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# Safety and Efficacy of Transplantation with Allogeneic Skin Tumors to Treat Chemically-Induced Skin Tumors in Mice

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Back	kground:	Transplantation with allogeneic c graft-versus-tumor (GVT) effect.	ells has become a p This study was aime	romising modality for cancer therapy, which can induce d at assessing the safety, efficacy, and tissue type GVT		
Material/Methods:		(tGVT) response of transplantation with allogeneic skin tumors to treat chemically-induced skin tumors in mice. FVB/N and ICR mice were exposed topically to chemicals to induce skin tumors. Healthy ICR mice were transplant- ed with allogeneic skin tumors from FVB/N mice to test the safety. The tumor-bearing ICR mice were transplanted				
	Results:	with, or without, allogeneic skin tu tumor volumes <i>in situ</i> , metastasis Transplantation with no more than After transplantation with allogen mors slightly at early stage, accon <i>vs.</i> 47.37%), while there were no <i>in situ</i> , metastasis tumors, and o	tumors to test the effi- tumors, overall surv n 0.03 g allogeneic sk neic skin tumors to npanied by fewer me statistically significa verall survival betwe	cacy. The body weights (BW), body condition scores (BCS), ival, and serum cytokines were measured longitudinally. the tumors from FVB/N mice to healthy ICR mice was safe. treat tumor-bearing mice, it inhibited the growth of tu- tastatic tumors at 24 days after transplantation (21.05% and differences in the values of BW, BCS, tumor volumes even the transplanted and non-transplanted groups. The		
Conclusions:		levels of serum interleukin (IL)-2 were significantly reduced in the controls ( <i>P</i> <0.05), but not in the recipients, which may be associated with the tGVT response. Our results suggest that transplantation with allogeneic skin tumors is a safe treatment in mice, which can induce short-term tGVT response mediated by IL-2.				
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# Background

Skin cancer is the most common type of cancer in the white population [1], and its incidence is rising rapidly in America and Europe. Cutaneous squamous cell carcinoma (cSCC) is a highly prevalent skin cancer [2]. Previous studies have suggested that the development of cSCC may be attributed to longterm ultraviolet exposure [3], chemical carcinogens contact [4], and immunosuppressive therapy in patients [5,6], which are always accompanied by various levels of immunosuppression. Therefore, enhancement of antitumor immunity has been an attractive strategy for intervention in patients with skin cancer.

There are numerous immunological strategies that can stimulate antitumor immunity [7,8], including tumor vaccination, cytokine-mediated immunotherapy, adoptive cellular immunotherapy, and others. Of particular interest is that transplantation with allogeneic cells is becoming a promising modality for cancer therapy. A previous study has shown that induction of bone marrow chimeras significantly reduces the size of mammary adenocarcinoma in mice [9]. Furthermore, transplantation with allogeneic hematopoietic stem cells or transfusion of allogeneic lymphocytes effectively eliminates leukemic cells and solid tumors [10,11], including renal cell carcinoma [12], breast cancer [13], and lung cancer [14]. However, these therapeutic strategies usually have severe adverse effects and may develop graft-versus-host disease [15,16].

Interestingly, vaccination with allogeneic or syngeneic melanoma cells has been demonstrated to induce graft-versus-tumor (GVT) effect, but does not cause severe adverse effects [17]. Furthermore, intra-tumoral injection with fusogenic membrane glycoproteins from allogeneic tumor cells results in obvious tumor regression [18]. However, injection with CD80 over-expressing allogeneic breast cancer cells did not achieve significant tumor regression [19]. Accordingly, we hypothesized that transplantation with MHC-mismatched tumors could induce stronger GVT responses with only minor adverse effects, which may serve as a new antitumor therapy. We named this antitumor effect using tumor tissue as "tissue type GVT" (tGVT). First, tumor cells are usually tolerant to host immunity, which can be broken down by allogeneic responses [20]. Second, the GVT response is accompanied by allograft rejection, which can inhibit the development of the cancer [21]. In addition, allogeneic tumor-infiltrating lymphocytes can mediate antitumor response in the non-allosensitized host [22]. Hence, we employed a transplantation model using highly immunogenic skin tumors as allogeneic tumor tissue to assess the tGVT effect in mice.

The levels of serum cytokines are valid biomarkers to predict and reflect alloreactivity [23] and they can be analyzed by cytometric bead array (CBA) technique [24]. Indeed, high levels of interleukin (IL)-2 and IL-4 are detected at the early stage of T cell activation and during acute rejection. The levels of serum IL-2 are correlated with the outcome of transplanted grafts, and their changes can be used to predict the risk of rejection [23]. Furthermore, elevated levels of serum IL-6 are associated with graft rejection [25]. IL-6 is required for the generation of Th17 cells [26], which mainly produce IL-17A and mediate rejection of allograft rejection [27]. Accordingly, the levels of serum cytokines may be appropriate biomarkers to assess the tGVT response.

In this study, we induced cSCC by sequential exposure to 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in FVB/N mice and ICR mice, and examined the effect of transplantation with allogeneic skin tumors from FVB/N mice to ICR mice with early stage of cSCC on the growth of tumors and cytokine responses. This study was aimed to evaluate the safety, therapeutic effect, and tGVT response of allogeneic tumor tissue transplantation.

### **Material and Methods**

#### Animals

Female ICR mice and FVB/N mice at 5–6 weeks of age were purchased from the Slac Laboratory Animal Center (Shanghai, China) and housed in a specific pathogen-free facility at the Laboratory Animal Center, Kunming Medical University. They were fed with a standard chow and water ad libitum, and kept on a 12:12 h light-dark cycle at 24±2°C with 50±10% relative humidity. The animal experimental protocols were approved by the Animal Research and Care Committee of Kunming Medical University, China.

#### Induction of the skin tumors

The mouse skin tumors were induced by a protocol of twostage skin carcinogenesis using DMBA as an initiator and TPA as a promoter, as described previously [28,29]. Briefly, individual ICR and FVB/N mice were tropically exposed to 0.025% DMBA in acetone (100  $\mu$ l/3 cm<sup>2</sup>, Sigma-Aldrich, St. Louis, MO) once on the shaved and depilated dorsal skin. One week later, the same skin area of individual mice was painted with 5  $\mu$ g TPA (Sigma-Aldrich, St. Louis, MO) in acetone twice within 1 week. The mice were monitored for the appearance of solid tumors. Individual mice with an induced tumor of 1 mm in a diameter or greater for >2 weeks were counted and used for the following experiments.

#### **Tumor scoring**

Following induction, the mice were monitored weekly for the development of tumors. The numbers of typical papillomas

(well-demarcated papules without erosion or ulceration) or carcinomas (poorly demarcated papules with erosion or ulceration) were counted, according to Girardi's method [30]. Tumor sizes were divided into 4 grades: >1 mm<sup>2</sup>,  $\geq$ 4 mm<sup>2</sup>,  $\geq$ 9 mm<sup>2</sup> and  $\geq$ 16 mm<sup>2</sup>.

#### **Body condition scoring**

The life quality of individual tumor-bearing mice was determined by body conditioning score (BCS) as described previously [31], using the following scores: 1. Muscle wasting is advanced, fat deposits are gone, and bones are very prominent; 2. The mouse is becoming thin and bones are prominent; 3. The mouse is in optimal condition and bones are palpable but not prominent; 4. The mouse is well-fleshed, and bones are barely felt; and 5. The mouse is obese, and bones cannot be felt at all.

#### **Cell counts**

To estimate the numbers of tumor cells for transplantation, the resected tumors from FVB/N mice were cut into small pieces, and different amounts of tumor tissues (0.015, 0.03, or 0.045 g) were digested with 0.1% collagenase, 75 U/ml of deoxyribonuclease I, and 0.2% hyaluronidase in PBS at 37°C for 3 h. The resulting cell suspension was washed and the number of tumor cells was counted in a blinded manner.

#### Histopathological analysis

When ulcerative lesions appeared, the tumors were subjected to biopsy. The biopsied tissue samples were fixed in 10% neutral buffered formaldehyde, and embedded in paraffin. The tissue sections (4  $\mu$ m) were stained with hematoxylin and eosin (HE) and examined in a blinded manner as described previously with minor modification [32]. Tumors with papillomatous structure, mild cytologic atypia of keratinocytes, and active mitosis limited in the basal and lower epidermis were classified as slight atypical. Tumors with similar architecture, moderately atypical keratinocytes, and active mitosis over the lower epidermis were classified as moderate atypical papillomas. Tumors with cytologic atypia of keratinocytes, and active mitosis spreading over the entire epidermis were termed severe atypical hyperplasia and SCC *in situ*.

#### Allogeneic tumor tissue transplantation

To assess the safety of transplantation with allogeneic cutaneous tumor tissues, healthy ICR mice were transplanted subcutaneously (near the inducible skin tumors) with 0.045, 0.03, or 0.015 g tumor tissues from FVB/N mice as the high-, medium-, or low-dose group (n=6 per group), respectively. Their body weights (BW), BCS, and the volumes of transplanted tissue masses were examined every other day up to 50 days after transplantation and the volumes were calculated according to the following formula:  $\frac{1}{2}$  (length×width<sup>2</sup>).

To examine the efficacy of allogeneic tumor transplantation, ICR mice were induced for skin tumors and when most induced tumors started to show visible erosion or ulceration, the mice were randomly divided into 2 groups. The experimental group was transplanted subcutaneously with 0.03 g skin tumors from FVB/N mice 3 times with an interval of 1–2 weeks; while the control mice received the sham surgery at each time point (n=19 per group). The characteristics of both groups of mice before the allogeneic tumor transplantation were evaluated (Table 1). Following transplantation with allogeneic skin tumors, BW, BCS, tumor volumes *in situ*, metastasis tumors, overall survival, and serum cytokines were measured longitudinally.

# Positron emission tomography/computed tomography (PET/CT) imaging

The potential tumor metastasis was examined by PET-CT, which was tested before and 24 days after transplantation using a combined PET/CT scanner (Biograph 16, Siemens, Erlangen, Germany), as described previously with minor modifications [33]. Briefly, the mice were fasted for 12 h and anesthetized intraperitoneally with pentobarbital sodium (40 mg/kg). Subsequently, individual mice were injected intravenously with approximately 7.4 MBq (200  $\mu$ Ci)/200  $\mu$ L fluorin-18-2-fluoro-2-deoxy-d-glucose (<sup>18</sup>F-FDG) and scanned for an increased rate of aerobic glycolysis at 45 min post-injection with a probe. Two-dimensional PET, CT, and merged PET/CT images were reconstructed and data were analyzed using AMIDE software.

#### Cytometric bead array

The levels of serum IL-2, IL-4, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF), IL-17A, and IL-10 in individual mice were measured before and one and 2 weeks after the first transplantation by CBA using a mouse Th1/Th2/Th17 cytokines kit (BD Biosciences, San Jose, CA) on an FACS Calibur flow cytometer. The data were analyzed using BD Biosciences CBA software.

#### Statistics

Continual data are expressed as means  $\pm$  standard deviations, medians, and inter-quartile ranges, dependent on their distribution. Categorical data are expressed as the real counts and percentages. The difference between groups over a period was analyzed by repeated measurement using a mixed-effects model or analyzed by *t* test, Wilcoxon rank sum test or Wilcoxon matched-pairs signed rank sum test, or Fisher's exact test. Survival curves were plotted using the Kaplan-Meier Table 1. Characteristics of ICR mice in the transplantation experiment.

	Experiment group N (%)	Control group N (%)
All cases	19	19
Body weight (g)	34.32±2.97	34.6±3.04
Body Conditioning Score	3.9±0.3	3.7±0.4
Tumor number ≥5 <5	5 (26.3) 14 (73.7)	4 (21.1) 15 (78.9)
Tumor Size Scoring T1/T2 ( $\geq$ 1 mm <sup>2</sup> ) T3 ( $\geq$ 9 mm <sup>2</sup> ) T4 ( $\geq$ 16 mm <sup>2</sup> )	3 (15.8) 7 (36.8) 9 (47.4)	7 (36.9) 2 (10.5) 10 (52.6)
Histopathological analysis Slight atypical hyperplasia Moderate atypical hyperplasia Severe atypical hyperplasia and Skin squamous cell carcinoma	6 (31.6) 11 (57.9) 2 (10.5)	8 (42.1) 9 (47.4) 2 (10.5)
Metastasis* Present Absent	4 (21.1) 15 (78.9)	3 (15.8) 16 (84.2)

\* Metastasis was evaluated mainly according to the results of PET-CT.

method and were compared by the log-rank test. Statistical analyses were performed using SPSS 19.0 statistic software (SPSS Inc., Chicago, IL). A p value of <0.05 was considered statistically significant.

# Results

#### Chemical induction of skin tumors in FVB/N mice

To induce skin tumors, FVB/N mice were sequentially painted with DMBA and TPA, and the development of tumors was monitored. We found that tumors began to occur at 8 weeks after induction and all mice developed the skin tumor at 16 weeks after induction (Figure 1A). Quantitative analysis indicated there were  $3.86\pm2.01$  papillomas per mouse at 25 weeks after induction (Figure 1A, 1B). Histologically, chemical exposure-induced cutaneous papillomas always showed visible erosion or ulceration (Figure 1C–1G), which included many neoplastic mitotic cells and atypical epithelium, as well as an asymmetric outline, the hallmarks of early-stage cSCC.

## Allogeneic tumor tissue transplantation from FVB/N mice to ICR mice produced short-term graft-versus-tumor effects but not long-term efficacy

To test the safety of allogeneic tumor transplantation, we first quantitatively assessed the numbers of tumor cells in tumors and found that the numbers of tumor cells were correlated linearly with the tumor weights (Figure 2A). Furthermore, we transplanted different doses of skin tumor tissues from FVB/N mice to healthy ICR mice and we found that transplantation with any of the doses of skin tumor tissues did not affect BW and BCS (Figure 2B, 2C). Measurements of tumors revealed that the transplanted tumor grew rapidly and the tumor volumes peaked at 8 days after inoculation and then gradually declined (Figure 2D). While most transplanted tumors disappeared after 20 days, only 1 tumor from the high-dose group grew back after transiently shrinking. These data suggest that transplantation with no more than 0.03 g tumor tissues was safe in mice.

Next, we tested whether transplantation with allogeneic skin tumors could inhibit the progression of chemical-induced skin tumors. ICR mice were chemically induced for the development of skin tumors and randomized into 2 groups (Table 1): the experimental and control groups (Figure 3). Following induction, all mice developed skin tumors at 12–13 weeks after induction and the mean numbers of papillomas were 3.95±2.32 and 3.58±2.04 per mouse in the experimental and control mice at 24 weeks after induction (Figure 4A, 4B). Histological analysis revealed that there was visible erosion or ulceration in the skin papilloma with different degrees of atypical hyperplasia (Figure 4C–4H).

Subsequently, the tumor-bearing ICR mice in the experimental group were subcutaneously transplanted 3 times with 0.03 g



Figure 1. Chemical induction of skin tumors in FVB/N mice using DMBA-TPA protocol and histopathological examination. (A, B) The growth kinetics of induced tumors and the mean numbers of induced papillomas in mice. Data are the mean values of 31 mice. (C, D) The representative images of FVB/N mice with tumors on the dorsal skin. (E) The representative tumor tissue in FVB/N mice. (F, G) HE staining showed skin severe atypical hyperplasia and squamous cell carcinoma. Pathologic tissue section No. FVB08. Bar: F, G, 20 μm.



Figure 2. Transplantation with allogenic skin tumors from FVB/N mice to healthy ICR mice. (A) A linear correlation was set up between the cell numbers and tumor tissue weights. (B) The changes in the body weights following transplantation with different weights of skin tumors. (C) The changes in the values of body conditioning scores. (D) The volumes of transplanted tumors in ICR mice. Data are expressed as the mean ±SD of each group (n=6 per group).



Figure 3. The illustration of experimental groups.

skin tumors from FVB/N mice, and the tumor-bearing ICR control mice received the sham surgery (Figure 5A, 5B). Following transplantation, the tumors transplanted the first and second time grew slowly and the tumor volumes peaked at 4 weeks after transplantation and then gradually disappeared (Figure 5C). Interestingly, the tumors transplanted the third time grew rapidly and peaked at 6 weeks after transplantation. Subsequently, most tumors disappeared except for 1 mouse with transplanted tumor, which grew continuously, and the mouse died on day 173. Further analyses indicated that BW, BCS, and tumor volumes were similar in the experimental and control mice (Figure 5D–5F). Furthermore, there was no significant difference in the survival periods between the 2 groups of mice (Figure 5H). However, PET-CT imaging revealed that there were 4 experimental mice with obvious metastatic tumors, while there were 9 control mice with metastatic tumors at 24 days after transplantation (Figure 5G, Table 2). Collectively, these data suggest that transplantation with allogeneic skin tumor inhibited the early growth and metastasis of skin tumors, but did not affect the overall survival of tumor-bearing mice.

# Cytokine changes after the allogeneic skin tumor tissue transplantation

To further understand the effect of transplantation with an allogeneic skin tumor, we tested the levels of serum cytokines by CBA. In comparison with that before transplantation, the levels of IL-2, IL-4, IL-6, and IFN- $\gamma$  in the control group decreased significantly at 1 week after transplantation, (*P*<0.05, Figure 6). In contrast, the levels of IL-2, IL-4, and IFN- $\gamma$  in the experimental group only decreased slightly, but IL-6 increased slightly. Two weeks after the transplantation, the level of IL-2



Figure 4. Chemical induction of skin tumors in ICR mice. (A, B) The growth kinetics of induced papillomas and the average numbers of papillomas per mouse. (C, D) The representative HE staining shows mild atypical hyperplasia. (PTS No. ICR1004). (E, F) The representative HE staining shows moderate atypical hyperplasia (PTS No. ICR2702). (G, H) The representative HE staining shows severe atypical hyperplasia and cutaneous squamous cell carcinoma *in situ* (PTS No. ICR0405) Bar: C–F, 20 µm. PTS – pathological tussue section



Figure 5. The effects of transplantation with allogeneic tumors in tumor-bearing ICR mice. (A) The schematic diagram of the transplantation procedure. (B) The average weights of transplanted tumor tissues from FVB/N mice. (C) The change in the volumes of transplanted tumors in ICR mice. (D) The changes in the body weights in ICR mice. (E) The changes in the values of BCS in ICR mice. (F) The volumes of endogenous skin tumors following transplantation with allogeneic skin tumors.
(G) The PET-CT images before and 24 days after allogeneic skin tumor transplantation. (H) The overall survival of mice.

 Table 2. PET-CT examination before and after allogenic skin tumor tissue transplantation.

	No. of cases	PET-CT assessment* N (%)	Р
Experiment group	19	4 (21.05)	0.085
Control group	19	9 (47.37)	

\* Progress 24 days after transplantation.





was still significantly lower (P<0.05), while IL-2 in the experiment group remained stable. The levels of TNF- $\alpha$  and IL-17A in these 2 groups increased significantly (P<0.05). Unexpectedly, the levels of IL-10 in the experiment group increased significantly (P<0.05), while they increased moderately in the control group. There was no significant difference between these 2 groups. Therefore, the short-term efficacy of tGVT may be correlated with the change of IL-2.

# Discussion

To evoke GVT immunity using allogeneic transplantation is an attractive method to treat cancer, and many experiments have verified the anticancer effect of GVT. While natural antitumor immunity exists in humans [20], transplantation with ethanol extracts of homologous tumors does not have a therapeutic effect [34]. Interestingly, the effectiveness of GVT was also observed in mammary carcinoma and renal cell carcinoma, which focusses increased attention on allogeneic transplantation [35,36]. In this study, we employed a protocol of mouse two-stage skin carcinogenesis using DMBA/TPA to induce skin tumors in 2 different strains of mice to test the tGVT effect of transplantation with allogeneic skin tumors on the growth of chemically-induced tumors in the recipients.

Previous studies have suggested that tumor growth is associated with immunosuppression [37,38], and high levels of novel antigens can be detected at early stages of chemically-induced tumors [37,39]. In our experiment, we found that all mice exposed to chemicals developed typical papillomas at 12–13 weeks after induction, similar to that of previous reports [40]. We chose to transplant mice with early-stage skin tumors 3 times to induce potent tGVT responses. We found that the transplanted tumors in the first and second transplantation disappeared after short-term increase, while in the third transplantation the transplanted tumor in 1 mouse continually grew until the mouse died. These data suggest that continual transplantation with the same allogeneic skin tumors may induce immune suppression and tolerance, which promotes the growth of transplanted allogeneic tumors in recipients.

There have been many attempts to treat solid tumors using allogeneic tumor cells or gene-modification allogeneic tumor cells [17,18]. In our study, we also found that transplantation with allogeneic skin tumors slightly reduced the volumes of endogenous skin tumors and the percentages of mice with metastatic tumors in the recipients for a short period of about 24 days after the transplantation, but with no long-term efficacy. These data indicate that transplantation with allogeneic skin tumors could temporarily inhibit the growth and metastasis of endogenous skin tumors in the recipients. Indeed, a previous study has shown that transfused donor WBCs exist for a short period in patients who received multiple blood transfusions [41] and activated antitumor T lymphocytes maintain in peripheral blood for a short period or even become anergic within 2 weeks following transition [42]. Apparently, transplantation with allogeneic skin tumors induced certain tGVT responses, which temporarily inhibited the growth and metastasis of endogenous skin tumor in the recipients.

The changes in the levels of serum cytokines may represent the degrees of acute and chronic allogeneic rejection response [23]. In this study, we found significantly reduced levels of IL-2, IL-4, IL-6, and IFN- $\gamma$  in the control mice, but not the recipients at 1 week after transplantation; as well as lower level of IL-2 in the control group, higher levels of IL-17 and TNF- $\alpha$  in the both groups of mice, and significantly higher levels of serum IL-10 in the recipients at 2 weeks after transplantation, similar to that of previous reports [23,43]. Our results showed that IL-2 decreased significantly in the control group, but were stable in the experimental group, which may be correlated with the short-term efficacy. TNF- $\alpha$  and IL-17 increased with the growth of the tumor in the 2 groups, supporting the notion that they may be tumor-promoting factors, linking inflammation and cancer [44,45], although high levels of TNF- $\alpha$  can inhibit tumor development [46], and IL-17 may take part in rejection reaction [26]. We also detected higher levels of serum IL-10 in the recipients. Although IL-10 has been considered an inhibitory cytokine associated with regulatory T and B cell responses, IL-10 can also promote CD8+ T cell responses [47]. IL-10 may be involved in the maintenance of CTL in the recipients. Therefore, the changes in the levels of serum cytokines may be associated with temporary inhibition of the growth and metastasis of endogenous skin tumors in the recipients.

#### Conclusions

Our data indicate that transplantation with no more than 0.03 g allogeneic skin tumors to healthy mice was safe, and transplantation with allogeneic skin tumors to tumor-bearing mice can temporarily inhibit the growth and metastasis of endogenous skin tumors in the recipients, which may be correlated with the tGVT response mediated by the alteration of IL-2. Our findings suggest that transplantation with allogeneic skin tumors may have a temporary antitumor effect correlated with tGVT, but there is still much work needed to magnify the tGVT response and improve the long-term efficacy.

#### **Conflicts of Interest**

All the authors have no financial conflicts of interest with regard to this work.

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