Generating specific homologous neutralizing-antibodies: a novel therapeutic strategy in cancer treatment

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Abstract

Current cancer therapeutic strategies still face great challenges especially due to tumor relapse, drug resistance, and low treatment efficiency. The reason is that some tumor cells are able to outsmart the host immune mechanisms and evade the immune system. In this study, using the mouse cutaneous squamous cell carcinoma (mCSCC) as an example, a new cancer immunotherapeutic strategy with homologous neutralizing-antibodies was established. The experiment is divided into three stages. In the first stage, mCSCC cells were isolated and cultured from DMBA/TPA-induced mCSCC. In the second stage, the expanded tumor cells were then injected into healthy mice in order to produce anti-tumor homologous neutralizing-antibodies. In the final stage, therapeutic serum was extracted from healthy mice and injected back into tumor mice. ELISA assay was used to analyze the levels of p53, Bcl-xL, NF-κB, and Bax. The findings demonstrated that the serum treatment reduced tumor volume while also reversing changes in p53, Bcl-xL, NF-κB, and Bax. In conclusion, a novel immunotherapeutic strategy was developed to treat mCSCC, although more study is required to fully understand the mechanism of this serum treatment.

Keywords: immunotherapeutic strategy; homologous neutralizing-antibodies; mouse cutaneous squamous cell carcinoma

1. Introduction

The challenges faced in the treatment of cancer today are mainly the tumor heterogeneity, resistance to therapy, and recurrences [1]. Current major strategies for cancer therapy include chemotherapy, radiotherapy, and immunotherapy. Despite the endeavors and achievements made in treating cancers during the past decades, resistance to classical chemotherapeutic agents and lack of specificity to reach target cells continues to be a major problem [2]. Some specific cell surface proteins have provided useful targets and biomarkers for advanced cancer therapies, but the tumor recurrence rate is still high. The primary reason is that tumor cells

express different biomarkers at different developmental stages [3]. In a tumor tissue entity, there will be a large number of tumor cells with different stages and various types. Some tumor cells can evade treatment targets when using chemotherapy and radiotherapy targeting one or several biomarkers [4]. These tumor cells that escape the elimination stage acquire genetic alterations and alter cell-surface antigen production to evade the immune system, especially the cancer stem cells with the ability to self-renew and differentiate [5]. Unexpectedly, these strategies might offer the evaded-tumor cells a favorable growth environment.

The immunotherapy is to enhance the patient's immune system to kill tumor cells [6]. Although the effectiveness of both active and passive cancer immunotherapy has significantly improved in recent years, it still cannot prevent tumor recurrence [7]. The main reason is that some tumor cells develop the ability to escape the patient's immune system by downregulating or losing the expression of the proteins recognized by the immune cells as antigens [8]. It actually provides suitable growth microenvironment for the immune-evaded tumor cells. Therefore, the above therapeutic method are unable to completely eradicate tumor cells with various types and stages. In this study, a novel tumor treatment strategy is designed by using the mouse cutaneous squamous cell carcinoma (mCSCC) as an example. The strategy achieve the goal of tumor treatment in three stages by isolating tumor cells, producing homologous neutralizing-antibodies, and killing the tumor cells.

2. Materials and methods

2.1 DMBA/TPA carcinogenesis

Fifty C57BL/6 male mice were equally randomly divided into tumor + serum treatment, tumor without serum treatment, control + serum treatment (control 1), control without serum treatment (control 2), and serum provider groups. One mice from tumor + serum treatment group was paired with one same blood type mice (type A or type B) from the serum provider

group. The mice in tumor + serum treatment and tumor without serum treatment groups receiving 7,12-Dimethylbenz (a) anthracene(DMBA)/12-O-Tetradecanoylphorbol-13-acetate/(TPA) treatment. Mice were shaved on the dorsal skin area. Two days later, mice were treated topically with 60 μg DMBA dissolved in 200 μl acetone to the naked backs. DMBA was administered to mice for two weeks and were further exposed to 2.5 μg TPA in 200 μl acetone once a week for a total of 10 weeks. DMBA (Lot: D3254) and TPA (Lot: P1585) were purchased from Sigma-Aldrich, China. Skin tumors were measured using a precision calliper allowing to discriminate size modifications >0.1 mm. The body weight were recorded weekly. Tumor volumes were measured the first day of treatment and every week until the end of the experiments with the formula $V=\pi \times [d^2 \times D]/6$, where d is the minor tumor axis and D is the major tumor axis [9]. Figure 1 shows a workflow of this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Guilin Medical University.

2.2 Cell preparation and serum injection

The preparation of single-cell suspensions from skin tumor tissues was used cell suspension preparation kit (Lot: KFS439, Beijing Baiaolaibo Technology Co, China) with a modification. Briefly, the dorsal skin tumor tissue were washed with PBS and cut into small fragments 1-2 mm in size in a Petri dish containing EDTA/Trypsin. Minced tumor pieces are transferred to a tube containing trypsin and incubated at 37°C for an hour with shaking. Add DMEM/10% FBS to the dish to recover all cells and tissue and pass through a 100-mm cell strainer. Centrifuge the cell suspension at 500×g for 5 min and plate out the recovered cells, ideally at densities of 1×10^5 per 100 mm dish in KC growth medium and incubate at 37°C in a 5% CO_2 incubator for 7 days with daily medium change (Figure 2A) (10). Each mice in tumor + serum treatment group were randomly paired with a mice in serum provider group. About 5×10^5 primary tumor cells suspended in PBS were injected into the tail vein of paired mice in

serum provider group. The 0.1 ml of mice whole blood from the serum provider group were collected from tail vein under ether anesthesia after 7 day injection. The serum is separated immediately by a brief centrifugation, and about 0.02-0.05 ml of serum can be separated at each time. The 0.02 ml serum were injected into the tail vein of its paired mice in tumor + serum treatment group once a week, three times in total (week 15 to 17).

2.3 Enzyme linked immunosorbent assay

Previous studies have shown that the level of p53, Bcl-xL, NF-κB, and Bax is associated with the occurrence, development, and metastasis of mCSCC [11-14]. Therefore, the concentration of p53, Bcl-xL, NF-κB, and Bax in the tissues were measured using ELISA assay in this study. Mouse p53 (Lot: ab224878), Bcl-xL (Lot: ab227899), NF-κB (Lot: ab176648), Bax (Lot: ab233624) ELISA Development Kit were purchased from Abcam, China. Briefly, the coated antibody was diluted, added into ELISA plate (100 μL/well) for 48 hr at 4 °C. Subsequently, the ELISA plate was washed for three times using tris-buffered saline (TBS), added with diluted sample (100 μL/well) for 90 min at 37 °C. After washing for three times, all samples was cultured with diluted enzyme labelled antibody (100 μl/well) for 60 min at 37 °C, washed for three times, then added with avidin-biotin-pcroxidasecomplex (ABC) developer (100 μL/well). After incubation in the dark for 30 min at 37 °C, 100 μl stop buffer was used to stop reaction. Then, the plates were read at 450 nm on a microplate reader (Thermo, China).

2.4 Statistical analysis.

Data are presented as mean± standard deviation (SD) from three independent experiments. Differences before and after treatment were analyzed using paired sample *t*-test using SPSS 16.0 (SPSS Inc., Chicago). *P* value less than 0.05 was considered statistically significant. All experiments were repeated at least three times.

3. Results

3.1 Monitoring body weight

At the beginning of the experiment, the average body weight of male C57BL/6 mice (6-8 weeks old) was 20.46 ±0.28 g (range: 20 to 21.3 g). The C57BL/6 male mice developed mCSCC on the back after 12 weeks of DMBA/TPA treatment. The average body weights in the DMBA/TPA-treated animals were 24.90 ±1.12 g and 26.65 ± 0.83 g in the control animals. At the end of experiment (week 17), the average body weights were 26.63 ± 1.36 g in the tumor + serum treatment, 27.6±1.2 g in the tumor without serum treatment, 28.47±0.85 in control 1, 28.5±0.79 g in control 2.

3.2 Serum treatment inhibits the growth of mCSCC

During the DMBA/TPA induction, the tumor grows gradually and reaches its maximal average size at 12 weeks, measuring 13.26 mm^3 in tumor + serum treatment group and 13.59 mm^3 in tumor without serum treatment group, respectively. In mice not given serum treatment, there were no appreciable changes in tumor size at week 17. The tumor size does, however, dramatically decrease to 8.63 mm^3 after 3 weeks of serum treatment, demonstrating that serum treatment can effectively reduce tumor size (Figure 2B).

3.3 Serum treatment reverses the expression of tumor related factors

According to the results of ELISA assay, p53, Bcl-xL, and NF-κB are highly expressed in mCSCC, whereas Bax is low expressed. After serum treatment, p53, Bcl-xL, and NF-B levels fell whereas Bax levels rose (Figure 3). This results indicates that serum treatment can effectively reverse the expression of tumor related factors.

4. Discussion

The principle behind developing this immunotherapeutic strategy is to treat various stages and types of tumor cells as a whole. Some tumor cells may escape the patient' immune system, but they can stimulate the production of homologous neutralizing-antibodies in healthy mice [15-16]. Following tumor cell isolation, cells of various growth stages and types were

expanded in culture medium. When these cells are injected into healthy mice, thousands of homologous neutralizing-antibodies against the corresponding antigens on the tumor cells are produced. The serum from the healthy mice's blood is then transfused back into the tumor mice to treat mCSCC (Figure 4). Because different stages of tumor cells have different surface biomarkers [17], we repeat the aforementioned serum treatment procedure once a week, three times in total (week 15 to 17). The findings revealed that the tumor size of mice was significantly reduced. To validate this treatment strategy, four mCSCC-associated proteins, p53, Bcl-xL, NF-κB, and Bax, were chosen as tumor biomarkers. In mCSCC, there was a significant increase in p53, Bcl-xL, and NF-κB and a decrease in Bax. Serum p53, Bcl-xL, NF-κB, and Bax antibodies were produced after tumor cells were injected into healthy mice. The tumor volume decreased after the serum treatment, which was accompanied by a reversed change of p53, Bcl-xL, NF-κB, and Bax. Unfortunately, one healthy mouse from serum provider group and one tumor mouse received serum treatment died during the study for unknown reasons.

This study has some limitations. Firstly, it is critical to consider the impact of tumor cells on healthy mice. Exogenous cells entering the body of mice can trigger an immune response, resulting in unpredictable outcomes [18]. Two mice died with a weight loss. The causes of this phenomenon must be investigated further. Second, in this study, only the blood type difference of mice (type A or type B) was considered, with no consideration given to other factors such as histocompatibility [19]. More factors should be considered in order to develop more effective immunotherapeutic strategy. In reality, it is still unclear how to classify mice's blood types. Third, while this treatment method has been successful in mice, more experiments must be conducted before it can be applied to humans. For example, injecting exogenous cells into the human body to produce therapeutic serum is currently unethical. The human body is far more complex than that of mice, and it is critical to

understand how many doses of tumor cells can be used, how many therapeutic neutralizing-antibodies have been produced? Is shortening or extending the time of cells expansion (7 days) or homologous neutralizing-antibodies production (7 days) more effective, and so on. Finally, there is a question. Is it desirable to employ serum treatments with heterologous neutralizing-antibodies made by other animals? If so, the outcome of treatment might be improved, but other issues, such animal selection, xeno-transplantation, and cross-species immune responses, would arise.

In summary, in this study, mCSCC cells were isolated and injected them into healthy mice to stimulate the production of various therapeutic homologous neutralizing-antibodies in serum. These neutralizing-antibodies in serum were then infused back into the tumor mice, achieving the goal of tumor reduction. Our research has explored a novel strategy for the treatment of tumors. However, some issues in the experiment require further investigation and resolution.

Funding

This research was supported by the grants from National Natural Science Foundation of China (No. 32260175)

Conflicts of Interest

The authors declare no conflicts of interest.

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Figure legend

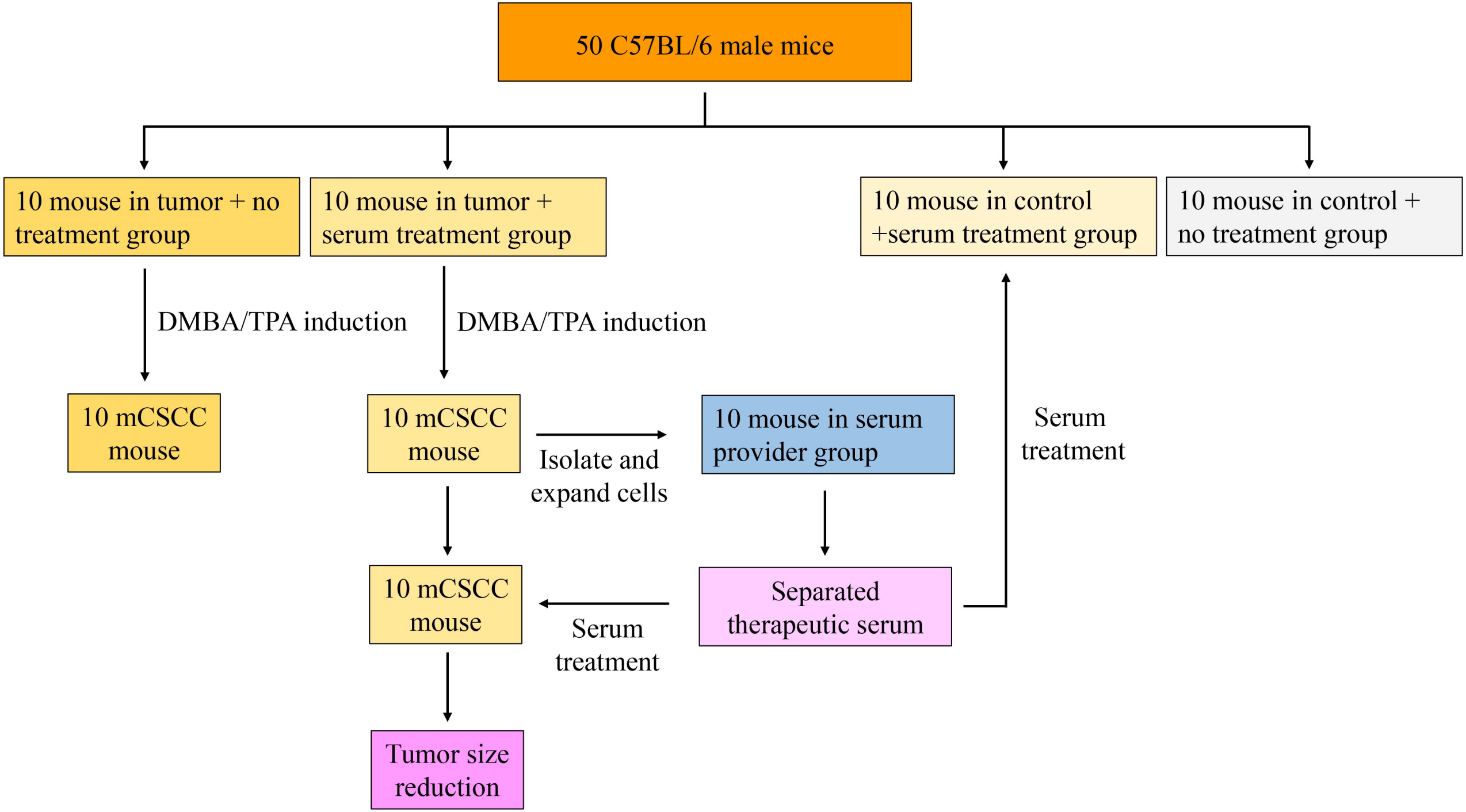
Figure 1. The workflow of this study.

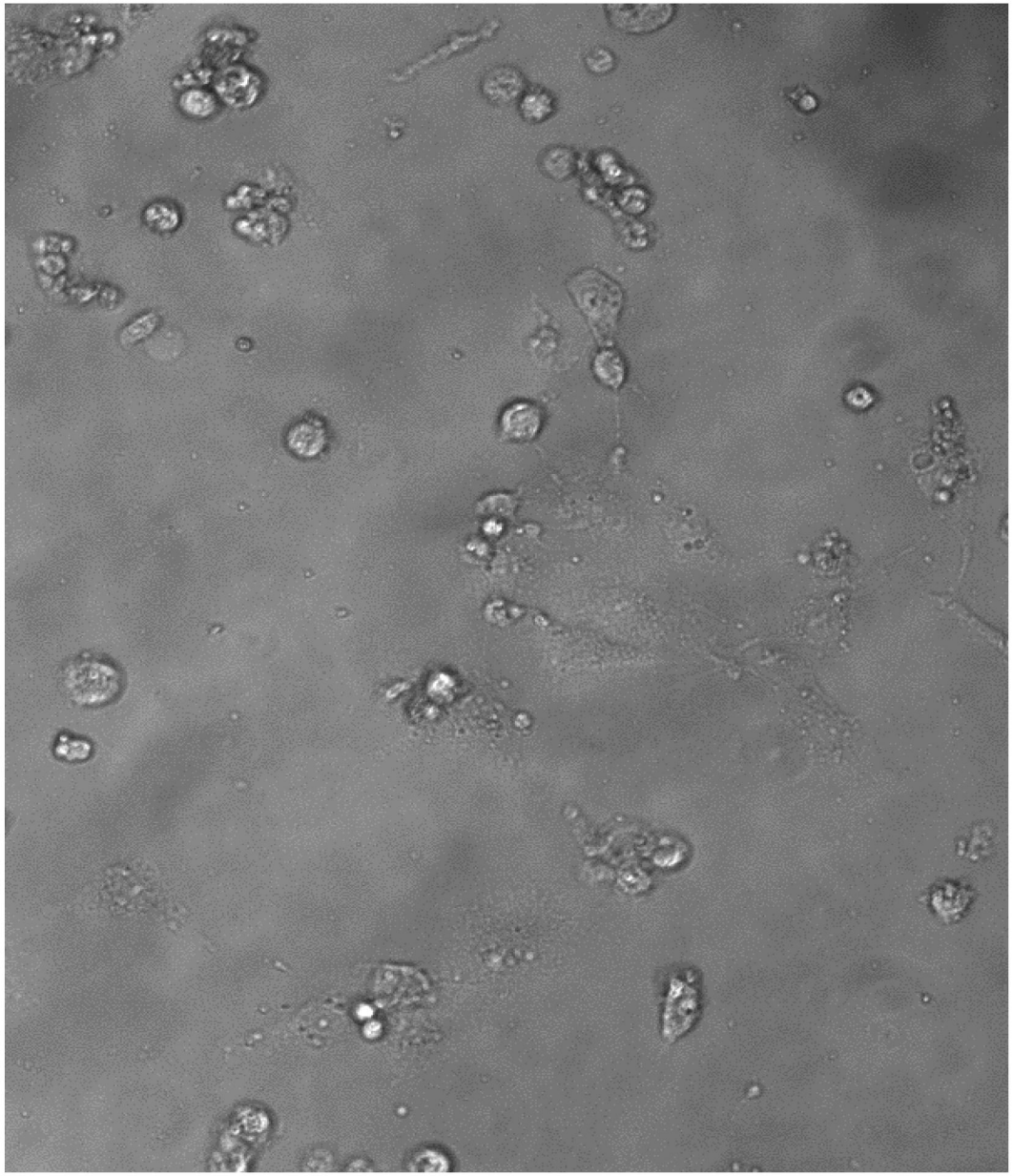
Figure 2. A. Isolated and cultured tumor cells. B. DMBA/TPA induces tumor growth and changes in tumor volume before and after serum treatment. Week 13 work for isolating and expanding the tumor cell. Week 14 work for injecting the tumor cells into the tail vein of paired mice in serum provider group to produce homologous neutralizing-antibodies.

$^{**}P < 0.01$

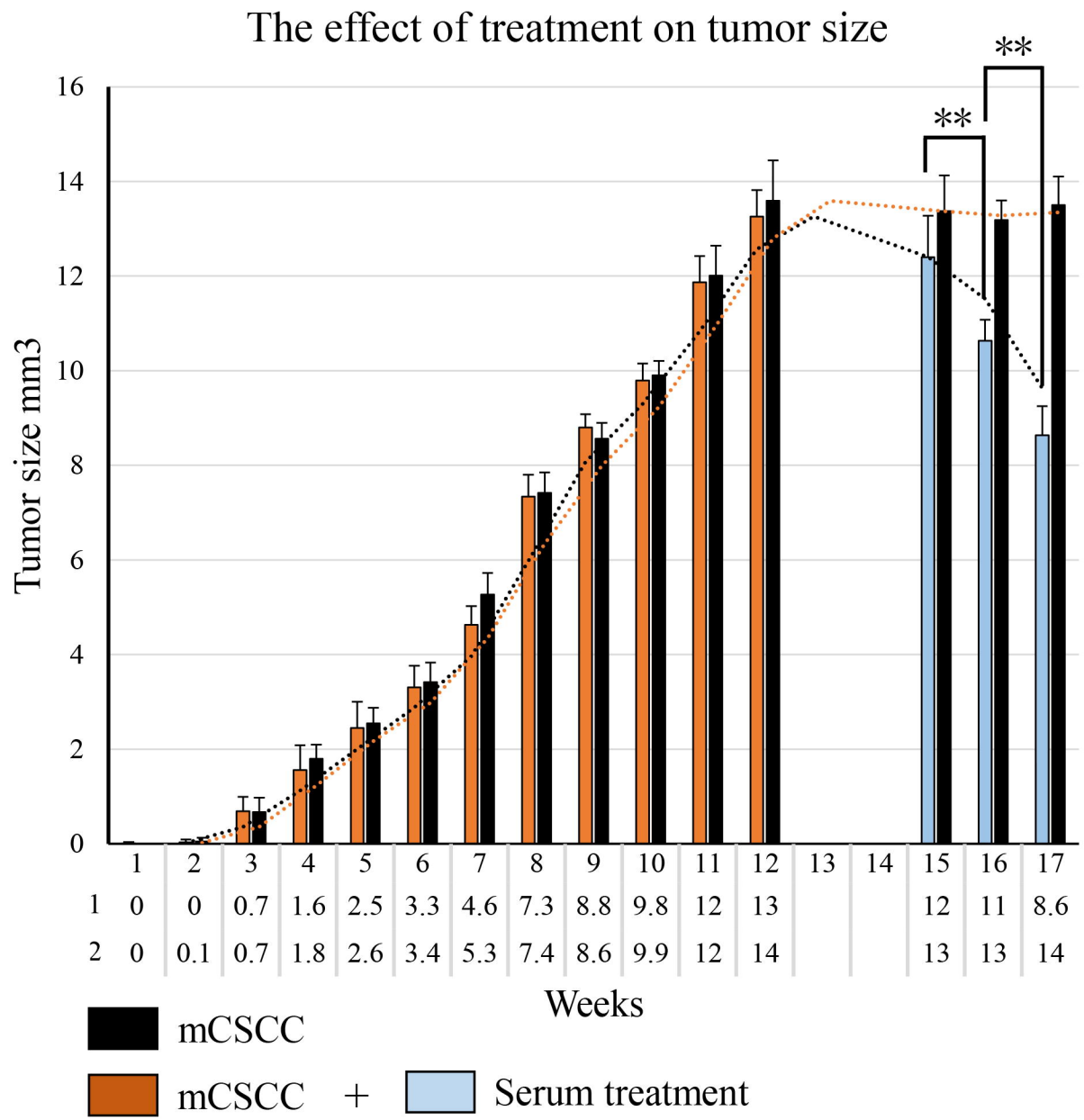
Figure 3. ELISA analysis showed the changes in the expression of p53, Bcl-xL, NF-κB, and Bax proteins before and after serum treatment. At week 12, the tumor volume reached its maximum value. The week 15, 16, and 17 are the first week, second week, and third week after serum treatment, respectively.

Figure 4. Schematic diagram of experimental design.


50 C57BL/6 male mice
10 mouse in tumor + no treatment group
10 mouse in tumor + serum treatment group
10 mouse in control +serum treatment group
10 mouse in control + no treatment group
DMBA/TPA induction
DMBA/TPA induction
10 mCSCC mouse
10 mCSCC mouse
Isolate and expand cells
10 mouse in serum provider group
Serum treatment
10 mCSCC mouse
Serum treatment
Separated therapeutic serum
Tumor size reduction

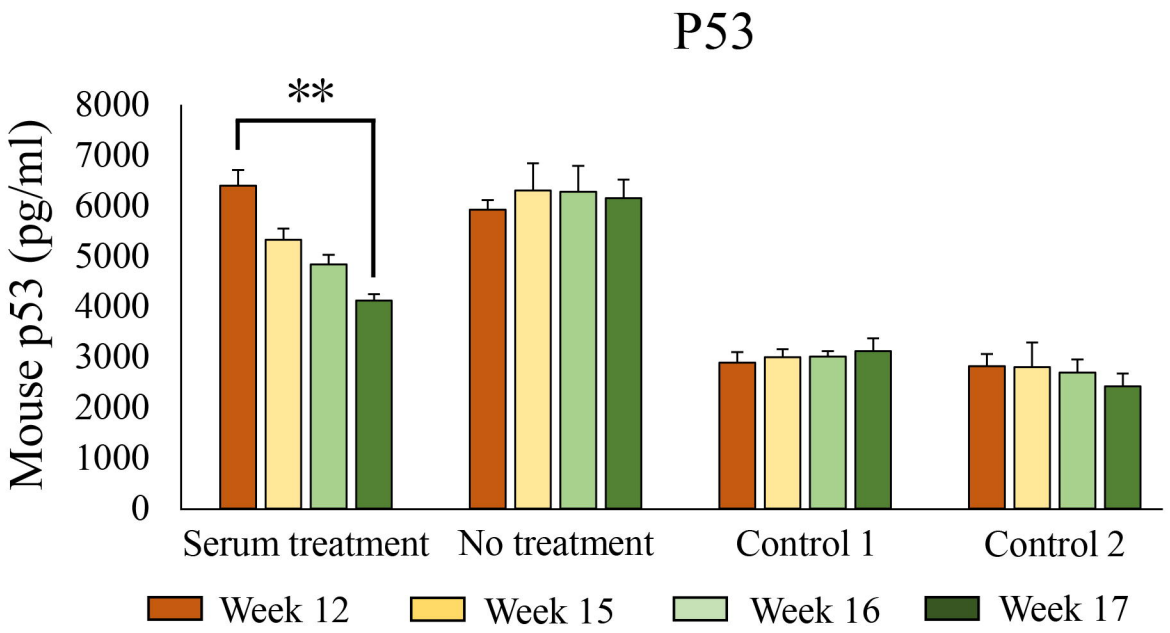


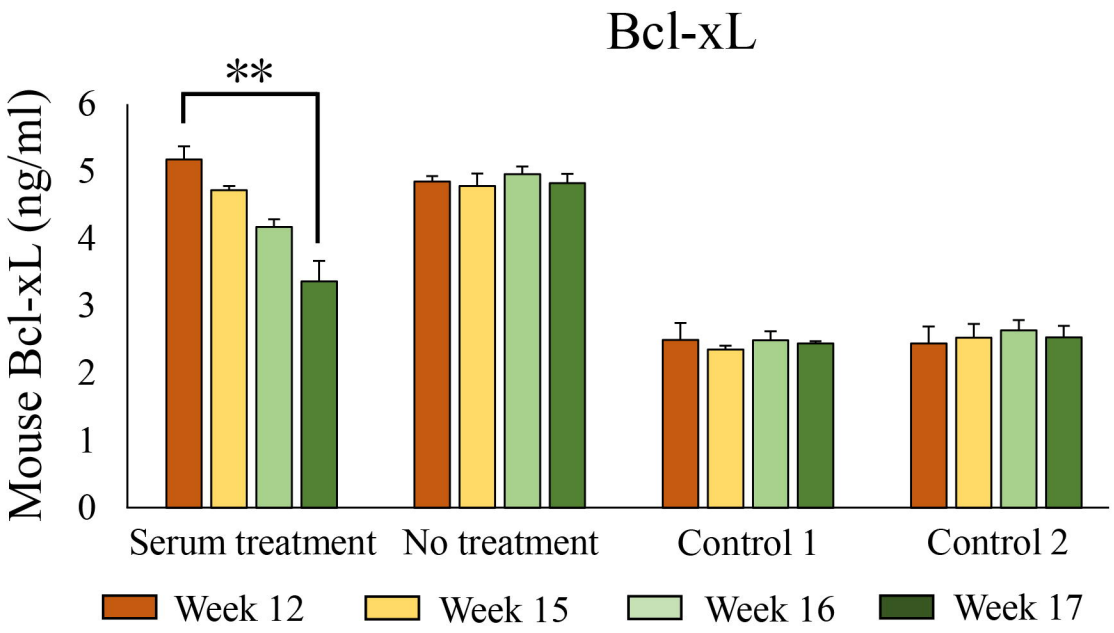
A

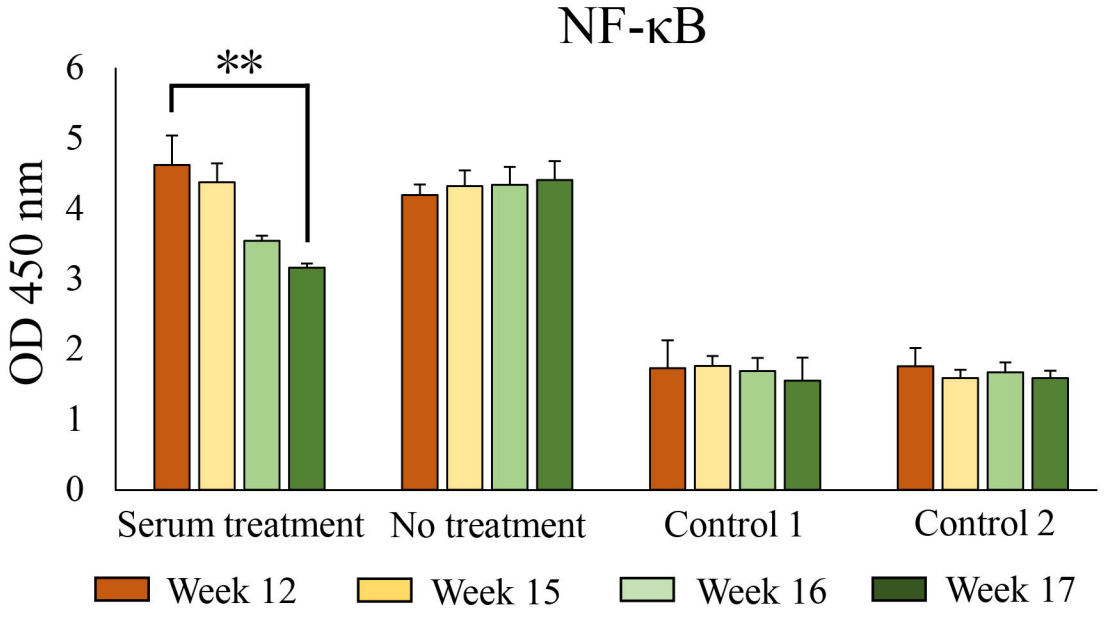


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0	0	0.7	1.6	2.5	3.3	4.6	7.3	8.8	9.8	12	13			12	11	8.6
2	0	0.1	0.7	1.8	2.6	3.4	5.3	7.4	8.6	9.9	12	14			13	13	14

B


P53
Mouse p53 (pg/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
**
Serum treatment
No treatment
Control 1
Control 2
Week 12
Week 15
Week 16
Week 17


Bcl-xL
Mouse Bcl-xL (ng/ml)
6
5
4
3
2
1
0
**
Serum treatment
No treatment
Control 1
Control 2
Week 12
Week 15
Week 16
Week 17


NF-κB
OD 450 nm
6
5
4
3
2
1
0
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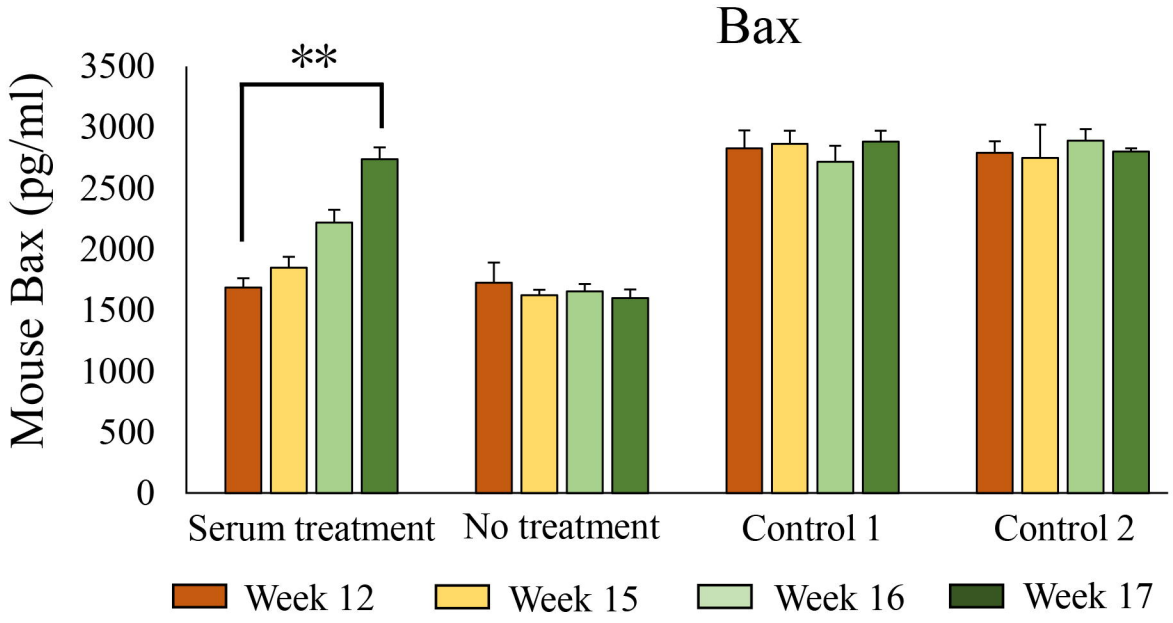
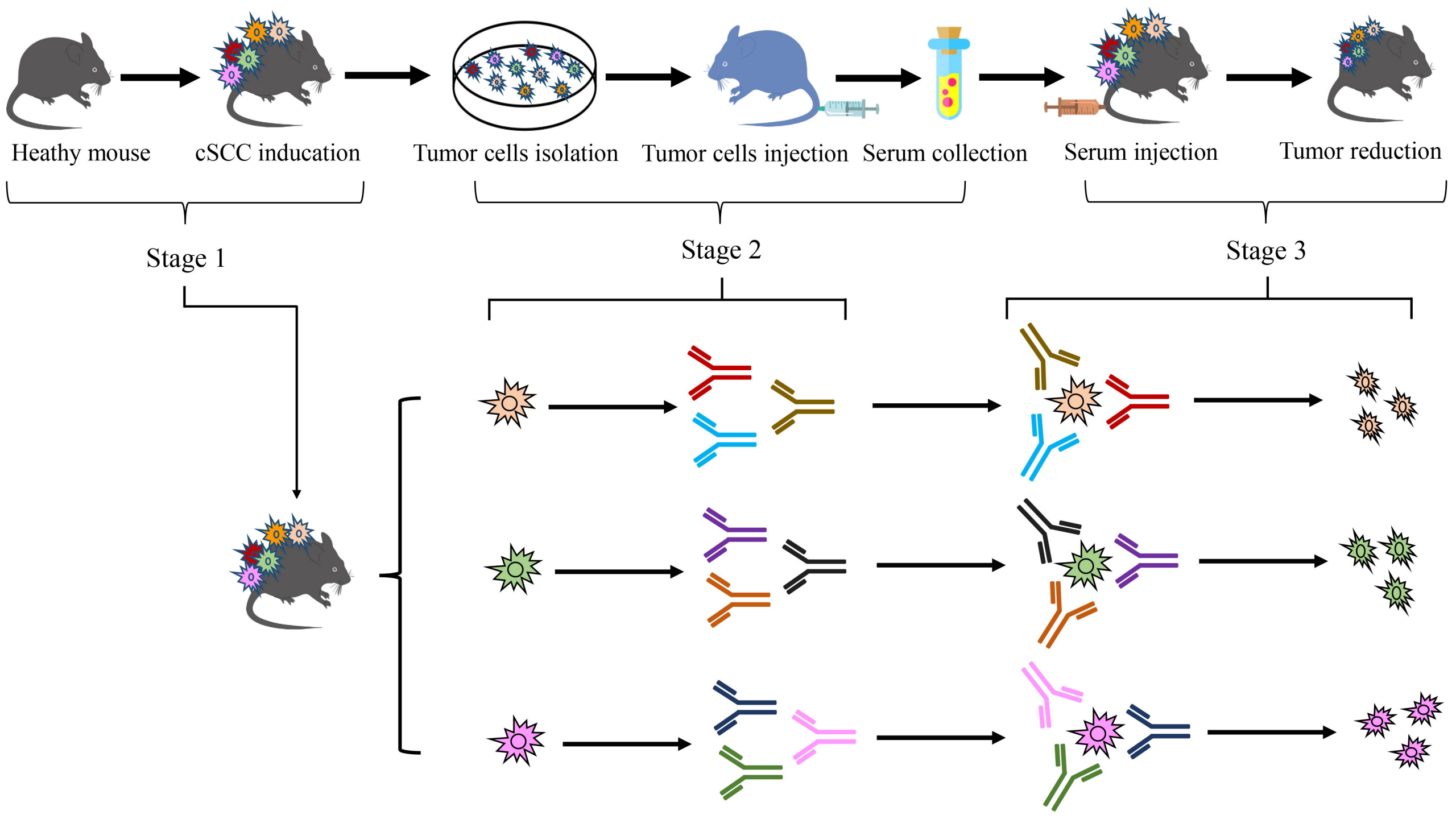
Serum treatment
No treatment
Control 1
Control 2
Week 12
Week 15
Week 16
Week 17


Bax
Mouse Bax (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
**
Serum treatment
No treatment
Control 1
Control 2
Week 12
Week 15
Week 16
Week 17


Heathy mouse
cSCC inducation
Tumor cells isolation
Tumor cells injection
Serum collection
Serum injection
Tumor reduction
Stage 1
Stage 2
Stage 3