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## ORIGINAL ARTICLE

# Age-related features of lung cancer treatment using reprogrammed CD8 positive T cells in mice subjected to injection of Lewis lung carcinoma cells

Evgenii Skurikhin<sup>1</sup>  | Mariia Zhukova<sup>1</sup> | Natalia Ermakova<sup>1</sup> | Edgar Pan<sup>1</sup>  |  
Darius Widera<sup>2</sup> | Lubov Sandrikina<sup>3</sup> | Lena Kogai<sup>3,4</sup> | Nikolai Kushlinskii<sup>5</sup> |  
Aslan Kubatiev<sup>1</sup> | Sergey Morozov<sup>1</sup> | Alexander Dygai<sup>1,3</sup>

<sup>1</sup>Institute of General Pathology and Pathophysiology, Moscow, Russia

<sup>2</sup>Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, Whiteknights Campus, Reading, UK

<sup>3</sup>Goldberg ED Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Centre of the Russian Academy of Sciences, Tomsk, Russia

<sup>4</sup>Ministry of Health of the Russian Federation, Siberian State Medical University, Tomsk, Russia

<sup>5</sup>Blokhin National Medical Research Center of Oncology, Moscow, Russia

## Correspondence

Evgenii Skurikhin and Edgar Pan, Institute of General Pathology and Pathophysiology, 125315 Moscow, Russia.

Email: [eskurikhin@inbox.ru](mailto:eskurikhin@inbox.ru); [artifexpan@gmail.com](mailto:artifexpan@gmail.com)

## Abstract

**Background:** Awareness of age-related features of carcinogenesis and the importance of cellular immunity is crucial for developing effective antitumor therapies for specific patient groups.

**Methods:** In this study, we examined different populations of cancer stem cells (CSCs) and circulating tumor cells (CTCs) in "young" (8–10 weeks) and "aged" (80–82 weeks) C57BL/6 male mice. We used an orthotopic model of Lewis lung carcinoma (LLC) to evaluate the effectiveness of cell therapy targeting lung cancer through reprogrammed CD8-positive T cells (rCD8+ T cells) in mice from two different ages.

**Results:** The findings revealed that tumor progression with age is primarily caused by impaired recruitment of T cells to the lungs. Additionally, a lower number of CTCs and CSCs were observed in younger mice compared to the older mice. The antitumor effect of rCD8+ T cells in aged mice was found to be inferior to that in young mice, which can be attributed to the reduced impact of therapy on specific CSCs populations.

**Conclusions:** These results offer new insights into the treatment of lung cancer using rCD8+ T cells. Considering the age-related characteristics influencing disease progression, this therapy has the potential to significantly enhance the effectiveness of treatment methods.

## KEYWORDS

aged and young mice, cancer stem cells, cell therapy, circulating tumor cells, reprogrammed CD8<sup>+</sup> T cells

## INTRODUCTION

Following breast cancer, lung cancer stands as the second most prevalent malignancy worldwide. The peril of morbidity and death from lung cancer increases with age. Regardless of the type of cancer, older people have a worse prognosis than younger individuals.<sup>1</sup> Unfortunately, most patients are diagnosed at an advanced stage.

Tumor initiation, metastasis, and especially recurrence often coincide with the presence of therapy-resistant cancer stem cells (CSCs).<sup>2</sup> For that reason, it is recommended to give particular consideration to novel strategies for the

diagnosis and treatment of lung cancer involving CSCs.<sup>3</sup> However, it is still unclear how the quantity of CSCs changes with aging and whether this correlates with a higher frequency of malignant tumors in elderly patients. Such uncertainties add complexity to the quest for and creation of treatment methodologies tailored to elderly patients.

Sox2 is highly expressed in CSCs and its increased expression indicates acquisition of chemoresistance by the tumor. However, Sox2 expression steadily decreases during the differentiation of progenitor cells into mature cells, and this decrease is also observed with age.<sup>2,4</sup> In addition to Sox2, oncogenesis markers such as EGFR, CD117, CD276,

CD90 and Axl are commonly utilized to identify lung CSCs.<sup>5</sup> There appears to be more information available concerning how the expression of some of these oncoantigens changes with age. For instance, a decrease in Axl levels was observed in aging mice.<sup>6</sup> In addition, the expression of EGFR, in particular EGFR VIII, decreases with aging.<sup>7</sup> According to research conducted by Darboe et al., the quantity of CD117<sup>+</sup> cells, which are defined as peripheral blood congenital lymphoid cells, decreases with aging.<sup>8</sup> In contrast, other studies have shown that aging mice demonstrate a notable reduction in the quantity of Lin<sup>−</sup>CD117<sup>+</sup> cells in the bone marrow.<sup>9</sup> Aging cells show an increase in PD-L1 expression. The quantity of PD-L1-positive cells is observed to rise with age.<sup>10</sup> All this data should be considered when studying the age-related characteristics of cancer pathogenesis and developing antitumor immunotherapy for elderly patients with lung cancer. Recently, there has been active investigation into the links between aging and the risk of tumor development. Alterations in the immune system with aging establish favorable conditions for tumor immune escape. The changes in the immune system are closely associated with a higher incidence of tumor development in older individuals compared to younger ones. Consequently, the weakened immune response against lung cancer is linked to a decline in the number of effector T cells and a simultaneous decrease in their cytostatic activity.<sup>11</sup> It is possible that this mechanism may contribute to tumor immune escape during aging and result in higher morbidity and mortality rates in elderly populations. Therefore, gaining an understanding of the role of T-cell-mediated immunity in tumor development and progression might help in the development of effective and safe methods for lung cancer therapy in older individuals. Previously, we demonstrated a high survival rate of in vitro reprogrammed mouse CD8-positive T cells (rCD8<sup>+</sup> T cells) in LLC culture.<sup>12</sup> In orthotopic and spontaneous metastatic lung cancer models, reprogrammed CD8<sup>+</sup> T cells effectively suppressed lung tumor growth and the development of metastatic disease in murine models. The primary targets for rCD8<sup>+</sup> T cells were CSCs. However, it should be noted that young animals were utilized to assess the effectiveness and safety of potential antitumor therapy approaches (such as chemotherapeutic agents and cell therapy) in this study and in studies conducted by other authors.<sup>13,14</sup>

In the present study, various cancer cell populations (including CSCs and CTCs) along with T cells were examined in “young” and “aged” male C57BL/6 mice using an orthotopic lung cancer model. Furthermore, we investigated the response characteristics of lung cancer to rCD8<sup>+</sup> T-cell therapy in “aged” mice in comparison to “young” ones.

## MATERIALS AND METHODS

### Animals

Male mice (strain C57BL/6) were acquired from the nursery of the Experimental Biological Models Department at the

Goldberg Research Institute of Pharmacology and Regenerative Medicine. The animals were maintained in accordance with the European Convention for the Protection of Vertebrates (Strasbourg, 1986) and the Principles of Good Laboratory Practice (OECD, ENV/MC/CUEM (98)17, 1997). A veterinary certificate is available to verify this information. The animal procedures and study design were approved by the Ethics Committee of the Goldberg Research Institute of Pharmacology and Regenerative Medicine (Protocol No. 189092021).

The mice were divided into groups based on their age: 40 “young” mice (8–10 weeks old) and 40 “aged” mice (80–82 weeks old). Each group consisted of 10 individuals. The groups were as follows: 1, intact “young” mice; 2, intact “aged” mice; 3, “young” mice with lung cancer; 4, “aged” mice with lung cancer; 5, “young” mice with lung cancer who were injected with naive CD8<sup>+</sup> T cells (nCD8<sup>+</sup> T cells); 6, “aged” mice with lung cancer who were injected with nCD8<sup>+</sup> T cells; 7, “young” mice with lung cancer who were injected with rCD8<sup>+</sup> T cells; 8, “aged” mice with lung cancer who were injected with rCD8<sup>+</sup> T cells.

### Lewis lung carcinoma cell line

The cell line of Lewis lung carcinoma (LLC) (strain C57BL) was used for both in vivo and in vitro experiments.

### Orthotopic model of Lewis lung carcinoma

Orthotopic lung cancer was induced by injecting 50  $\mu$ L of a suspension of LLC cells ( $1.5 \times 10^6$  cells per mouse) into the left lung, between the sixth and seventh ribs.<sup>15,16</sup> All manipulations were performed under inhalation anesthesia. At 7 days after LLC cell inoculation, animals were sacrificed by CO<sub>2</sub> overdose.

### Design of the experiment

The design of the experiment is presented in Figure 1. CD8<sup>+</sup> T cells were isolated from bone marrow and reprogrammed. Subsequently, we assessed the initial histological parameters of the lungs, as well as the number of various populations of T cells, CTCs, and CSCs in the lungs and blood of “aged” and “young” mice. Next, we created a lung cancer model in both “young” and “aged” mice. On the seventh day following the LLC injection, we evaluated the histological profile of the lungs in animals of different age groups, as well as the quantity of different types of T cells and CSCs in the lungs and blood. In the end of the research, we performed cell therapy in “aged” and “young” mice with lung cancer on days 4 and 6. After completion, we assessed the effectiveness of rCD8<sup>+</sup> T-cell therapy compared to nCD8<sup>+</sup> T-cell therapy using histological and cytometric indicators.

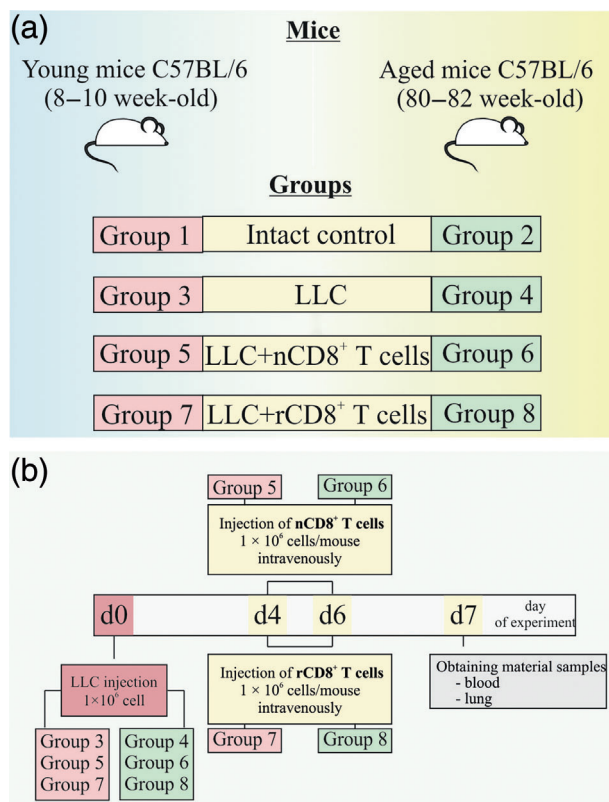


FIGURE 1 Design of the experiment.

## Isolation of mononuclear cells

Isolation of mononuclear cells from tissues (bone marrow, blood, lungs) was carried out using standard methods with a density medium Histopaque-1077.<sup>17,18</sup>

## Magnetic separation of CD8<sup>+</sup> T-cells

Mononuclear cells isolated from bone marrow were subjected to magnetic separation to enrich the cell population with naive CD8<sup>+</sup> T cells (CD8<sup>+</sup>CD3<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup>). EasySep™ Mouse Naive CD8<sup>+</sup> T Cell Isolation Kit was used for this procedure with using the StemCell Technologies manufacturer protocol.

## Reprogramming of bone marrow CD8<sup>+</sup> T cells and CD8<sup>+</sup> T-cell injection

After magnetic separation, naive CD8<sup>+</sup> T cells were reprogrammed using nivolumab and a MEK inhibitor.<sup>19</sup> Nivolumab, PD-1 inhibitor (Bristol-Myers Squibb) and MEK12 inhibitor (Sigma-Aldrich) were utilized for reprogramming. The phenotype of rCD8<sup>+</sup> T cells was analyzed using Cytation 5 (BioTek Instruments) as described previously.<sup>19</sup> To estimate stability of the population, exhaustion of rCD8<sup>+</sup> T cells was carried out in vitro.<sup>20</sup> Naive, non-reprogrammed CD8<sup>+</sup> T cells (nCD8<sup>+</sup> T cells) were used as control.

## CD8<sup>+</sup> T-cell injection

To investigate the migration of CD8<sup>+</sup> T cells into the lungs of recipient mice, both intact mice and mice with LLC were intravenously injected with Carboxyfluorescein Succinimidyl Ester labeled reprogrammed and non-reprogrammed CD8<sup>+</sup> T cells at a concentration of 1 × 10<sup>6</sup> cells per mouse in 0.1 mL of Phosphate-Buffered Saline (PBS).

On the fourth and sixth days of the experiment, CD8<sup>+</sup> T cells were intravenously injected into recipient mice with LLC at a dose of 1 × 10<sup>6</sup> cells per mouse in 0.1 mL of PBS to determine their antitumor and antimetastatic activity.

## Lung tissue histology

Lung tissue samples were sectioned into 5-μm thick slices and then stained with hematoxylin and eosin after standard processing through increasing concentrations of alcohol to xylene and being embedded in paraffin blocks.<sup>18,21</sup>

## Staining and flow cytometry

Mononuclear cells from the lungs and blood were stained for surface and intracellular markers as described earlier.<sup>19</sup> Briefly, to block the Fc-receptors, cells were preincubated with anti-CD16/CD32 antibodies (unconjugated). Then, the cells were incubated with relevant antibodies for 30 min in the dark, washed twice, fixed, and stained with intracellular antibodies (Sox2 or Ki67). Flow cytometry was conducted using a FACS Canto II flow cytometer. Data were analyzed using FACS Diva analysis software (BD Biosciences).

The T-cell and CSC panels were categorized into subpopulations based on their specified markers. These subpopulations included CSCs (Axl + CD90+, Axl + CD90 + Sox2+, Axl + Sox2+, CD90 + Sox2+, CD117 + Sox2+, EGF + Sox2+, CD276 + Sox2+, PD-1+, PD-L1+, PD-1 + PD-L1+, PD-L1 + Ki67+, PD-1 + Ki67+, CD3-CD4-CD8-PD-L1+) and T-cells (CD3 + CD8+, CD3 + CD8 + PD-L1+, CD3 + CD8 + PD-1+, CD3 + CD8 + PD-1hi, CD8 + CCR7-, CD8 + CCR7+, CD3-CD4+, CD3 + CD4+, CD3 + CD4 + PD-1+, CD3 + CD4 + PD-L1+, CD3 + CD4 + CD8+, CD95-CD62L-CD8-CCR7+, and CD8 + CD62L + CCR7 + CD95hi).

## Data analysis

The statistical analysis was conducted using the Mann-Whitney U test with SPSS version 12.0 software (SPSS Inc.). The arithmetic mean (*M*), standard error of the mean (*m*), and the *p* value (*p*) were calculated. A *p* value of less than 0.05 was considered statistically significant when comparing the two values.

## RESULTS

### Histological examination of the lungs

#### The quantity of inflammatory cells in the lungs increases with age

Differences in the structure of the lungs of “aged” (group 2) and “young” (group 1) animals were not revealed (Figure 2). However, “aged” mice exhibited small lymphocytic-neutrophilic infiltrates in the lungs, along with perivascular and peribronchial accumulation of inflammatory cells, which were not observed in the lungs of “young” animals. In addition, despite a growth in body weight, the lungs of “aged” animals demonstrated a decrease in weight compared to “young” mice (Table 1).

#### In lung cancer, histopathological changes in the lungs of “aged” mice are more significant than in “young” mice

Against the background of LLC formation in the lungs of animals in both age groups, lymphocytic-neutrophilic infiltration and well-vascularized large tumor nodules were detected on day 7 (Figure 2). The tumor nodules were composed of atypical cells exhibiting cellular and nuclear polymorphism. Among the atypical cell population, giant multinucleated cells were observed, along with numerous cells undergoing a mitotic process. Numerous small necrotic areas were observed within the tumor tissue. Notably, the severity of lymphocytic-neutrophilic infiltration, necrotic changes, and tumor size were greater in “aged” animals of group 4 compared to “young” animals of group 3 (Figure 2). Furthermore, “aged” mice with lung cancer displayed thickening of the alveolar septa and focal edema in the lung tissue.

#### The antitumor effect of rCD8<sup>+</sup> T cells in “aged” mice was inferior to that in “young” mice

Injection of rCD8<sup>+</sup> T cells had a minimal impact on lymphocytic-neutrophilic infiltration in the lungs, but it significantly decreased the quantity of tumor emboli in the vessels, as well as perivascular and peribronchial metastases. In “young” mice of group 7, the tumor size was smaller compared to untreated mice in group 3 (Figure 2 and Table 1).

Following the administration of rCD8<sup>+</sup> T cells, hyperemia of the microvascular vessels and persistent peribronchial lymphocytic-macrophage infiltrates were observed in the lungs of “aged” mice in group 8. Multiple small tumor nodules were found in the lung parenchyma of six out of 10 “aged” mice in group 8. However, the therapy reduced the size of the lung tumor relative to “aged” mice in group 4 (Figure 2 and Table 1). Nevertheless, the inhibitory effect of rCD8<sup>+</sup> T cells on tumor size in “aged” mice of group 8 was inferior to that in “young” mice of group 7.

Furthermore, we compared the effects of rCD8<sup>+</sup> T cells and nCD8<sup>+</sup> T cells in mice from two age groups using an orthotopic LLC model. In “young” mice of group 5, nCD8<sup>+</sup> T cells exhibited an inhibitory effect on tumor emboli, metastases, and tumor size; however, their effect was less pronounced than that of rCD8<sup>+</sup> T cells (Figure 2 and Table 1). In contrast, in “aged” mice of group 6, nCD8<sup>+</sup> T cells significantly increased tumor size and inflammatory infiltration in the lungs compared to groups 4 and 8.

### T-cell cytometry

#### The population of circulating T cells in the blood decreases and their number increases in the lungs with age

In the peripheral blood and lungs of “young” (group 1) and “aged” (group 2) animals, T cells of various phenotypes were studied: CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>-</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, CD95<sup>-</sup>CD62L<sup>-</sup>CD8<sup>-</sup>CCR7<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> (Figure 3). We found that the count of T cells in the blood of “aged” mice was less than that of “young” animals. On the contrary, in the lungs, the count of populations of T cells increased (Figure 4).

#### LLC-induced recruitment of T cells to the lungs is impaired with age

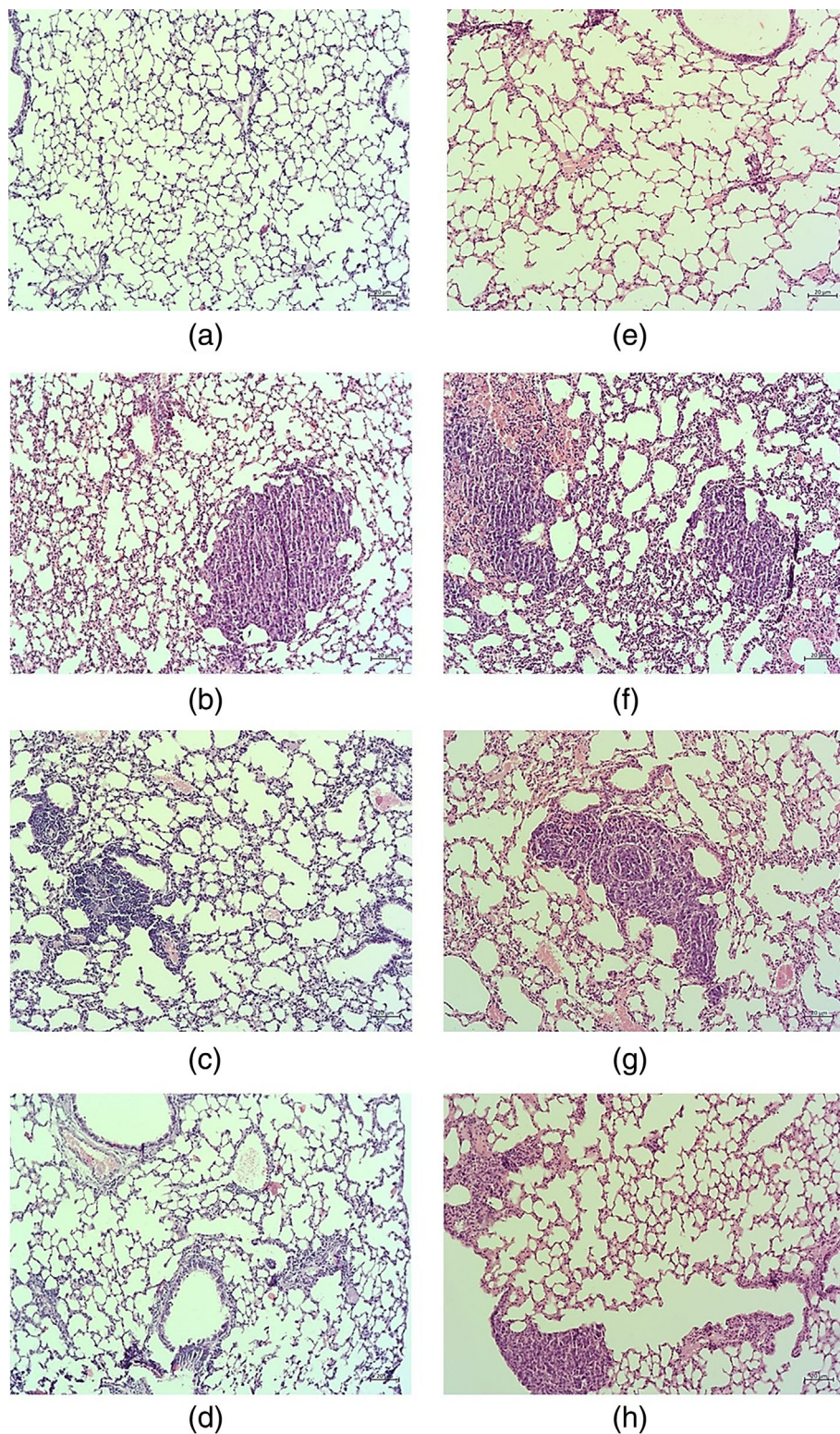
Modeling lung cancer caused an increase in the count of all populations of T cells in the blood of “aged” animals of group 4 relative to “young” animals of group 3 (Figure 5).

At the same time, the quantity of most T-cell populations (CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup>) in the lungs increased (Figure 6). CD8<sup>+</sup>CCR7<sup>-</sup> T cells were the exception as the content of these did not change and the proportion of pro-inflammatory CD3<sup>-</sup>CD4<sup>+</sup> T cells was reduced.

In “aged” mice with lung cancer (group 4), there was a decline in the quantity of several T-cell populations in the blood compared to “aged” mice in group 2 (Figure 5). However, the exception was cells with the immunophenotype CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD95<sup>-</sup>CD62L<sup>-</sup>CD8<sup>-</sup>CCR7<sup>+</sup>, and CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, as their numbers remained unchanged. In the lungs of “aged” mice in group 4, a decrease in CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cells was observed (Figure 6). Conversely, there was an increase in T cells with the CD3<sup>+</sup>CD8<sup>+</sup>CD279<sup>hi</sup> and CD3<sup>-</sup>CD4<sup>+</sup> phenotypes.



**FIGURE 2** Micrographs of lung sections obtained from “young” (a–d) and “aged” (e–h) male C57BL/6 mice: intact control (a, e); with Lewis Lung Carcinoma (LLC) (b, f); with LLC treated with naive CD8<sup>+</sup> T cells (c, g); and with LLC treated with reprogrammed CD8<sup>+</sup> T cells (d, h) on day 7. Tissues were stained with hematoxylin-eosin. Scale bar: 20  $\mu$ m.



However, the numbers of T cells with the CD8<sup>+</sup>CCR7<sup>-</sup> and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> phenotypes did not show any change.

Compared with “young” mice, the content of all studied populations of T cells in the blood of “aged” animals increased significantly.

In lung cancer, rCD8<sup>+</sup> T-cell therapy has a more pronounced stimulatory effect on T cells in “aged” animals than in “young” animals

Despite variations in the initial T-cell content in the blood of “young” and “aged” experimental animals, the response

**TABLE 1** Effect of rCD8<sup>+</sup> T-cell therapy on body and lung weight, and tumor volume in “young” and “aged” C57BL/6 male mice in LLC (orthotopic model), day 7 ( $M \pm m$ ).

Group/characteristic	Body weight (g)	Lung weight (mg)	Tumor volume (mm <sup>3</sup> )
<b>“Young” mice</b>			
Group 1 (intact control)	27.97 $\pm$ 0.32	198.05 $\pm$ 15.21	0
Group 3 (LLC)	21.02 $\pm$ 0.77 <sup>a</sup>	174.33 $\pm$ 12.96	4.62 $\pm$ 3.85 <sup>a</sup>
Group 5 (LLC + nCD8 <sup>+</sup> T cells)	21.43 $\pm$ 0.45 <sup>a</sup>	183.33 $\pm$ 10.27	2.53 $\pm$ 0.12 <sup>a</sup>
Group 7 (LLC+ rCD8 <sup>+</sup> T cells)	21.50 $\pm$ 0.37 <sup>a</sup>	194.00 $\pm$ 8.66 <sup>a</sup>	1.95 $\pm$ 2.33 <sup>a,c,d</sup>
<b>“Aged” mice</b>			
Group 2 (intact control)	40.58 $\pm$ 1.31 <sup>b</sup>	117.6 $\pm$ 7.93 <sup>b</sup>	0
Group 4 (LLC)	37.15 $\pm$ 0.77 <sup>a</sup>	144.6 $\pm$ 9.93 <sup>a</sup>	10.54 $\pm$ 2.21 <sup>a</sup>
Group 6 (LLC + nCD8 <sup>+</sup> T cells)	32.8 $\pm$ 1.04 <sup>a</sup>	278.67 $\pm$ 20.67 <sup>a,c</sup>	42.22 $\pm$ 8.34 <sup>a,c</sup>
Group 8 (LLC+ rCD8 <sup>+</sup> T cells)	34.1 $\pm$ 0.97 <sup>a</sup>	210.67 $\pm$ 5.17 <sup>a,c</sup>	4.19 $\pm$ 1.21 <sup>a,c,d</sup>

Abbreviations: LLC, Lewis lung carcinoma; nCD8<sup>+</sup> T cells, naive CD8-positive T cells; rCD8<sup>+</sup> T cells, reprogrammed CD8-positive T cells.

<sup>a</sup>For comparison with the intact mice by Mann–Whitney *U* test ( $p < 0.05$ ).

<sup>b</sup>For comparison with the “young” mice by Mann–Whitney *U* test ( $p < 0.05$ ).

<sup>c</sup>For comparison with the mice with LLC by Mann–Whitney *U* test ( $p < 0.05$ ).

<sup>d</sup>For comparison with the mice with LLC treated with naive CD8<sup>+</sup> T cells by Mann–Whitney *U* test ( $p < 0.05$ ).

to the introduction of rCD8<sup>+</sup> T cells was similar across different age groups of animals. Following cell therapy, the majority of T-cell populations in the blood increased, with the exceptions being CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup> T cells in “young” mice of group 7 and CD95<sup>+</sup>CD62L<sup>+</sup>CD8<sup>+</sup>CCR7<sup>+</sup> cells in “aged” mice of group 8, which showed a decrease in numbers compared to untreated mice (Figure 7).

In the lungs, the results differed from those observed in the blood. The content of various T-cell populations in the “young” mice lungs in group 7 decreased in comparison to animals in group 3. Conversely, in the lung tissue of “aged” mice in group 8, the T-cell content was higher than in animals of group 4. The exception to this pattern was the decrease in CD3<sup>+</sup>CD8<sup>+</sup>CD279<sup>hi</sup> cells in “aged” mice following rCD8<sup>+</sup> T-cell therapy (Figure 8). When comparing the results between “aged” and “young” animals, it was found that the stimulatory effect of rCD8<sup>+</sup> T cells in “aged” mice of group 8 was superior to that in “young” mice of group 7 (Figures 7 and 8).

Furthermore, a comparison was made between the effects of rCD8<sup>+</sup> T cells and nCD8<sup>+</sup> T cells. In contrast to rCD8<sup>+</sup> T cells, injections of nCD8<sup>+</sup> T cells had no significant influence on the majority of T-cell populations in young mice of group 5 in comparison to group 3 (Figures 7 and 8). The exceptions were an increase in CD3<sup>+</sup>CD8<sup>+</sup>CD279<sup>hi</sup> and CD8<sup>+</sup>CCR7<sup>+</sup> cells circulating in the blood, as well as tissue CD3<sup>+</sup>CD4<sup>+</sup> cells. In “aged” mice of group 6, when nCD8<sup>+</sup> T cells were administered, similar changes were observed compared to the administration of rCD8<sup>+</sup> T cells in “aged” mice of group 8.

## Cytometric examination of cancer cells

The levels of CTCs and CSCs expressing Axl, EGF, PD-L1, and PD-1 increase with age

So far, specific markers or combinations of markers for CSCs have not been definitively distinguished. Additionally,

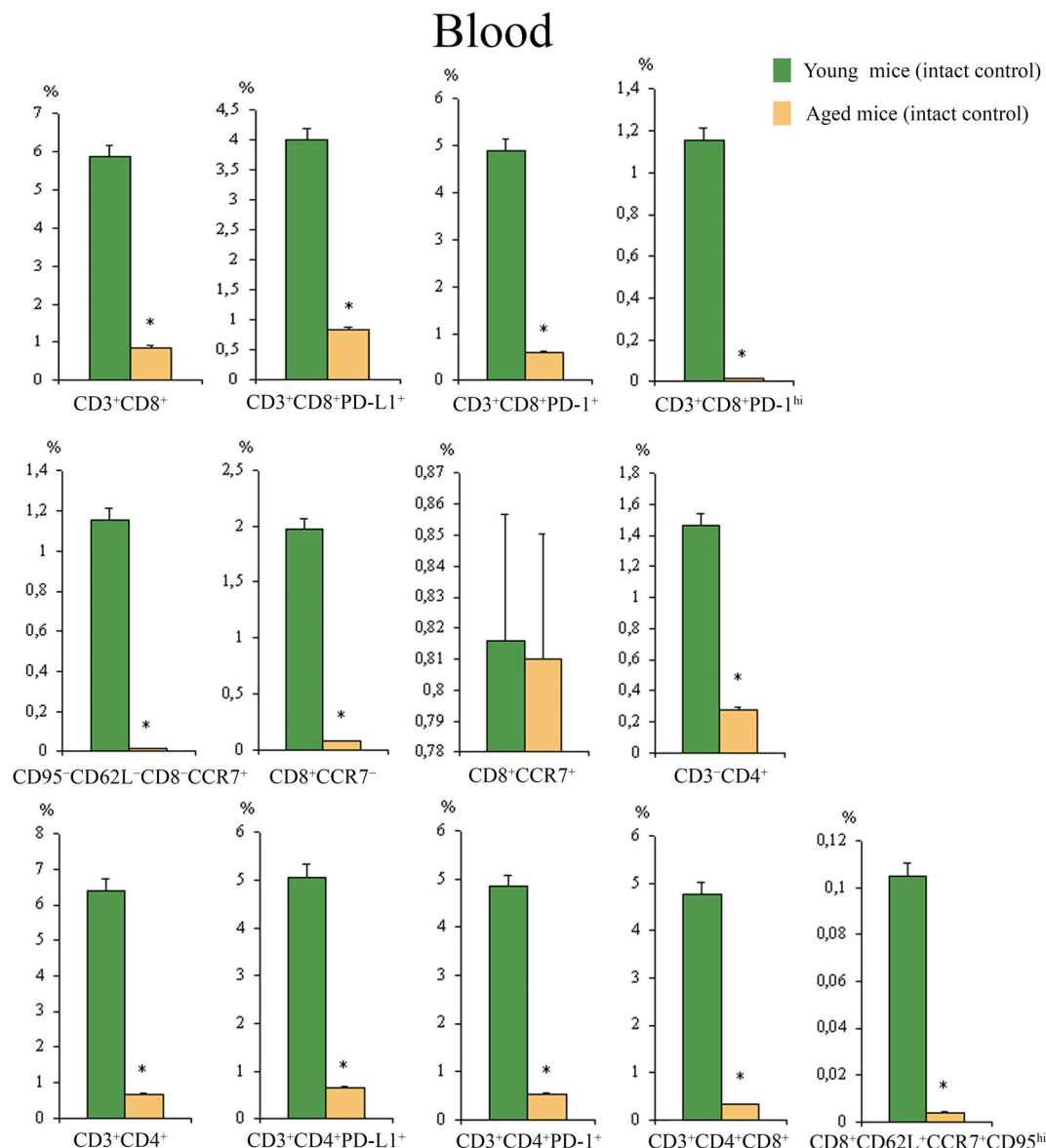
some of these markers are also expressed on mononuclear cells of healthy animals.<sup>22</sup> It is possible that the expression of these markers indicates the presence of somatic cells with potential cancerous transformation or inactive CSCs. The content of CSCs and the level of expression of oncogenic markers may undergo changes with age, which could potentially reflect the risks of tumor growth. In this study, we examined age-related variations in the content of cells expressing CSC markers, such as CD90, CD117, PD-L1, PD-1, CD276, Axl, EGF, Sox2, and Ki67. Histological examination did not reveal any tumor nodes or lung metastases in the lungs of “aged” and “young” intact animals (groups 2 and 1, respectively) (Figure 2 and Table 1). However, cytometric analysis of the blood in “aged” mice showed an increased count of cancer cells with the phenotypes EGF<sup>+</sup>Sox2<sup>+</sup>, PD-L1<sup>+</sup>, PD-1<sup>+</sup>PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup> compared to “young” animals. Additionally, the number of cells exhibiting the phenotypes Axl<sup>+</sup>CD90<sup>+</sup>, CD45<sup>+</sup>Axl<sup>+</sup>, and EGF<sup>+</sup>CD117<sup>+</sup>, as well as those with the phenotypes CD276<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, and Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup> increased in “aged” mice (Figure 9).

In the lungs of “aged” mice, the quantity of cells with the phenotypes Axl<sup>+</sup>CD90<sup>+</sup>, PD-1<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD117<sup>+</sup>EGF<sup>+</sup>, and PD-1<sup>+</sup>PD-L1<sup>+</sup> was reduced, whereas the number of CD45<sup>+</sup>Axl<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, and CD117<sup>+</sup>Sox2<sup>+</sup> cells increased in comparison to “young” mice (Figure 10).

The development of lung cancer in “aged” and “young” mice is determined by different populations of CSCs and CTCs

An elevated count of CSCs expressing the phenotypes EGF<sup>+</sup>Sox2<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>PD-L<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup> was observed in





**FIGURE 3** Number of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD95<sup>-</sup>CD62L<sup>-</sup>CD8<sup>-</sup>CCR7<sup>+</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>-</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> T cells in the blood of intact “young” (group 1) and “aged” (group 2) mice of the C57BL/6 line (% of the total number of labeled mononuclear cells). \*In comparison with the intact “young” mice (group 1) by Mann–Whitney test ( $p < 0.05$ ).

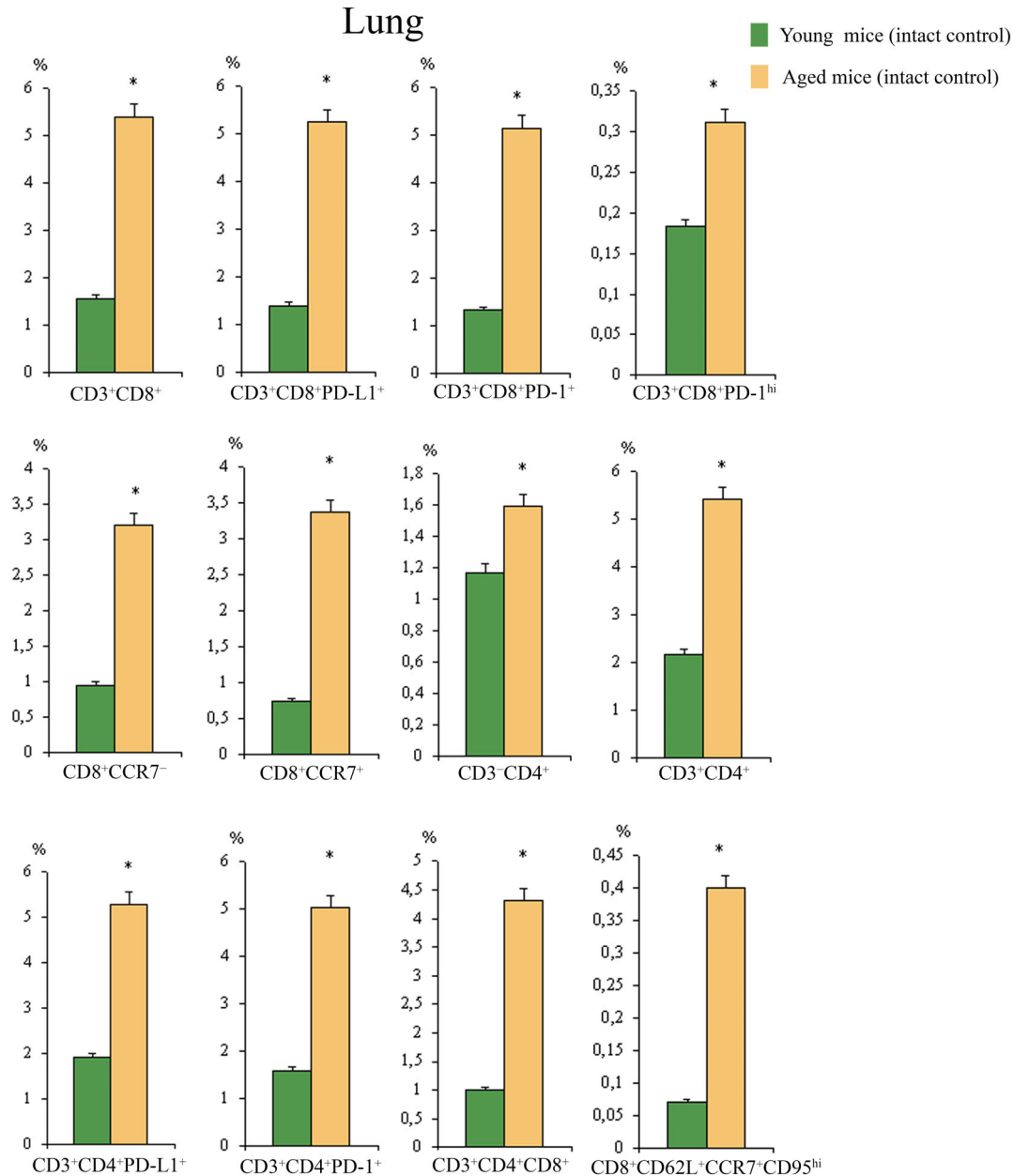
the blood of “young” mice from group 3 (LLC) compared to group 4 (Figure 11). At the same time, an increase in CD45<sup>-</sup>Axl<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, and PD-1<sup>-</sup>PD-L<sup>+</sup> cancer cells was detected in the lungs. The most pronounced changes were observed in CD45<sup>-</sup>Axl<sup>+</sup> cancer cells (Figure 12).

A similar change was detected in the blood of “aged” animals with LLC. The number of CSCs with the phenotypes Axl<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, and CD90<sup>+</sup>Sox2<sup>+</sup> increased compared to the control group. However, the content of Axl<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, and CD90<sup>+</sup>Sox2<sup>+</sup> cells in the blood of “aged” mice was higher relative to “young” animals, whereas the number of PD-L1<sup>+</sup>Ki67<sup>+</sup> cells was lower (Figure 11). Additionally, in “aged” mice of group 4 (LLC), an elevation in the level of CD117<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>,

CD276<sup>+</sup>Sox2<sup>+</sup>, and Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup> cells was observed (Figure 12).

In lung cancer, the inhibitory effect of rCD8<sup>+</sup> T cells in “young” mice affects the vast majority of populations of CTCs and CSCs, in “aged” mice—cancer cells expressing markers Axl, CD90 in combination with Sox2

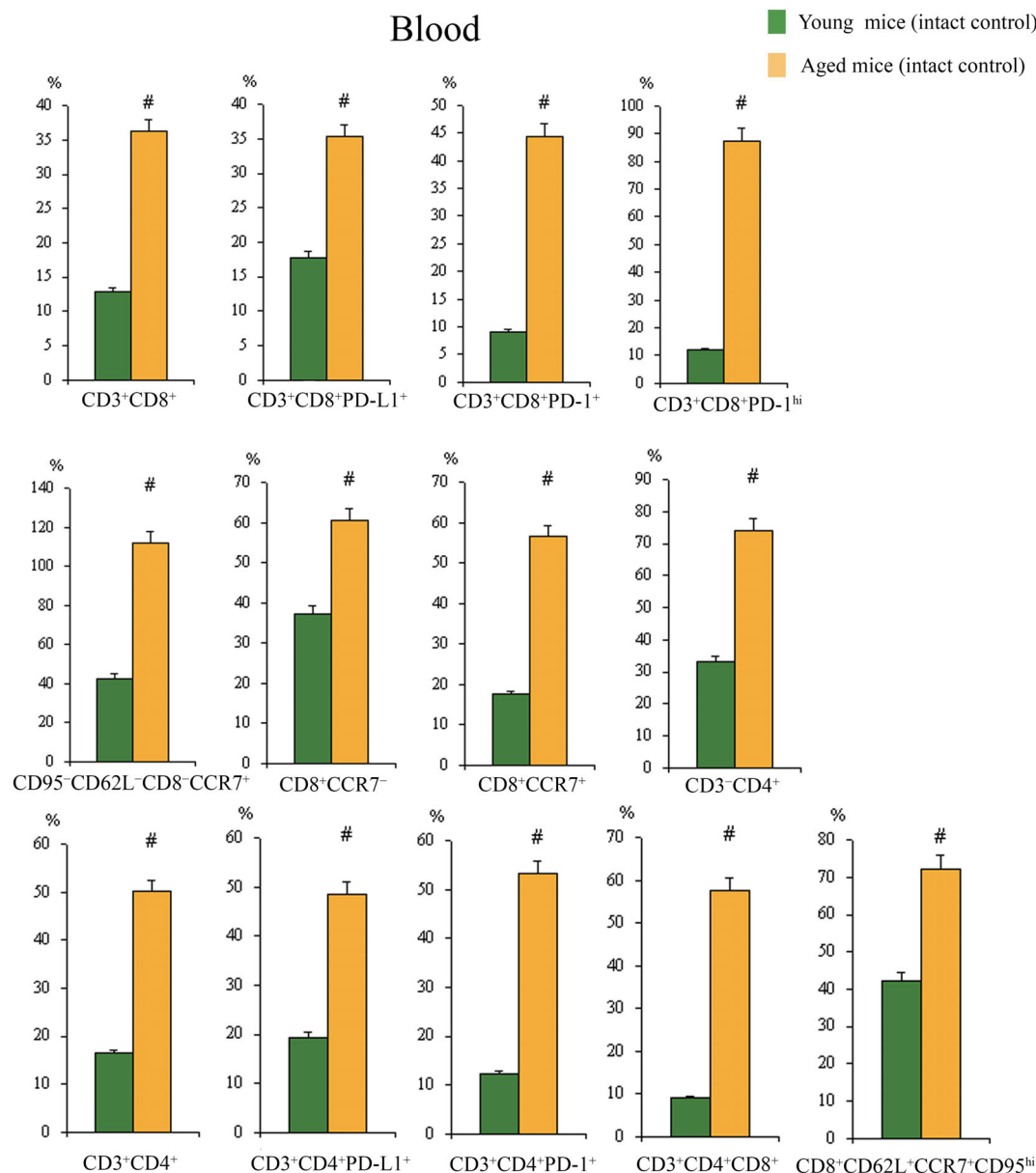
The injection of rCD8<sup>+</sup> T cells resulted in a decrease in the count of all studied populations of CSCs and CTCs in the lungs and blood of “young” animals in group 7 in comparison to group 3 (Figure 13). However, there was a rise of



**FIGURE 4** Number of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>-</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> T cells in the lungs of intact “young” (group 1) and “aged” (group 2) mice of the C57BL/6 line (% of the total number of labeled mononuclear cells). \*In comparison with the intact “young” mice (group 1) by Mann-Whitney test ( $p < 0.05$ ).

the quantity of circulating blood cells expressing the CD45<sup>-</sup>Axl<sup>+</sup> and CD276<sup>+</sup>Sox2<sup>+</sup> phenotypes. Furthermore, we examined the influence of naive CD8<sup>+</sup> T cells in young mice with LLC (group 5) in comparison with the corresponding control group (group 3). Following the injection of nCD8<sup>+</sup> T cells a slight reduction in the population of CTCs and CSCs expressing CD117 and EGF alone or in combination with each other, or in combination with Sox2, was observed in the blood and lungs of “young” animals in group 5 (Figure 14). However, there was an elevation in the number of cancer cells expressing Axl, CD90, PD-L1, PD-1,

CD276, and Ki67. In contrast to “young” mice in group 7, the injection of rCD8<sup>+</sup> T cells into “aged” mice in group 8 reduced the number of some populations of CSCs and CTCs only compared to “aged” mice in group 4. In the blood, this reduction was observed in Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, and PD-1-PD-L1<sup>+</sup> cells, while in the lungs, it was observed in PD-1-PD-L1<sup>+</sup> cells (Figures 13 and 14). However, the number of many CSCs and CTCs increased, including CD45<sup>-</sup>Axl<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, and CD117<sup>+</sup>EGF<sup>+</sup> cells in the blood, as well as CD45<sup>-</sup>Axl<sup>+</sup>,



**FIGURE 5** Number of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD95<sup>-</sup>CD62L<sup>-</sup>CD8<sup>-</sup>CCR7<sup>+</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>-</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> T cells in the blood of “young” (group 3) and “aged” (group 4) C57BL/6 mice with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to intact mice.

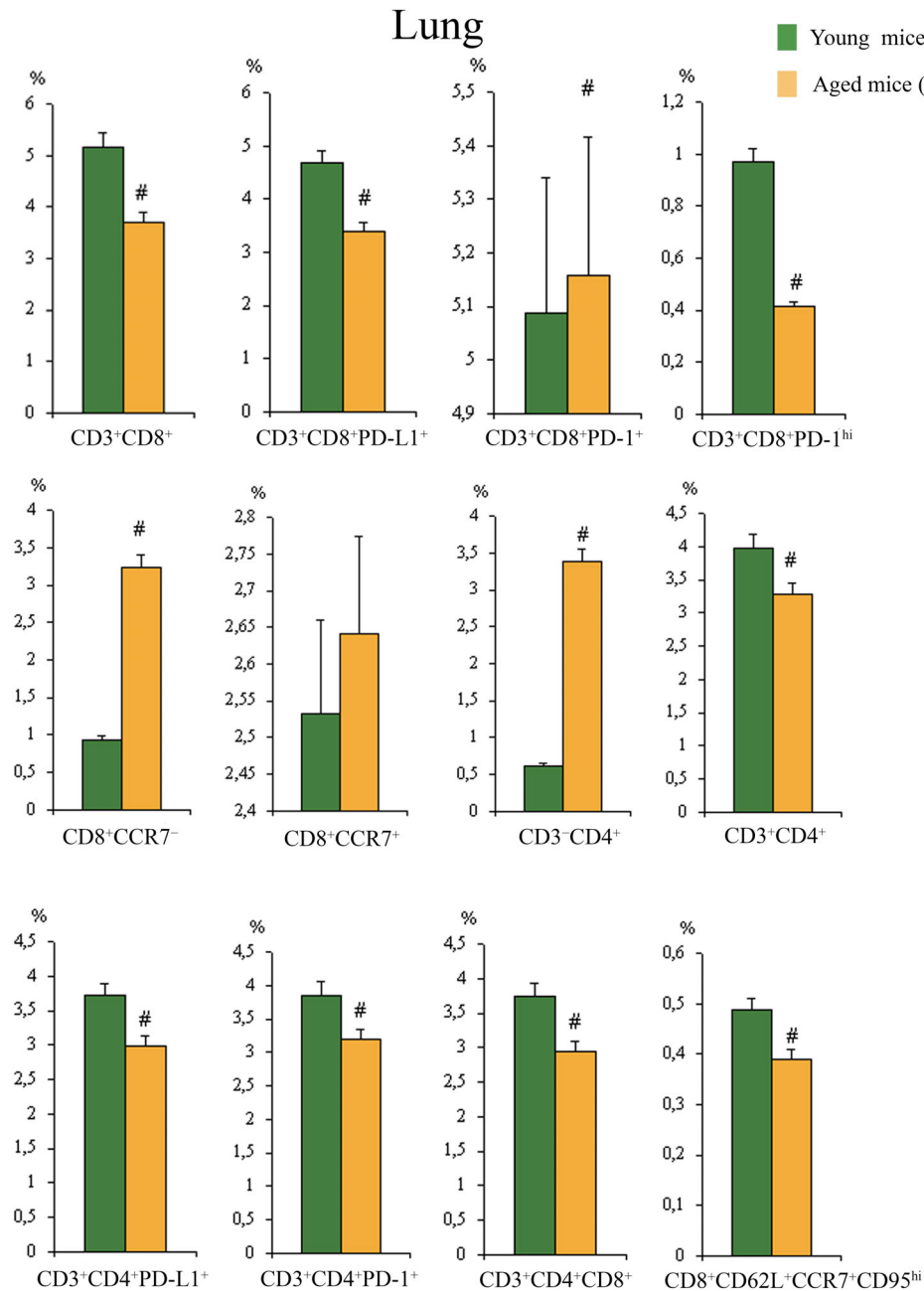
#For comparison with the “young” mice with LLC (group 3) by Mann-Whitney test ( $p < 0.05$ ).

PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>PD-L1<sup>+</sup>, and CD117<sup>+</sup>EGF<sup>+</sup> cells in the lungs.

After injections of nCD8<sup>+</sup> T cells, the distribution pattern of CSCs and CTCs in the lungs and blood of “aged” mice with lung carcinoma was comparable to that after the injection of rCD8<sup>+</sup> T cells. However, naive cells had a lesser inhibitory effect on Axl<sup>+</sup>CD90<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, and CD90<sup>+</sup>Sox2<sup>+</sup> blood cells compared to reprogrammed cells (Figure 13). In the lungs, nCD8<sup>+</sup> T cells decreased the number of CD45<sup>-</sup>Axl<sup>+</sup> and CD276<sup>+</sup>Sox2<sup>+</sup> cells, whereas this effect was not noticed with rCD8<sup>+</sup> T cells (Figure 14). Additionally, the main stimulatory effect of nCD8<sup>+</sup> T cells was associated with cells expressing PD-1 and PD-L1 markers.

## DISCUSSION

Despite the significant contribution of cancer diseases to morbidity and mortality rates, there is a lack of studies examining the influence of age on malignant neoplasm development and the antitumor immune response. Understanding the mechanisms of aging and its impact on carcinogenesis is critical for enhancing current methods and developing new effective and safe approaches for cancer treatment in elderly patients. This study aimed to investigate age-related differences in the quantity of oncogenic cells (CSCs and CTCs) and T cells involved in the antitumor immune response using an orthotopic lung cancer model.



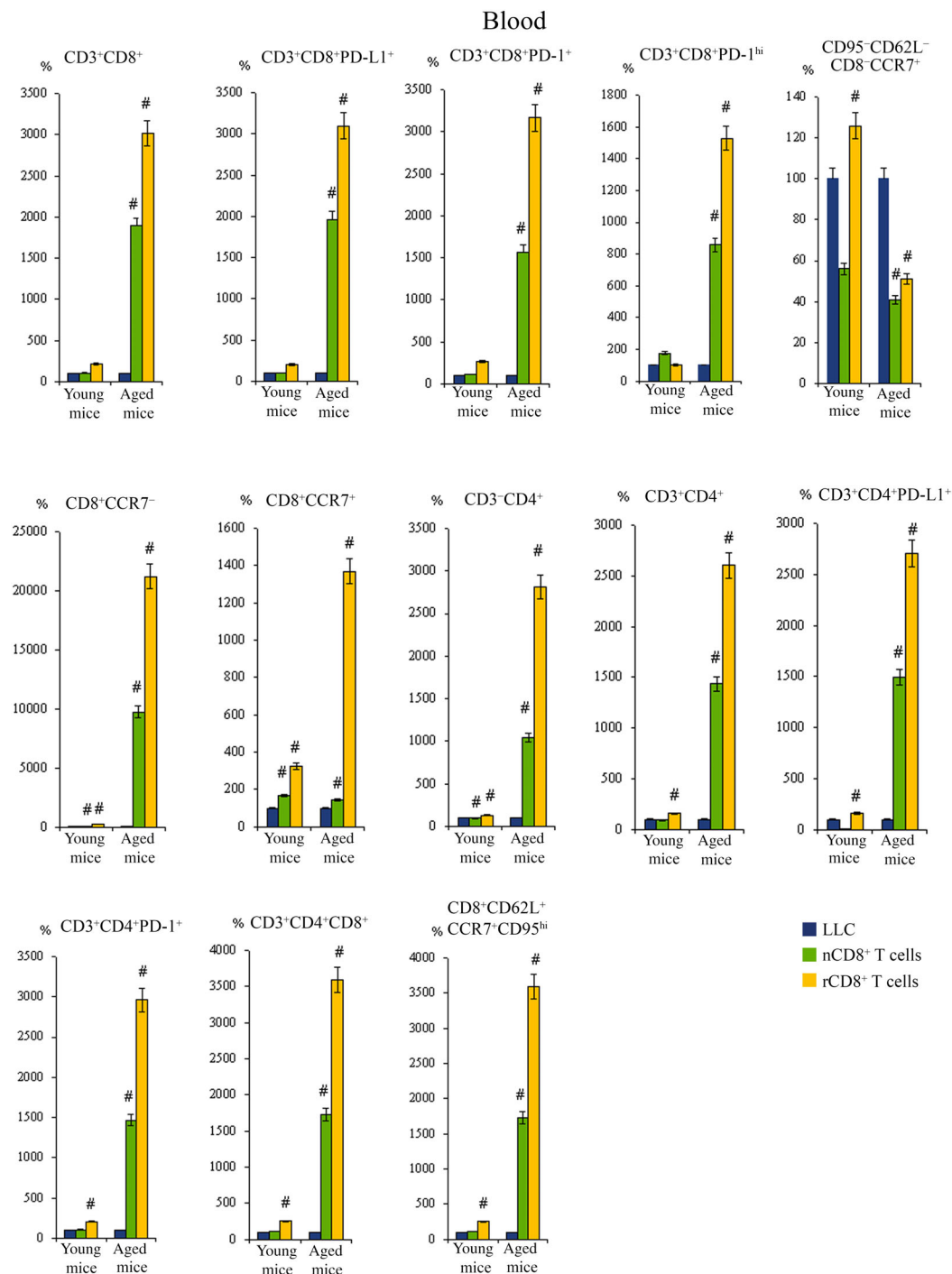
**FIGURE 6** Number of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>-</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> T cells in the lungs of “young” (group 3) and “aged” (group 4) C57BL/6 mice with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to intact mice. #For comparison with the “young” mice with LLC (group 3) by Mann-Whitney test ( $p < 0.05$ ).

Additionally, the influence of rCD8<sup>+</sup> T-cell therapy was investigated in “aged” mice in comparison to “young” mice.

In the initial stage of this study, we examined age-related differences in lung tissue and the content of various T-cell populations. Histological analysis revealed no significant differences in lung structure between “young” mice (group 1) and “aged” mice (group 2) (Figures 1 and 2). However, histological slides of group 2 mice exhibited small lymphocytic-neutrophil infiltrates in the pulmonary parenchyma, as well as perivascular and peribronchial accumulation of inflammatory cells.

The cytometric evaluation of CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells considered the heterogeneity within each population. Our findings demonstrated that the quantity of CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells, along with their constituent T-cell subpopulations (CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>), was higher in the lungs of “aged” animals in group 2 in comparison to “young” animals in group 1. Conversely, the opposite pattern was observed in the



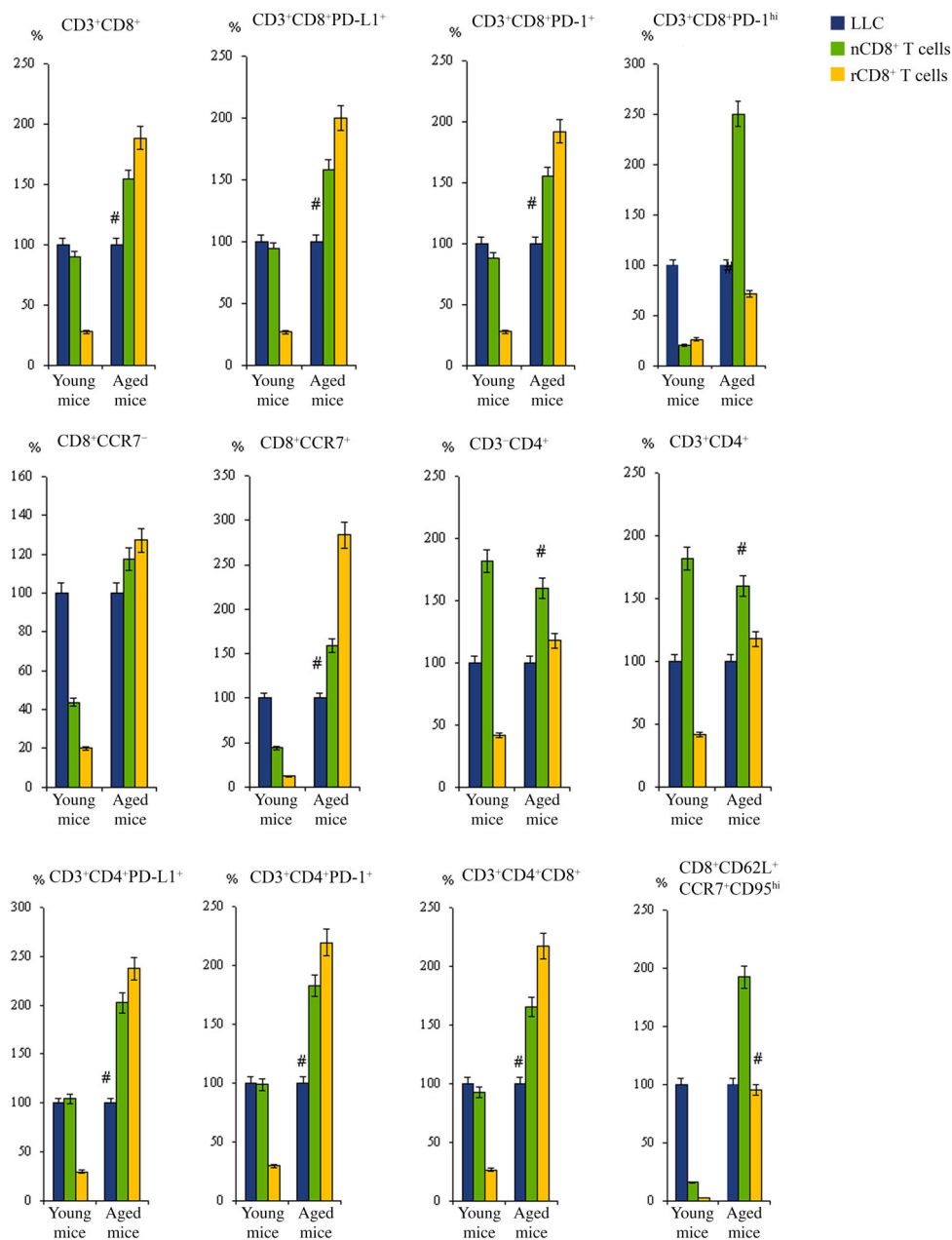


**FIGURE 7** The effect of nCD8<sup>+</sup> T cells and rCD8<sup>+</sup> T cells cell therapy on the number of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD95<sup>-</sup>CD62L<sup>-</sup>CD8<sup>-</sup>CCR7<sup>+</sup>, CD8<sup>+</sup>CCR7<sup>-</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> T cells in the blood of “young” (groups 5 and 7) and “aged” (groups 6 and 8) mice of the C57BL/6 line with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to mice with LLC. #For comparison with “young” (group 3) and “aged” (group 4) mice with LLC, respectively, according to the Mann–Whitney test ( $p < 0.05$ ).

blood (Figures 3 and 4). When examining the expression of various oncoantigens, we conducted assays on distinct populations of CSCs and CTCs in the lungs and blood of mice in groups 1 and 2 (Figures 9 and 10). The PD-1 receptor and its PD-L1 ligand are parts of the protein system that regulate immune responses.<sup>23</sup> Increased expression of these

molecules by cancer cells serves as one of the mechanisms through which tumors avoid detection by the immune system.<sup>24</sup> EGF, CD274, and Ki67 play pro-oncogenic roles and their involvement in cancer resistance to chemotherapy has been established.<sup>25,26</sup> In the lungs and blood of “aged” mice, the number of cancer cells expressing PD-L1 and PD-1 was

## Lung

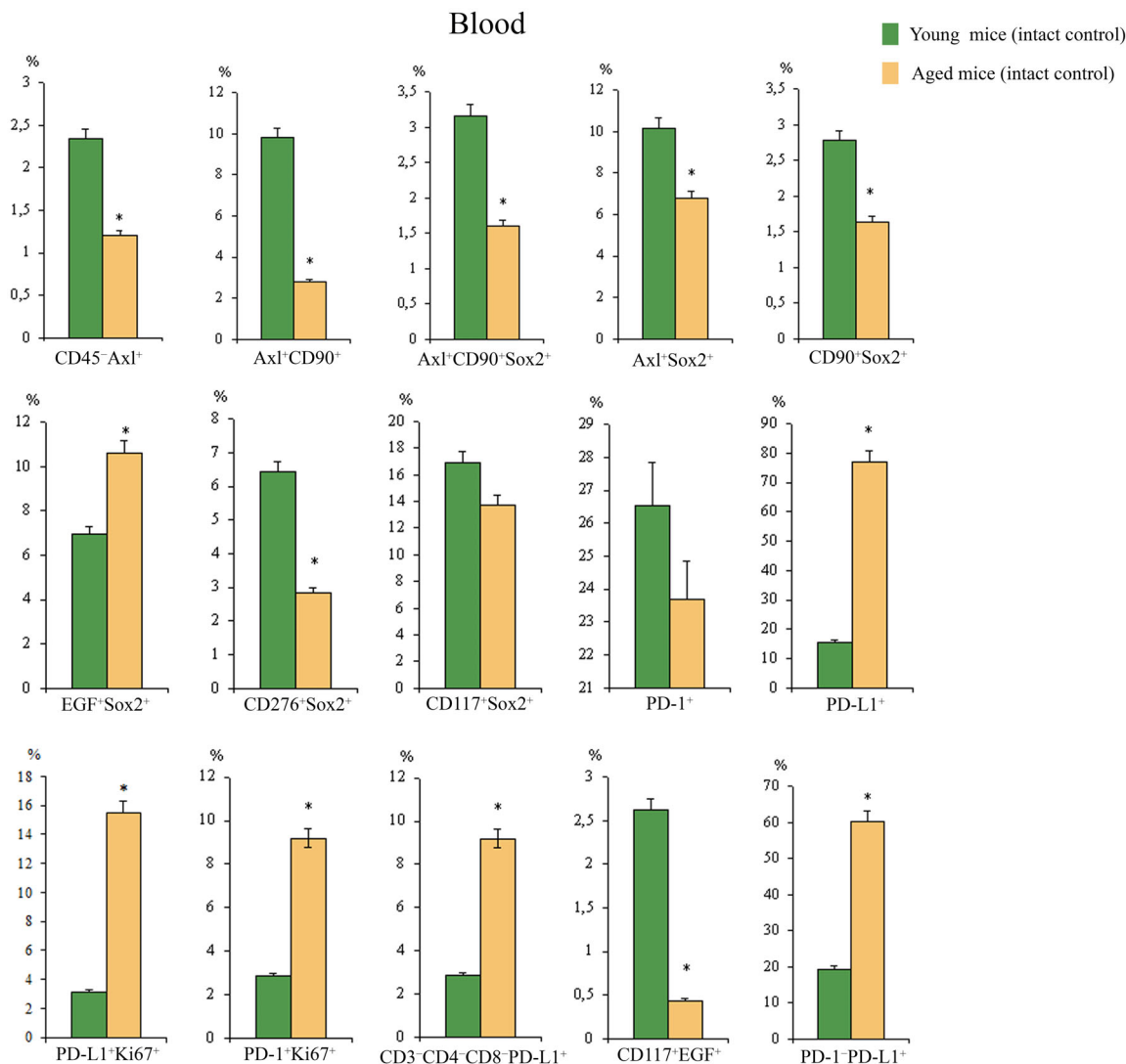


**FIGURE 8** The effect of nCD8<sup>+</sup> T cells and rCD8<sup>+</sup> T cells cell therapy on the number of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup>, CD95<sup>+</sup>CD62L<sup>+</sup>CD8<sup>+</sup>CCR7<sup>+</sup>, CD8<sup>+</sup>CCR7<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup>CCR7<sup>+</sup>CD95<sup>hi</sup> T cells in the lungs of “young” (groups 5 and 7) and “aged” (groups 6 and 8) mice of the C57BL/6 line with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to mice with LLC. #, for comparison with “young” (group 3) and “aged” (group 4) mice with LLC, respectively, according to the Mann-Whitney test ( $p < 0.05$ ).

higher compared to “young” mice. Additionally, an increase in the quantity of cells expressing EGF, Ki67, and PD-L1 markers was noticed in the blood of “aged” mice. This increase in cells expressing these markers may indicate the development of metastatic disease in “aged” mice. Moreover, age-related inflammatory remodeling of lung tissue contributes to an increased risk of tumor progression by creating an inflammatory microenvironment favorable for the induction of carcinogenesis (Figures 1, 3, and 4).

Cellular immunity mediated by CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells plays a critical role in protecting the body against tumors.<sup>12</sup> The observed increase in the quantity of various T-cell populations in the lung tissue with age suggests age-linked remodeling of the immune system

aimed at reducing the risk of lung cancer induction. It is known that the aging immune system has a higher proportion of highly differentiated CD8 and CD4 T cells compared to younger ages. At the same time, there is a decline in the reservoir of naïve T cells, accompanied by an exponential reduction in thymic activity due to aging.<sup>27</sup> It can therefore be anticipated that the older immune system will maintain its efficiency at the basal level or may even be more efficient, but it will significantly lose its efficiency post-stimulation with oncoantigens due to the decreased reserve of undifferentiated T cells. Thus, when performing oncological diagnostics in older patients, it is important to consider the expression levels of oncoantigens such as Axl, EGF, PD-1, and PD-L1. The early diagnosis and treatment of lung



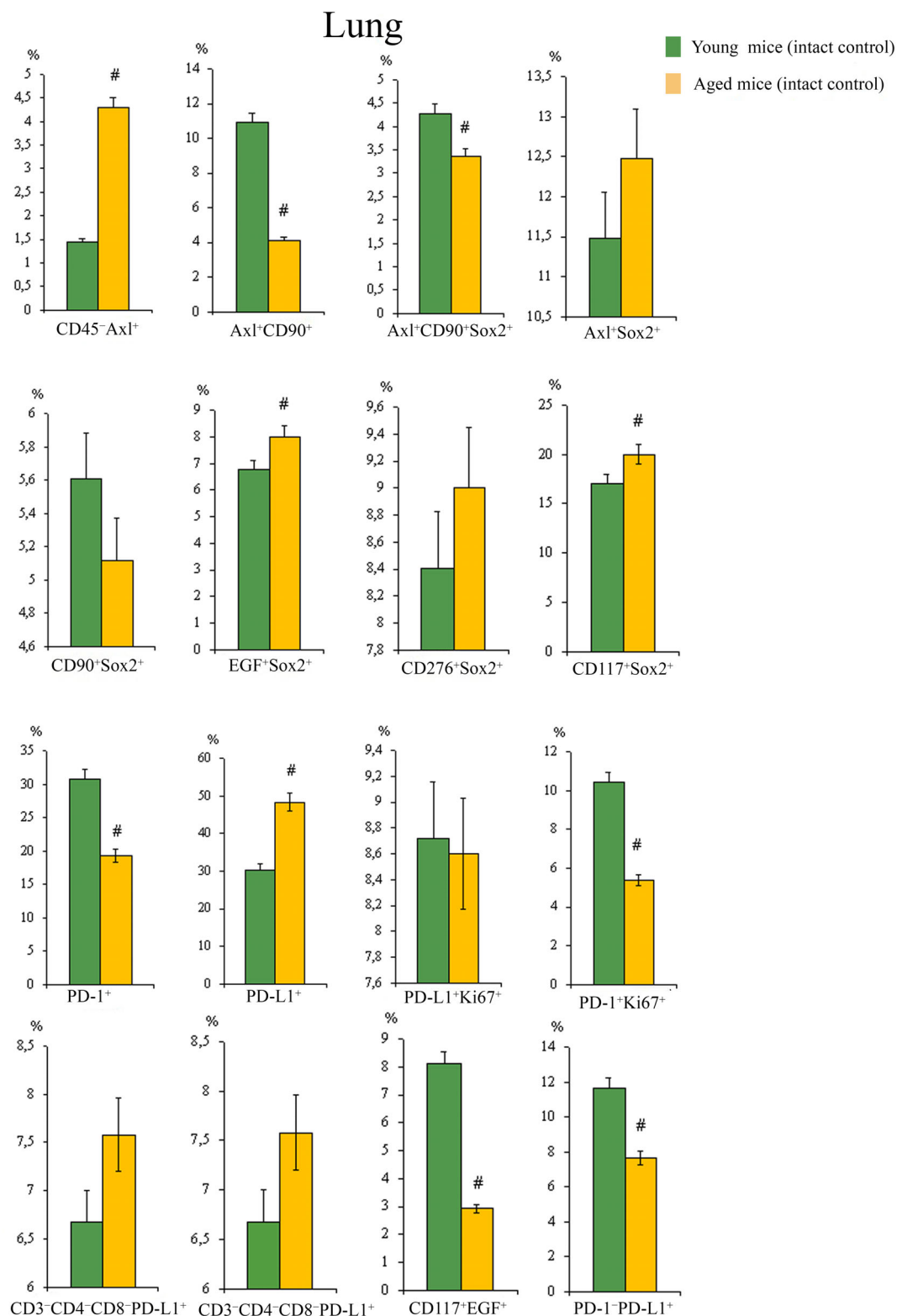
**FIGURE 9** Number of CD45<sup>+</sup>Axl<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD117<sup>+</sup>EGF<sup>+</sup>, and PD-1<sup>+</sup>PD-L1<sup>+</sup> cancer cells in the blood of intact “young” (group 1) and “aged” (group 2) mice of the C57BL/6 line (% of the total number mononuclear cells). \*For comparison with intact “young” mice (group 1) according to the Mann-Whitney test ( $p < 0.05$ ).

cancer and metastatic disease in these patients could be improved by evaluating CSCs and CTCs, along with the simultaneous examination of CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells to detect any aberrant responses of undifferentiated and differentiated cells.

Age-linked changes in the content and ratio of immune cells and cancer cells contribute to the specific characteristics of LLC-induced lung cancer in mice aged 80–82 weeks. Therefore, the severity of histopathological changes in lung tissue and the size of the tumor were greater in “aged” animals of group 4 in comparison to “young” animals of group 3 (Figure 2). These results closely align with findings from prior studies performed with “aged” mice of the FVB and C57BL/6 lines, which demonstrated more active tumor growth and metastasis in the prostate compared to young individuals.<sup>28,29</sup> In contrast to our studies, Bianchi-Frias

et al. and Boulefour<sup>28,29</sup> modeled orthotopic lung cancer with TRAMP-C2 cells.

Due to the prominent role of CTCs in tumor development and metastasis,<sup>30</sup> we deemed it necessary to compare the levels of these cells in “young” (group 3) and “aged” (group 4) mice during the modeling of orthotopic lung cancer. In the lung tissue of “aged” mice with lung cancer, we observed an elevated number of cells expressing Axl<sup>+</sup>CD90<sup>+</sup>, PD-1<sup>+</sup>, PD-1<sup>+</sup>PD-L1<sup>+</sup>, and PD-1<sup>+</sup>Ki67<sup>+</sup> compared to “young” animals (Figure 12). Our findings regarding the accumulation of tumor cells expressing PD-L1 align with existing literature, which reports the age-related accumulation of PD-L1<sup>+</sup> cells that are not immune cells.<sup>10</sup> A decrease in the number of CD3<sup>+</sup>CD4<sup>+</sup>PD-1<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup>PD-L1<sup>+</sup> T cells, as well as CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup> T cells in the lungs and blood of

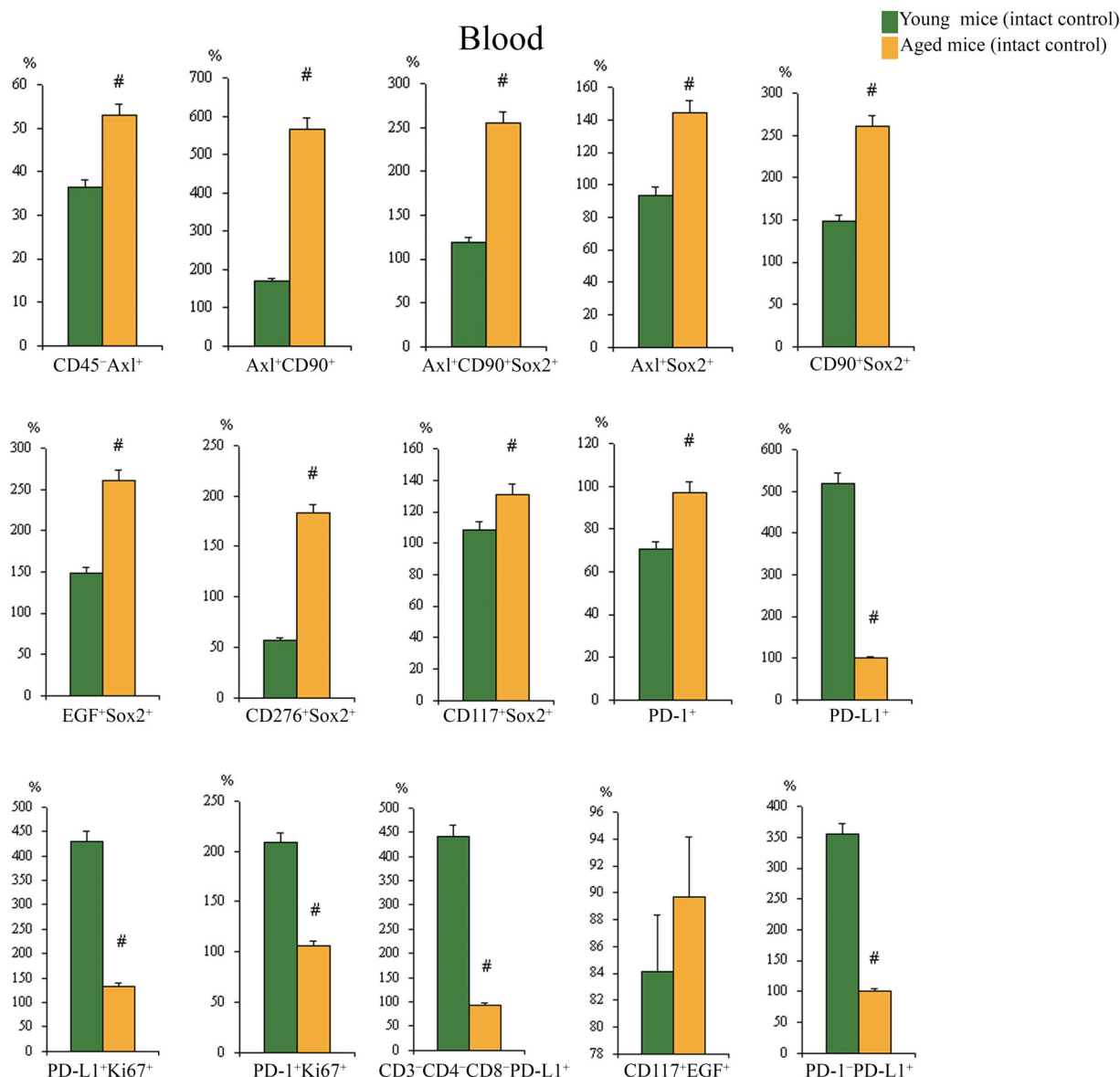


**FIGURE 10** Number of CD45<sup>+</sup>Axl<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>PD-L1<sup>+</sup>, CD117<sup>+</sup>EGF<sup>+</sup>, and PD-1<sup>-</sup>PD-L1<sup>+</sup> cancer cells in the lungs of intact “young” (group 1) and “aged” (group 2) mice of the C57BL/6 line (% of the total number mononuclear cells). \*, for comparison with intact “young” mice (group 1) according to the Mann-Whitney test ( $p < 0.05$ ).

“aged” animals with LLC suggests the potential significance of the PD-1/PD-L1 signaling pathway in cancer progression (Figures 5 and 6).

Of particular note is the increase in CD3<sup>-</sup>CD4<sup>+</sup> T cells in the lung tissue of “aged” mice with lung cancer in comparison to the intact control (Figure 6). CD3<sup>-</sup>CD4<sup>+</sup> T cells





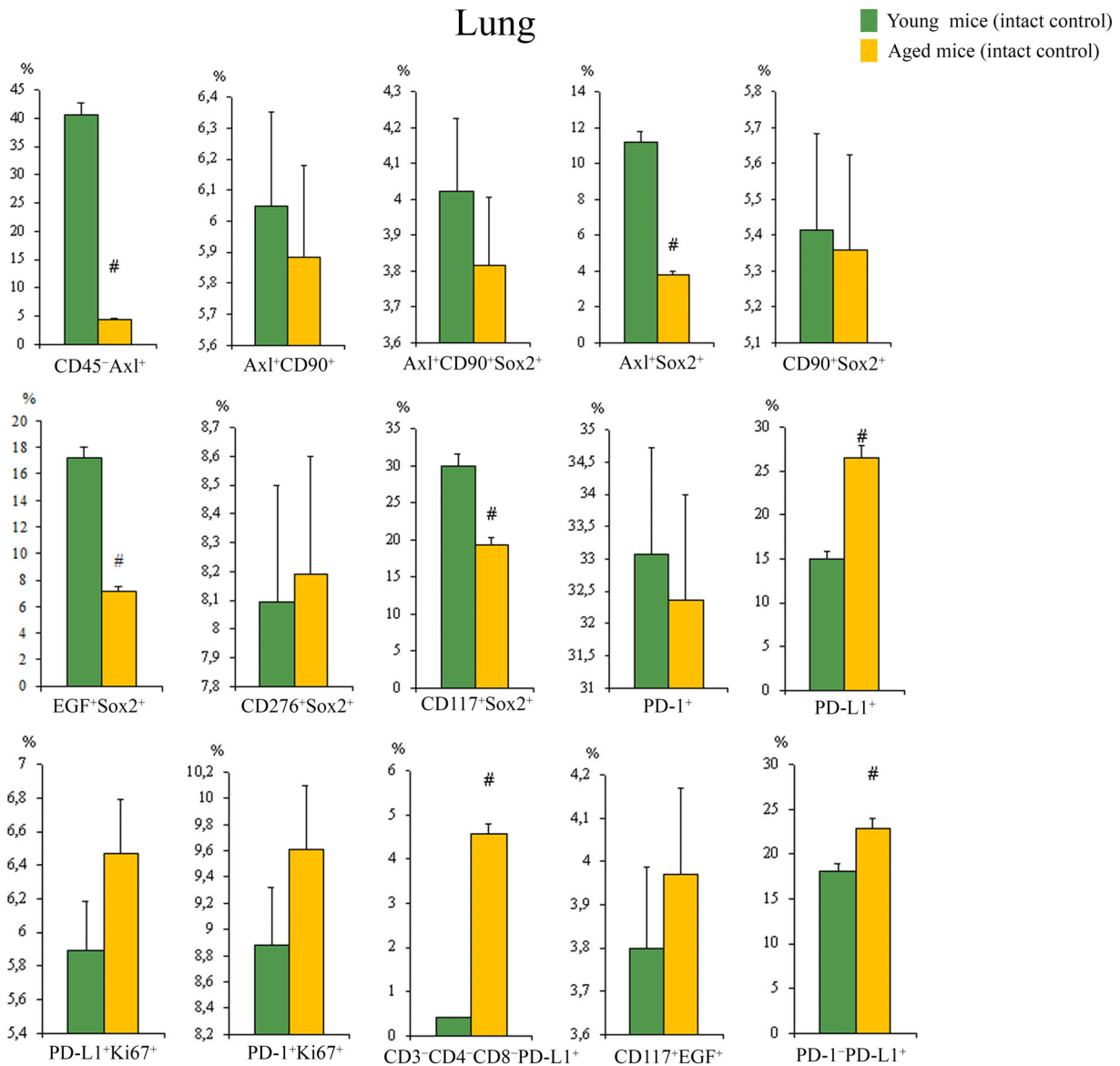
**FIGURE 11** Number of  $CD45^{-}Axl^{+}$ ,  $Axl^{+}CD90^{+}$ ,  $Axl^{+}CD90^{+}Sox2^{+}$ ,  $Axl^{+}Sox2^{+}$ ,  $CD90^{+}Sox2^{+}$ ,  $EGF^{+}Sox2^{+}$ ,  $CD276^{+}Sox2^{+}$ ,  $CD117^{+}Sox2^{+}$ ,  $PD-1^{+}$ ,  $PD-L1^{+}$ ,  $PD-L1^{+}Ki67^{+}$ ,  $PD-1^{+}Ki67^{+}$ ,  $CD3^{-}CD4^{-}CD8^{-}PD-L1^{+}$ ,  $CD117^{+}EGF^{+}$ , and  $PD-1^{-}PD-L1^{+}$  cancer cells in the blood of “young” (group 3) and “aged” (group 4) mice of the C57BL/6 line with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to intact mice. #, for comparison with “young” mice with LLC (group 3) according to the Mann-Whitney test ( $p < 0.05$ ).

are considered pro-inflammatory T-cells. An important characteristic of  $CD3^{-}CD4^{+}$  T cells is their activity, which is influenced by the levels of inflammatory and homeostatic cytokines.<sup>31</sup> It is possible that in “aged” mice, a decrease in the sensitivity of  $CD3^{-}CD4^{+}$  T cells to external stimuli was observed. The rise in  $CD3^{-}CD4^{+}$  T-cell count within this context may serve as a compensatory reaction to their diminished functions.

In contrast to the “aged” mice of group 4, the lungs of “young” mice in group 3 exhibited a notable increase in CSC populations expressing the phenotypes  $CD45^{-}Axl^{+}$ ,  $CD117^{+}Sox2^{+}$ , and  $EGF^{+}Sox2^{+}$ . Conversely, the lungs of “young” mice exhibited a decrease in CSCs expressing the phenotypes  $Axl^{+}CD90^{+}$ ,  $PD-L1^{+}$ ,  $PD-L1^{+}Ki67^{+}$ ,

$CD3^{-}CD4^{-}CD8^{-}PD-L1^{+}$ , and  $CD117^{+}EGF^{+}$ . Hence, the distinct characteristics of lung cancer development in “young” mice may be attributed to a different ratio of CSCs compared to “aged” mice.

In response to tumor formation, we observed the recruitment of  $CD3^{+}CD8^{+}$  and  $CD3^{+}CD4^{+}$  T cells from the blood into the lungs. Naïve T cells ( $CD8^{+}CD197^{+}$ ,  $CD8^{+}CD62L^{+}CD197^{+}CD95^{hi}$ ), cells expressing PD-1 and PD-L1 ( $CD3^{+}CD8^{+}PD-L1^{+}$ ,  $CD3^{+}CD8^{+}PD-1^{+}$ ,  $CD3^{+}CD4^{+}PD-1^{+}$ ,  $CD3^{+}CD4^{+}PD-L1^{+}$ ,  $CD3^{+}CD8^{+}PD-1^{hi}$ ), and  $CD8^{+}CD62L^{+}CCR7^{+}CD95^{hi}$  T cells played a significant role in “young” animals of group 3. The quantity of these cells in the lungs of “young” mice with lung cancer exceeded several times the count in the intact control group



**FIGURE 12** Number of CD45<sup>+</sup>Axl<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD117<sup>+</sup>EGF<sup>+</sup>, and PD-1<sup>+</sup>PD-L1<sup>+</sup> cancer cells in the lungs of “young” (group 3) and “aged” (group 4) mice of the C57BL/6 line with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to intact mice. #For comparison with “young” mice with LLC (group 3) according to the Mann–Whitney test ( $p < 0.05$ ).

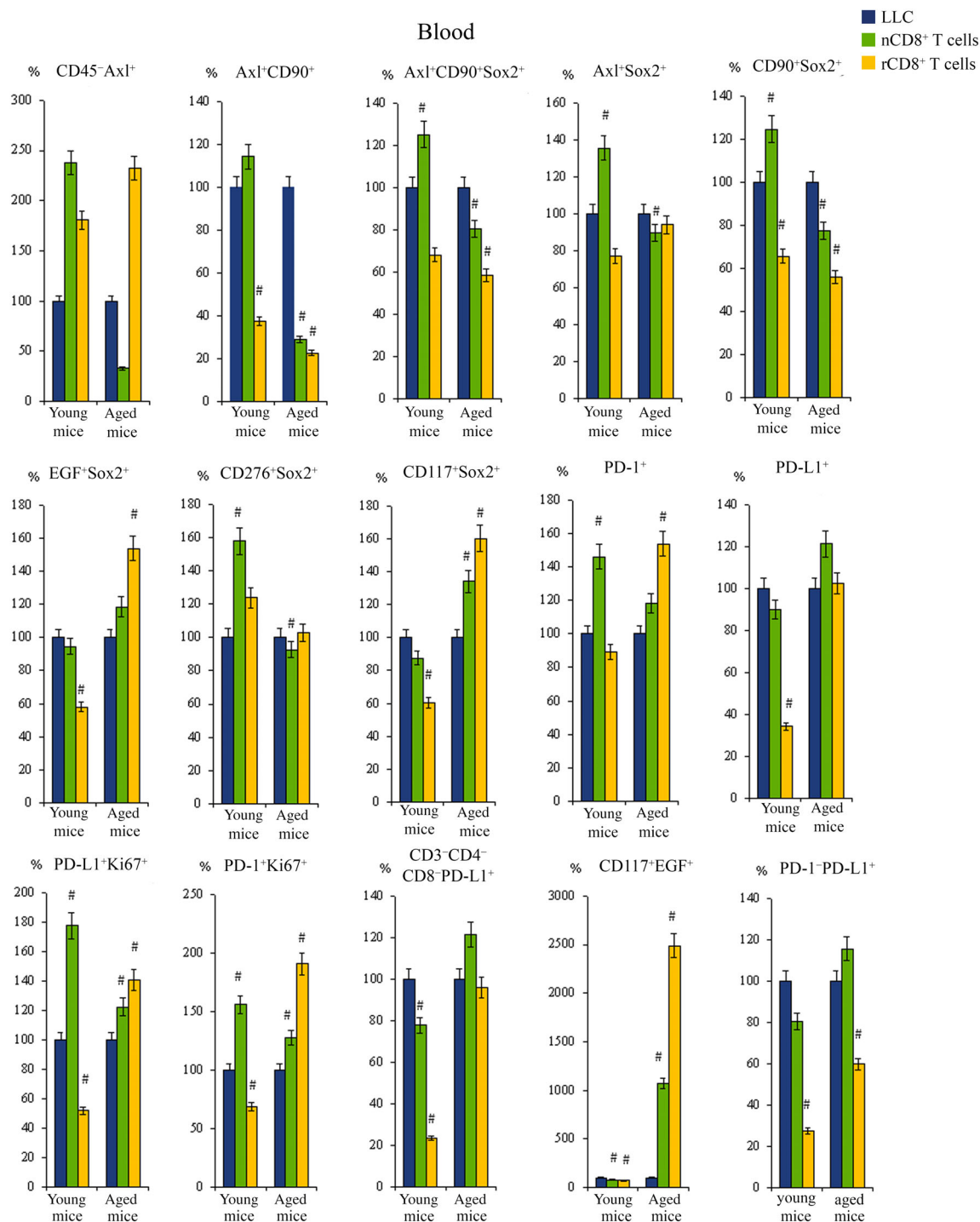
(Figure 6). However, in “aged” animals of group 4, tumor formation did not lead to a significant increase in T cells in the lung tissue.

The dysfunction and changes in the ratio of different T-cell populations (differentiated and undifferentiated) may contribute to different patterns of tumor development in “aged” and “young” experimental animals. Moreover, the inhibitory role of PD-1/PD-L1 on T cells increases with age, therefore, for older patients, immunotherapy targeting checkpoints such as PD-1 and PD-L1 can be an effective therapeutic approach for lung cancer with a good response rate and fewer side effects compared to chemotherapy.

Previously, we demonstrated the anti-tumor and anti-metastatic effects of rCD8<sup>+</sup> T cells in vitro and in vivo in

the C57BL/6 male mice aged 8–10 weeks (“young” mice).<sup>32</sup> CD8<sup>+</sup> naive T cells were obtained from the bone marrow of C57BL/6 mice aged 8–10 weeks. The reprogramming process involved the use of MEK and PD-1 inhibitors, alongside targeted training with LLC cell antigens. Positive results from the introduction of rCD8<sup>+</sup> T cells in “young” mice prompted us to investigate the effects of cell therapy in “aged” mice (80–82 weeks) with LLC. Simultaneously, it is recommended to assess the antitumor effects of rCD8<sup>+</sup> T cells in mice of various age groups.<sup>32</sup>

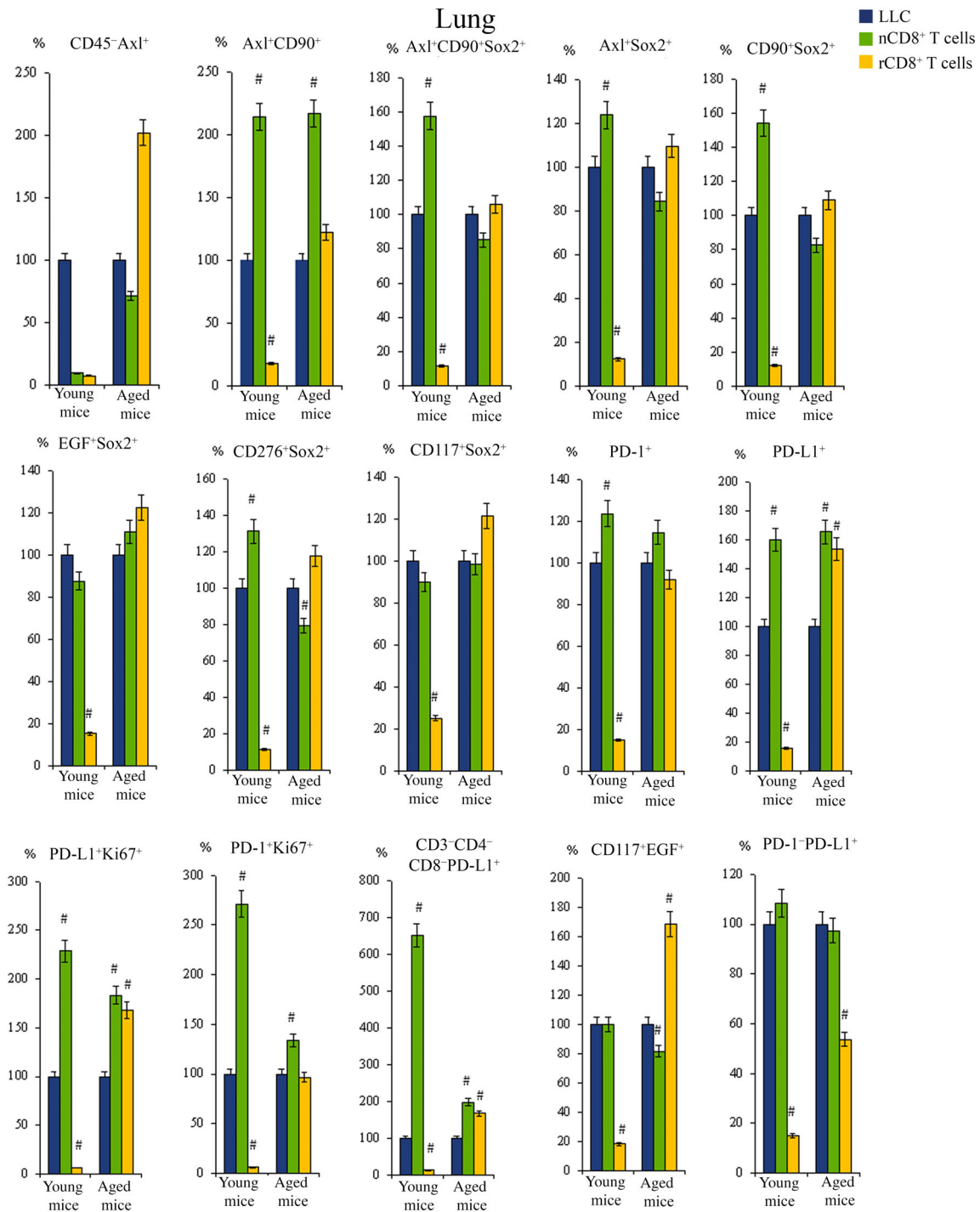
The results of cell therapy demonstrated a reduction in tumor size in the lungs of “aged” mice with Lewis lung carcinoma in group 8 compared to the corresponding pathological control group (Figure 2). However, the antitumor



**FIGURE 13** The effect of rCD8<sup>+</sup> T-cell and nCD8<sup>+</sup> T-cell cell therapy on the number of CD45<sup>+</sup>Axl<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD117<sup>+</sup>EGF<sup>+</sup>, and PD-1<sup>+</sup>PD-L1<sup>+</sup> cancer cells in the blood of “young” (groups 5 and 7) and “aged” (groups 6 and 8) mice of the C57BL/6 line with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to mice with LLC. #For comparison with “young” (group 3) and “aged” (group 4) mice with LLC, respectively, according to the Mann–Whitney test ( $p < 0.05$ ).

effect of rCD8<sup>+</sup> T cells in “aged” mice of group 8 was inferior to that in “young” mice of group 7. Thus, the treated “aged” mice of group 8 still had a significant tumor volume, multiple small tumor nodules, and pulmonary metastases, whereas this was not observed in “young” mice of group

7 under the same conditions. Additionally, cell therapy led to a substantial increase in CD3<sup>+</sup>CD8<sup>+</sup> T cells and CD3<sup>+</sup>CD4<sup>+</sup> T cells, as well as their individual subpopulations (including those expressing PD-L1 and PD-1) in the blood and lung tissue of “aged” mice (group 8) compared to



**FIGURE 14** The effect of rCD8<sup>+</sup> T-cell and nCD8<sup>+</sup> T-cell therapy on the number of CD45<sup>+</sup>Axl<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, EGF<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, CD117<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>PD-L1<sup>+</sup>, CD117<sup>+</sup>EGF<sup>+</sup>, and PD-1<sup>+</sup>PD-L1<sup>+</sup> cancer cells in the lungs of “young” (groups 5 and 7) and “aged” (groups 6 and 8) mice of the C57BL/6 line with Lewis lung carcinoma (LLC). The number of cells is expressed in % relative to mice with LLC. #For comparison with “young” (group 3) and “aged” (group 4) mice with LLC, respectively, according to the Mann–Whitney test ( $p < 0.05$ ).

the pathological control (Figures 7 and 8). This T-cell response to the introduction of rCD8<sup>+</sup> T cells in “aged” mice significantly exceeded the response observed in “young” mice and requires further detailed investigation.

In contrast to “young” mice in group 7, where the introduction of rCD8<sup>+</sup> T cells decreased the levels of all studied populations of CSCs and CTC in the blood and lung tissue, the response of cancer cells in “aged” mice of group 8 to cell



therapy was ambiguous. According to our data, the introduction of rCD8<sup>+</sup> T cells reduces the number of cells with PD-1-PD-L1<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, and PD-1<sup>+</sup> phenotypes in the lungs. However, it increases the number of cells with CD45<sup>-</sup>Axl<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, and CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>PD-L1<sup>+</sup> phenotypes (Figures 13 and 14). A similar effect in “aged” mice can be explained by senescent cell accumulation responsible for tissue aging in the tissues. These cells exhibit metabolic activity and release various substances such as inflammatory mediators, cytokines (IL-6, TNF $\alpha$ , IL-8, TGF- $\beta$ , IL-10), chemokines, metalloproteases, growth factors, and reactive oxygen species. The presence of an immunosuppressive microenvironment makes it easier for cancer cells to develop. In a mouse model, aging stromal cells were found to stimulate the accumulation of immunosuppressive myeloid cells through IL-6. Hyperproduction of this cytokine is observed in invasive forms of cervical carcinoma in humans.<sup>27</sup>

The baseline synthesis of pro-inflammatory cytokines, including IL-6, TNF $\alpha$ , IL-8, TGF $\beta$ , and IL-10, by aging immune cells often elevates over time. This leads to a perversion of the immune response to stimulation or therapy, weakening the immune control of Axl and PD-L1<sup>+</sup> cancer cells. Consequently, these cells form a pool of cancer cells resistant to rCD8<sup>+</sup> T cells. It is possible that due to this mechanism, a tumor develops in the lungs of “aged” mice.

In conclusion, we evaluated the influence of nCD8<sup>+</sup> T-cell therapy in orthotopic lung cancer. As shown above, in “aged” mice (group 2), the initial levels (before lung cancer modeling) of immune cells (the total population of CD3<sup>-</sup>CD4<sup>+</sup> cells in the lungs; CD8<sup>+</sup>CCR7<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>PD-1<sup>hi</sup> cells in the blood) were higher than in “young” mice (group 1) (Figures 3 and 4). In this model situation, the additional administration of nCD8<sup>+</sup> T cells to “aged” mice with LLC increases the activity of inflammation. The expected result of this is an increase in CSCs in the blood (CD45<sup>-</sup>Axl<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>) and lungs (Axl<sup>+</sup>CD90<sup>+</sup>, Axl<sup>+</sup>CD90<sup>+</sup>Sox2<sup>+</sup>, Axl<sup>+</sup>Sox2<sup>+</sup>, CD90<sup>+</sup>Sox2<sup>+</sup>, CD276<sup>+</sup>Sox2<sup>+</sup>, PD-1<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, PD-1<sup>+</sup>Ki67<sup>+</sup>, CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>PD-L1<sup>+</sup>) (Figures 13 and 14), leading to tumor progression in the lungs of “aged” mice in group 6 in comparison to group 2 (Figure 2). Similar effects, albeit less pronounced due to the low initial levels of immune cells, CTC, and CSCs, were detected in “young” mice of group 5 relative to group 3. These data provide additional confirmation of the advantage of rCD8<sup>+</sup> T-cell therapy over nCD8<sup>+</sup> T-cell therapy.

We acknowledge the limitations of this study and recognize that the obtained results can serve as a complement to the existing understanding of oncogenesis in laboratory animals. Specifically, the orthotopic LLC model demonstrates an increase in the level and alteration in the ratio of individual CD3<sup>+</sup>CD8<sup>+</sup> T-cell and CD3<sup>+</sup>CD4<sup>+</sup> T-cell subpopulations, including those associated with inflammation, at 80–82 weeks of age in male mice of the C57BL/6 line. These immune system changes potentially enhance inflammatory

mechanisms and contribute to tumor formation and development. Cancer cells expressing PD-L1, Axl, EGF, PD-L1, and Ki67 are subjects of further investigation to determine their role as risk factors for lung cancer in older patients.

Based on the results of this study, another assumption is that the PD-1/PD-L1 signaling pathway predominantly determines the aggressiveness of orthotopic lung cancer in mice aged 80–82 weeks. In older animals, rCD8<sup>+</sup> T-cell therapy only partially prevents tumor growth. Recognizing the need for improvement in the treatment strategy using rCD8<sup>+</sup> T cells for lung cancer, we conclude that enhancing treatment effectiveness in older patients is achievable by selectively targeting specific populations of CSCs, particularly those expressing Axl and PD-L1. Combining agents that target specific populations of CSCs, such as reprogrammed T cells, with traditional chemotherapy may improve the survival of elderly patients. However, as mentioned earlier, this issue requires further investigation. Additionally, we propose monitoring therapy effectiveness by evaluating the content of CSCs, as demonstrated in previous experiments.<sup>32</sup>

In this regard, it is necessary to continue the search for reliable diagnostic and prognostic biomarkers for the timely detection of malignant neoplasms in the elderly, as well as to enhance existing approaches to diagnosis and treatment.

## CONCLUSIONS

The current research demonstrates that the increased aggressiveness of LLC-induced lung cancer in “aged” mice is attributed to a reduction in T-cell populations and elevated levels of CTCs and CSCs expressing Axl, PD-1, and PD-L1. CD8<sup>+</sup> T cells obtained from the bone marrow of young mice, programmed through the combined use of MEK inhibitor targeting the MAPK/ERK signaling pathway and nivolumab, a monoclonal antibody blocking immune checkpoints, and trained on LLC cells, demonstrate reduced cancer activity in “aged” mice with LLC. However, the antitumor effect of rCD8<sup>+</sup> T cells in “aged” mice is inferior to that observed in “young” mice. This disparity can be attributed to a significant population of CTCs and CSCs that are resistant to cell therapy, exhibiting the phenotypes CD45<sup>-</sup>Axl<sup>+</sup>, PD-L1<sup>+</sup>, PD-L1<sup>+</sup>Ki67<sup>+</sup>, and CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>PD-L1<sup>+</sup>. Combining chemotherapy with rCD8<sup>+</sup> T-cell therapy may offer improved efficacy in eliminating EGF, PD-L1, PD-1, and Ki67 positive cancer cells.

## AUTHOR CONTRIBUTIONS

**Evgenii Skurikhin:** Conceptualization; resources; data curation; writing—original draft preparation; writing—review and editing; supervision; project administration; funding acquisition. **Mariia Zhukova:** software; validation; investigation; writing—original draft preparation; writing—review and editing; visualization. **Natalia Ermakova:** methodology; software; validation; investigation; visualization. **Edgar Pan:** methodology; software; validation; formal analysis;

investigation; data curation; visualization. **Darius Widera:** writing—review and editing. **Lubov Sandrikina:** investigation. **Lena Kogai:** investigation. **Nikolai Kushlinskii:** resources; writing—review and editing; funding acquisition. **Aslan Kubatiev:** resources. **Sergey Morozov:** resources; funding acquisition. **Alexander Dygai:** resources; writing—review and editing; supervision; project administration; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

## DATA AVAILABILITY STATEMENT

The data presented in this publication can be accessed by contacting the corresponding author, subject to a reasonable request.

## ORCID

Evgenii Skurikhin  <https://orcid.org/0000-0001-7445-4767>

Edgar Pan  <https://orcid.org/0000-0002-2163-7647>

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