from mice. The amount of cancer stem cells (CSC) decreased in the blood and lung of mice treated with rs$CD8^+$ T-cells. A morphological study revealed a decrease in the number of metastases in the lung tissue. The antitumor effects of rs$CD8^+$T-cells are based on the activation of the host immune response by increasing the populations of $CD8^+$ and $CD4^+$ T-cells and apoptosis of CSCs.

Keywords: reprogrammed spleen $CD8^+$ T-cells; Lewis lung carcinoma; cancer stem cell; $CD8^+$ and $CD4^+$ T-cells; antimetastatic activity

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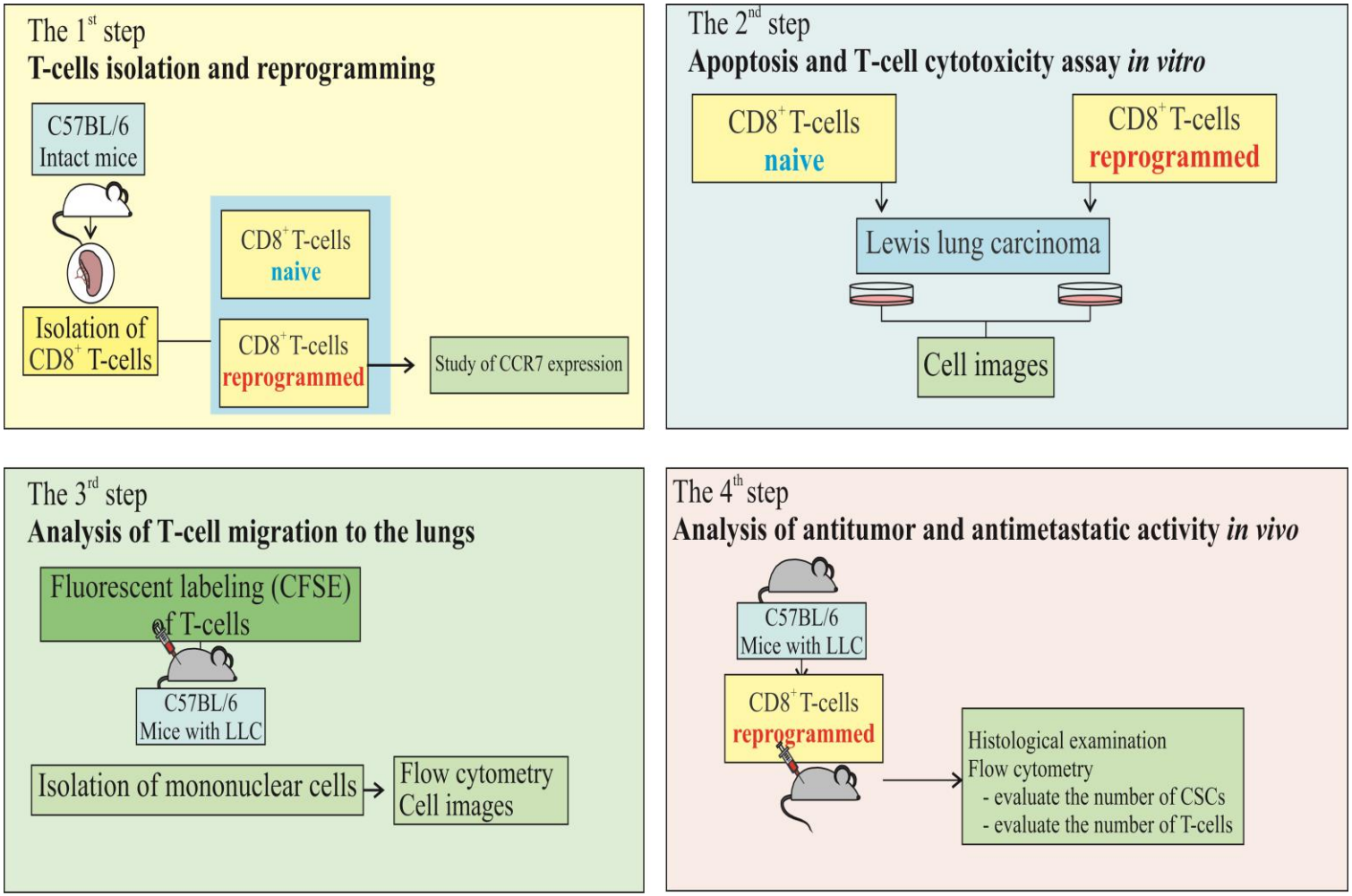
The experimental design of the investigation



The study was conducted according to the guidelines of the European Convention on the protection of vertebrates used in experiments for other scientific purposes and approved by the Institutional Ethics Committee of Goldberg ED Research Institute of Pharmacology and Regenerative Medicine, Tomsk NRMC (protocol code 189092021).

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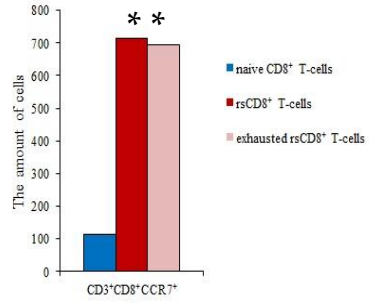
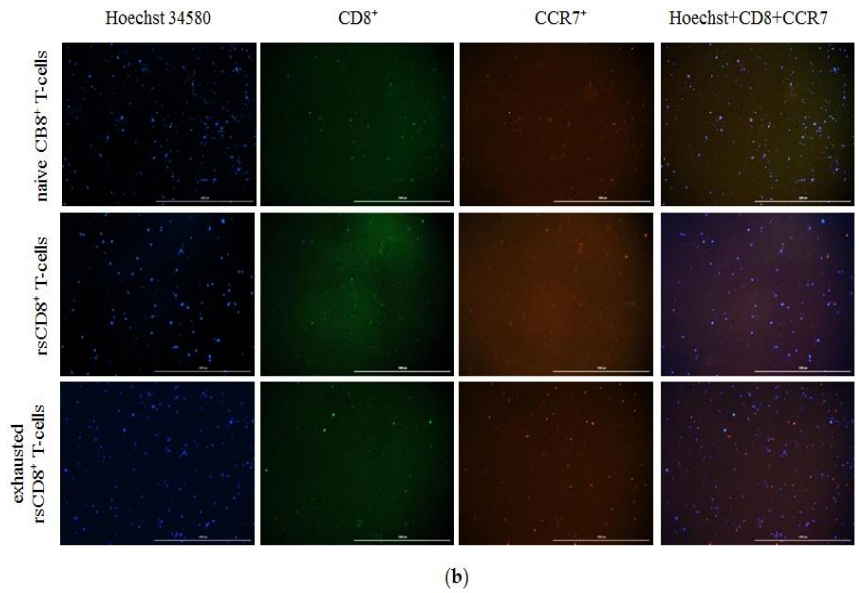
Results and Discussion

Study of Detection of the CCR7 Expression



(a)



(b)

(a) The count of naive, reprogrammed, and exhausted reprogrammed $CD8^+$ T-cells of the spleen of C57BL/6 mice expressing the CCR7 marker in T-cell culture; **(b)** 20× images of T-cells stained with: Hoechst (blue) to identify cell nuclei; CD8 FITC (green); CCR7 AF555 (red); (Hoechst$^+$CD8$^+$ CCR7) composite image using all three colors. Determination of the percentage of cells $CD3^+CD8^+CCR7^+$ was made by assessing the ratio of cells counted in green and red channel to total cells counted in the blue (DAPI) channel. All scale bars are 1000 µm. *—for comparison with the naive spleen T-cells by Mann–Whitney test ($p < 0.05$).

The rsCD8$^+$ T-cells had higher expression levels of chemokine receptor CCR7 than naive spleen CD8+ T-cells in CD8$^+$ T-cell culture.

Exhaustion did not cause changes in the CCR7 expression by rsCD8$^+$ T-cells, which indicates that the changes induced by the MEK inhibitor and nivolumab are stable.

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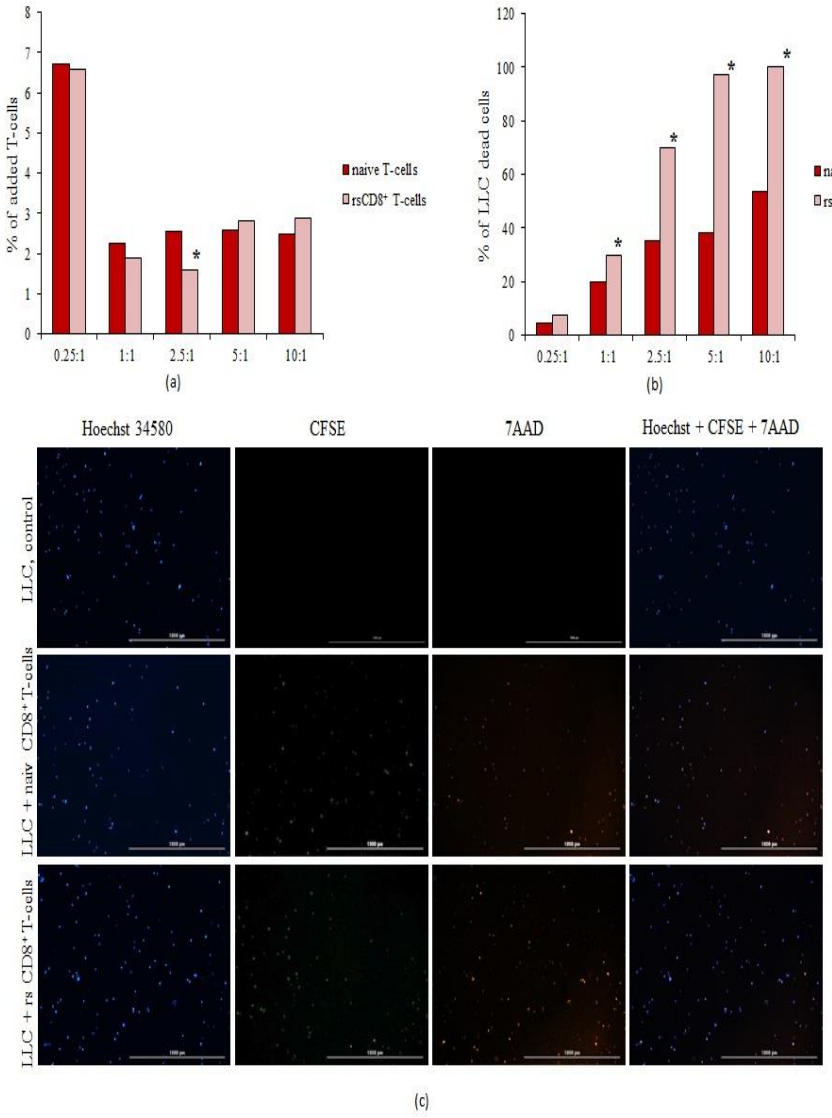
Results and Discussion

Study of Detection of the Cytotoxicity and Apoptosis of rsCD8+T-cells in Vitro



The rsCD8$^+$ T-cells were more resistant to the cytotoxic effect of cancer cells in comparison with naive CD8$^+$ T-cells.

The rsCD8$^+$ T-cells are more stable under cultivation: 0.79% of the cells were in apoptosis.

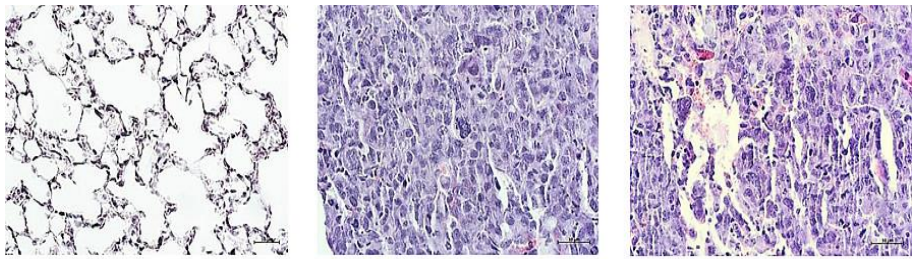
Cytotoxicity of rsCD8$^+$ T-cells was higher in comparison with that of naive CD8$^+$ T-cells in the same ratios.

The rsCD8$^+$ T-cells, when treated with MEKi and nivolumab *in vitro*, were resistant to the cytotoxic effect of cancer cells and distinct from naive CD8$^+$T-cells.

Cytotoxicity and apoptosis of naive and reprogrammed spleen CD8$^+$ T-cells in LLC culture. (**a**) The count of apoptotic tumor LLC cells after co-cultivation with reprogrammed spleen CD8$^+$ T-cells (% from dead cells of LLC culture); (**b**) The count of apoptotic tumor LLC cells after cocultivation with spleen CD8$^+$ T-cells (% from added cells); (**c**) Hoechst (blue) to identify cell nuclei; 7AAD (red); (Hoechst+7AAD+) composite image using all two colors. Determination of the percent of died cells of LLC Hoechst+7AAD+ was made by assessing the ratio of cells counted in blue and red channel to total cells of LLC without green channel. All scale bars are 1000 μm. * - for comparison with naive CD8+ T-cell by Mann–Whitney test ($p < 0.05$).

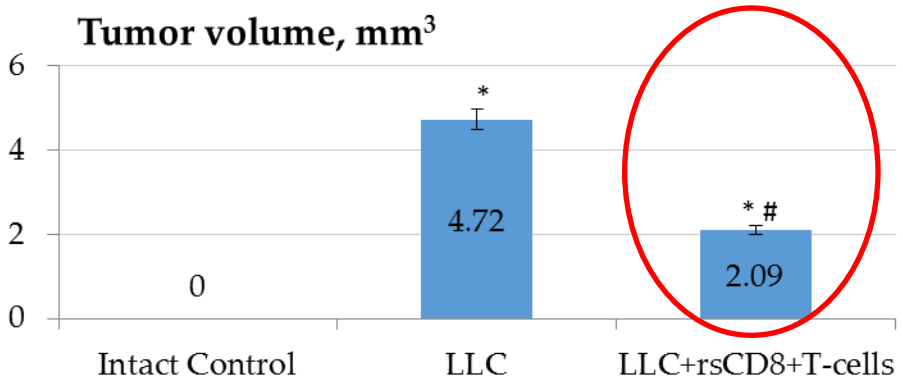
Results and Discussion

Lung Histology of Mice with Lewis Lung Carcinoma after rsCD8⁺ T-cell Therapy



Micrographs of lung sections obtained from male C57BL/6 (**a**) mice of intact control; (**b**) mice with LLC; (**c**) mice with LLC treated with rsCD8⁺T-cells on d7. Tissues were stained with hematoxylin-eosin. ×400. Scale bar 10 μm.

We evaluated the lung histology on d7 in response to treatment with LLC injection. Vehicle-treated lung of mice displayed the lung tumors characterized by scattered tumor cells with cellular and nuclear polymorphism. Multinucleated giant cells are organized in clusters or aligned along the alveolar walls. The histological picture of the lungs of LLC mice treated with rsCD8⁺T-cells only displayed growth deceleration, but not regression of tumor (Figure C), showed a decrease in the number of tumor emboli in the vessels, and perivascular and peribronchial metastases.

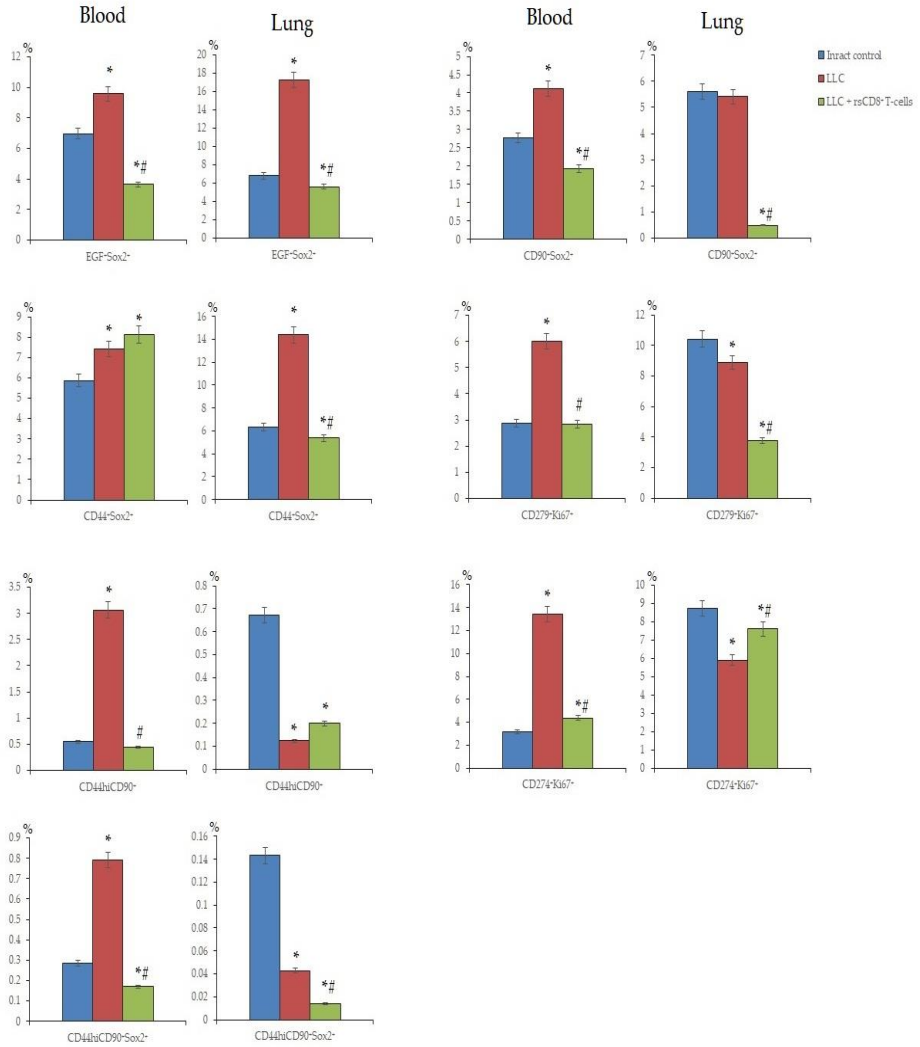


Therapy with rsCD8⁺T-cells caused an increase in tumor growth inhibition index (TGII). The value of TGII after cell therapy with rsCD8⁺T-cells was 54.7 %. Moreover, we observed an increase in tumor volume and the average number of metastases.

*—for comparison with the intact group by Mann–Whitney test ($p < 0.05$); #—for comparison with the mice with LLC by Mann–Whitney test ($p < 0.05$).

Results and Discussion

Effect of rsCD8⁺ T-cells on Cancer Cells and Cancer Stem Cells in the Lungs and Blood of Mice with Lewis Lung Carcinoma



*—for comparison with the intact group by Mann–Whitney test ($p < 0.05$); #—for comparison with the mice with LLC by Mann–Whitney test ($p < 0.05$).

Phenotype of cancer cells and cancer stem cells:
EGF⁺Sox2⁺, CD44⁺Sox2⁺, CD90⁺Sox2⁺,
CD44^{hi}CD90⁺, CD44^{hi}CD90⁺Sox2⁺,
CD279⁺Ki67⁺, CD274⁺Ki67⁺

We observed a significant increase in the number of CSCs with different phenotypes in the lungs of mice on the d7 after LLC injection: EGF⁺Sox2⁺, CD44⁺Sox2⁺, CD274⁺Ki67⁺. At the same time, the all populations of cancer cells were increased in the blood of mice with LLC in the compared with the intact control on the d7.

The rsCD8⁺ T-cells injection significantly reduced the number of cancer cells and CSCs population (EGF⁺Sox2⁺, CD44^{hi}CD90⁺Sox2⁺, CD90⁺Sox2⁺, CD279⁺Ki67⁺) in the blood and lungs of mice with LLC on the d7. However, the population of proliferating CD274⁺ cells in the lung was increased after rsCD8⁺ T-cell injection. At the same time, the number of CD44⁺Sox2⁺ cells in the blood and the number of CD44^{hi}CD90⁺ cells in the lung were changed weakly.

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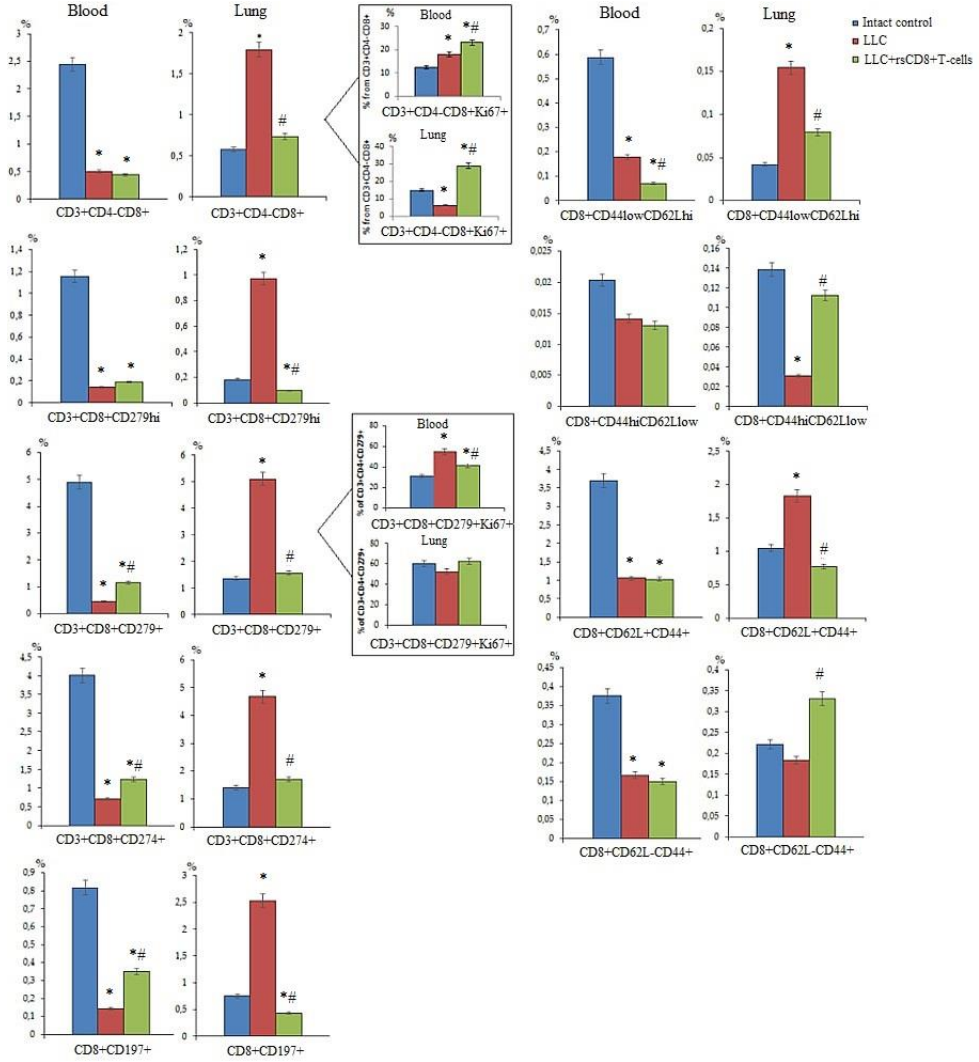
Results and Discussion

Effect of rsCD8⁺ T-cells on the Content of CD8⁺ T-cells in the Blood and Lungs of Mice with Lewis Lung Carcinoma



Phenotype of CD8 T-cells:
$CD3^+CD4^-CD8^+$, $CD3^+CD8^+PD\text{-}1^+$, $CD3^+CD8^+PD\text{-}L1^+$, $CD3^+CD8^+PD\text{-}1^{hi}$, $CD8^+CD197^+$, $CD8^+CD62L^-CD44^+$, $CD8^+CD62L^{hi}CD44^{low}$, $CD8^+CD62^+CD44^+$, $CD3^+CD4^-CD8^+Ki67^+$ and $CD3^+CD8^+PD\text{-}1^+Ki67^+$.

Administration of rsCD8⁺T-cells caused an increase in a significant number of CD8⁺T-cell populations ($CD8^+CD197^+$, $CD3^+CD4^-CD8^+Ki67^+$, $CD3^+CD8^+PD\text{-}1^+$, $CD3^+CD8^+PD\text{-}1^+$) in the blood of mice with LLC compared to untreated mice with LLC.

After rsCD8⁺T-cell therapy, the CD8⁺T-cell population in the lungs of recipient mice with LLC was lower in comparison with the animals of LLC group without treatment. At the same time, populations of proliferating $CD3^+CD4^-CD8^+$ cells and effector CD8⁺T-cells ($CD8^+CD62L^{hi}CD44^{low}$ and $CD8^+CD62^+CD44^+$) in the lung were increased after rsCD8+ T-cell administration.

*—for comparison with the intact group by Mann–Whitney test ($p < 0.05$); #—for comparison with the mice with LLC by Mann–Whitney test ($p < 0.05$).

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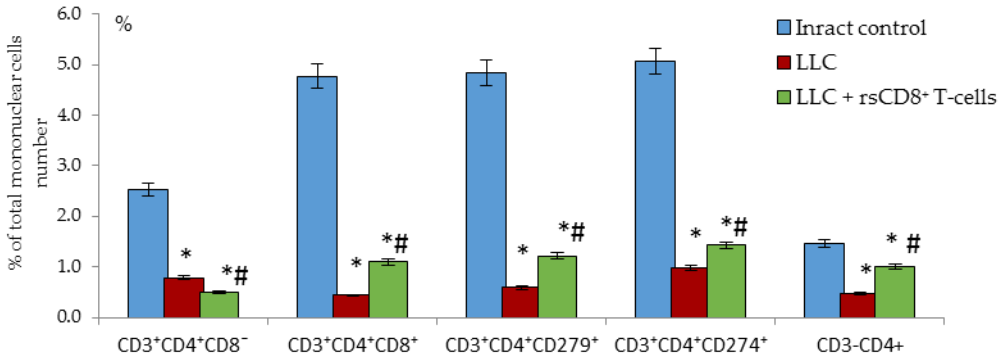
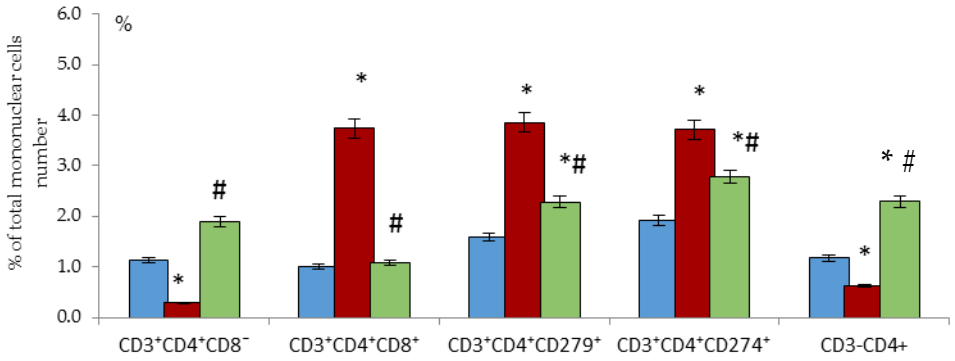
Results and Discussion

Effect of rsCD8$^+$ T-cells on the Content of CD4$^+$ T-cells in the Blood and Lungs of Mice with Lewis Lung Carcinoma

Blood



Lung



*—for comparison with the intact group by Mann–Whitney test ($p < 0.05$); #—for comparison with the mice with LLC by Mann–Whitney test ($p < 0.05$).

Phenotype of CD4 T-cells: CD3$^-$CD4$^+$, CD3$^+$CD4$^+$CD8$^+$, CD3$^+$CD4$^+$CD8-, CD3$^+$CD4$^+$PD-L1(CD274)$^+$, CD3$^+$CD4$^+$PD-1(CD279)$^+$, proliferating cells: CD3$^+$CD4$^+$PD-1$^+$, CD3$^+$CD4$^+$CD8$^-$

Administration of rsCD8$^+$T-cells caused an increase in a significant number of CD4$^+$T-cell populations (CD3$^-$CD4$^+$, CD3$^+$CD4$^+$CD8$^+$, CD3$^+$CD4$^+$PD-L1(CD274)$^+$, CD3$^+$CD4$^+$PD-1(CD279)$^+$, proliferating cells: CD3$^+$CD4$^+$PD-1$^+$ and CD3$^+$CD4$^+$CD8$^-$) in the blood of mice with LLC compared to untreated mice with LLC (d7). At the same time, the content of CD3$^+$CD4$^+$CD8$^-$ cells decreased even more during treatment in the blood.

The rsCD8$^+$ T-cells injection significantly reduced the number of T-cells with phenotype CD3$^+$CD4$^+$CD8$^+$, CD3$^+$CD4$^+$PD-L1$^+$, CD3$^+$CD4$^+$PD-1$^+$ and proliferating CD3$^+$CD4$^+$PD-1$^+$ cells in the lungs. At the same time, the number of CD4$^+$ T-cell populations with phenotype CD3$^+$CD4$^+$CD8$^-$, CD3$^-$CD4$^+$ and proliferating CD3$^+$CD4$^+$CD8$^-$ increased in the lungs of mice with LLC. We explain this by increased migration of these populations of CD4$^+$ T-cells from the blood to the lungs in response to rsCD8$^+$T-cells injection.

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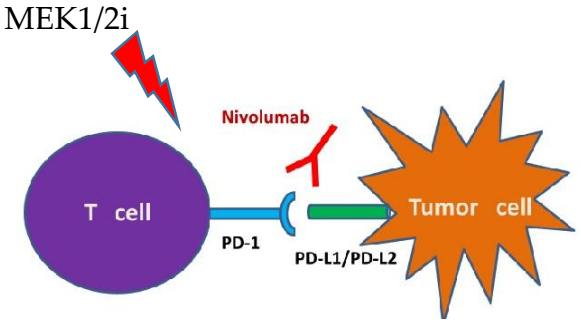
Conclusions

Prior our studies have analyzed the impacts of reprogramming by MEKi and blockade of the PD-1 on activity of $CD8^+$T-cells derived from bone marrow or blood. In this study we observed that reprogramming induced the activity of $CD8^+$T-cells isolated from spleen. We presented evidence that reprogramming may have favorable $CD8^+$T-cells impacts and enhances the antitumor activity of $CD8^+$T-cells in the LLC orthotopic model.

This strategy generated $CD8^+$T-cells with higher efficacy for cell therapy. The effect of reprogrammed spleen $CD8^+$T-cell therapy has been associated with the activation of the host immune response by increasing the populations of $CD8^+$ and $CD4^+$ T-cells in mice, and the effect of rs$CD8^+$T-cells and mouse effector $CD8^+$ T-cells on cancer cells and CSCs.



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Thank you for attention!

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