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Skin Allografting Activates Anti-tumor Immunity and Suppresses Growth of Colon Cancer in Mice

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Abstract

INTRODUCTION: The tumor cells could escape from the immune elimination through the immunoediting mechanisms including the generation of immunosuppressive or immunoregulative cells. By contrast, allograft transplantation could activate the immune system and induce a strong allogenic response. The aim of this study was to investigate the efficacy of allogenic skin transplantation in the inhibition of tumor growth through the activation of allogenic immune response. **METHODS**: Full-thickness skin transplantation was performed from C57BL/6 (H-2^b) donors to BALB/c (H-2^d) recipients that were receiving subcutaneous injection of isogenic CT26 colon cancer cells (2×10^6 cells) at the same time. The tumor size and pathological changes, cell populations and cytokine profiles were evaluated at day 14 post-transplantation. **RESULTS**: The results showed that as compared to non-transplant group, the allogenic immune response in the skin-grafting group inhibited the growth of tumors, which was significantly associated with increased numbers of intra-tumor infiltrating lymphocytes, increased populations of $CD11c^+$MHC-classII$^+CD86^+$ DCs, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD19^+$ B cells, as well as decreased percentage of $CD4^+CD25^+Foxp3^+$ T cells in the spleens. In addition, the levels of serum IgM and IgG, tumor necrosis factor (TNF)-α and interferon (IFN)-γ were significantly higher within the tumor in skin transplant groups than that in non-transplant group. **CONCLUSIONS**: Allogenic skin transplantation suppresses the tumor growth through activating the allogenic immune response, and it may provide a new immunotherapy option for the clinical refractory tumor treatment.

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Introduction

It has been generally well-known that tumors are genetically unstable [1]. During the process of the tumor formation and development, tumor-specific antigens could be produced due to gene mutation and some other reasons [2,3], and then tumor cells become the targets of the immune cells [4], which is termed as immune elimination. However, with the tumors' continuous development and variation, as well as under the immune selection, more variants appeared in tumors. At this time, the tumors and the immune system are in the phase of immune equilibrium, some tumors are eliminated, but the other tumors with low immunogenicity survived and then they enter the phase of immune escape. Simultaneously, these tumors can also change their microenvironment to establish an immunosuppressive network by both inducing the production of immunosuppressive or immunoregulative cells, including tolerogenic dendritic cells (Tol-DCs), tumor associated macrophages, regulatory T cells (Tregs), regulatory B cells (Bregs) [5–7], and secreting cytokines, such as interferon (IL)-10 [8], transforming growth factor (TGF)-β [9], and vascular endothelial growth factor (VEGF) [10]. The whole cascade network mentioned above is also called tumor immunoediting [11]. Eventually, the tumors could escape from the immune clearance, expand and metastasize to other parts of the body.

The activation of the immune system has been pursued as an important strategy in the development of cancer treatment. In 1890, Coley et al. found that erysipelas could induce the immune system to combat the tumors, and up to now, immunotherapy has been developed gradually as an option for the treatment of cancer [12–14]. The anti-tumor effect of erysipelas is mainly through the activation of the immune system by the bacteria such as *Streptococcal*. Similarly, there are many other ways to activate the immune system. As we know, allograft transplantation could induce allogenic immune response effectively as it induces a strong immune-mediated transplant rejection. Numerous types of immune cells, antibodies and cytokines would be activated and produced against the allograft [15,16]. It had been proposed in theory that the immune cells that are activated by the allografts could also act on the tumors, as both of them have allogeneic or new antigens [17], and this proposal was first examined in a mouse skin transplantation model [18]. In addition, one clinical study shows some benefit of this strategy in some patients with hormone-refractory prostate cancer [19].

In comparison with other tissues the skin has the higher antigenicity [20] that could induce a strong allogenic immune response. At the same time, the skin transplantation has the several advantages, for examples, the skin grafts are easy to access, the operative procedure is simple with less trauma, and the observation of the rejection is easy. The skin allografts began to fell of between post-operative days 10–14. The objective of our current study was to investigate the inhibiting effect of allogenic skin transplantation on the growth of the CT26 murine colon cancer cells in mice.

Materials and Methods

Animals

Female adult BALB/c (H-2^d) and C57BL/6 (B6) (H-2^b) mice with 6–8 weeks old weighting 18-20 g were purchased from China Food and Drug Inspection Institute (Beijing, China). The mice were housed in the animal facility under a conventional experimental environment at Tianjin General Surgery Institute (Tianjin, China), and provided with water and chow ad libitum. All the experiments were performed on the basis of protocols approved by the Animal Care and Use Committee of Tianjin Medical University (Tianjin, China), according to the Chinese Council on Animal Care guidelines.

Growth of CT26 Cancer Cells

The CT26 murine colon cancer cell line is derived from a BALB/c mouse and was purchased from the Tumor Center of Chinese Academy of Medical Sciences (Beijing, China). Cancer cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (HyClone Laboratories, Logan, UT, USA) medium supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin (Gibco, Shanghai, China) in a 37 °C 5% CO_2 incubator. Cells at 75–80% confluence were used for the experiments. Serum-free media (100 μl) containing 2×10^6 cells (cell viability ≥95%) were slowly injected subcutaneously into the right neck of mice by a 30-gauge needle. Tumor growth rates were evaluated at intervals of 3 days after tumor initiation, and was determined by the volume of tumors using the formula $V = 0.5 \times L \times W^2$ [21], whereas L was the length of the tumor and W the width of the tumor.

Allogenic Skin Transplantation and Experimental Groups

Full-thickness skin grafts from B6 mice were collected and cut into pieces measuring 1×1 cm^2, and then the grafts were transplanted onto the back of BALB/c recipient mice [22] at the same day of tumor cells injection. There were four experimental groups (n = 6, each group): (1) normal control group, without tumor cells injection or skin transplantation; (2) skin transplant alone group, with skin transplantation only; (3) tumor alone group, with tumor cells injection only; and (4) tumor with skin transplant group, with both tumor cells rejection and skin transplantation at the same day.

Histological examination

To examine the suppressive effect of skin transplantation on the proliferation of tumor cells, the tumors from both tumor alone group and tumor with skin transplant group were collected at day 14 post-transplanted and fixated in 10% formalin. These tissues were then embedded in paraffin and sectioned at 4 μm for hematoxylin and eosin (H&E) staining. The infiltration of immune cells in tissue sections were examined under light microscopy.

Fluorescence-Activated Cell Sorting (FACS) Analysis

The spleens from mice in each group were collected at day 14 post-transplantation, grinded and passed through sterilized meshes (100 meshes) to obtain a homogeneous cell suspension. After the red blood cells were lysed in a lysis solution, the splenocytes were washed and suspended in phosphate buffered solution (PBS). FACS analysis was performed as previously described [23], to determine the phenotype of immune cells in splenocytes based on the positive stain with antibodies against CD3*e*, CD4, CD8*a*, CD11c, CD19, CD25, CD86, Foxp3, or MHC class II. The levels of circulating $CD3e^{+-}$ IgM^+ and $CD3e^+IgG^+$ antibodies in the sera of BALB/c mice were also measured by using FACS [24]. The sera were 1:20 diluted in PBS and incubated with splenocytes (5×10^5 cells) of B6 mouse at 37 °C for 30 min. Then after being washed, the splenocytes were double stained with anti-mouse CD3*e* antibody and antibodies against either IgM or IgG. All fluorescent-labeled antibodies were purchased from *e*Bioscience (*e*Bioscience, San Diego, CA, USA). The percentage of

each phenotype of immune cells or antibodies was analyzed by using Flowjo software.

Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of tumor necrosis factor (TNF)-α and interferon (IFN)-γ in BALB/c mice in each group were measured by the ELISA kit (*e*Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The optical density (OD) value was measured through the Microplate Reader (Tecan, Männedorf, Switzerland).

Statistical Analysis

The experimental data were presented as mean ± standard error of the mean (SEM). The differences between two groups were analyzed using independent- sample t test and the differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) following by post hoc analysis with the least significant difference (LSD) test. $P < .05$ was considered statistically significant.

Results

Skin Transplantation Inhibited Tumor Proliferation and Increased Intra-Tumor Immune Cell Infiltration

All the skin allografts had fallen off because of the rejection. In order to evaluate the suppressive effect of allogenic skin transplantation on the proliferation of tumors, the tumor size or volume was calculated at intervals of 3 days after tumor initiation. As shown in Figure 1, *A* and *B*, the tumor volume in tumor with skin transplant group was reduced significantly as compared to the tumor alone group at days 8, 11 and 14 (day 8: 57.67 ± 7.10 mm^3 vs. 107.24 ± 10.00 mm^3, $P < .001$; day 11: 128.67 ± 20.01 mm^3 vs. 216.68 ± 29.87 mm^3, $P < .001$; day 14: 198.64 ± 31.59 mm^3 vs. 352.19 ± 32.39 mm^3, $P < .001$). In addition, the H&E staining of the tumors showed that more lymphocytes infiltrated in the tumor with skin transplant group than that in the tumor alone group (Figure 1*C*). These results demonstrate that allogenic skin transplantation could increase intra-tumor immune cell infiltration, and inhibit the growth of tumors.

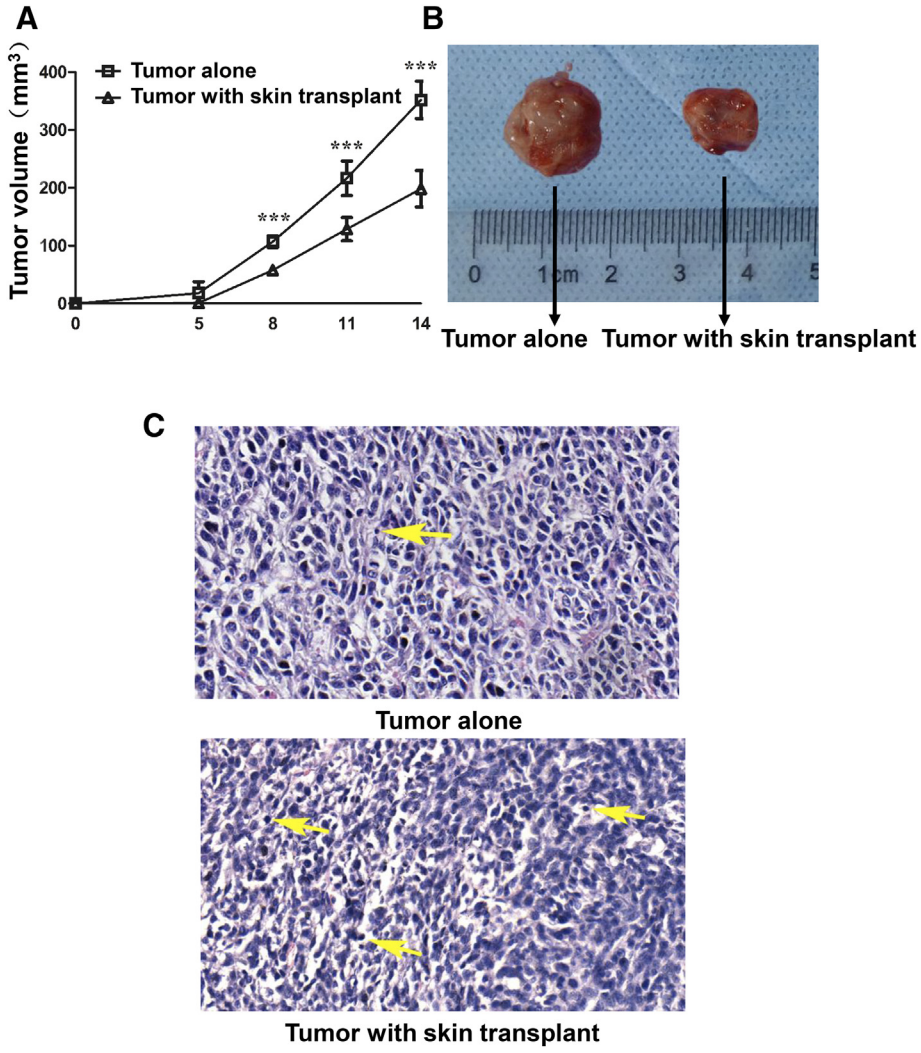

Figure 1. Skin transplantation inhibited tumors proliferation and increased immune cells infiltration. (a) The trend of tumor volume in both tumor alone group and tumor with skin transplant group at post-transplanted day 8, 11 and 14. Values were presented as mean ± SEM, statistical analysis was done by independent-sample t test, n = 6. *** indicated $P < .001$. (b) Gross pathological of tumors at post-transplanted day 14. (c) Histology of tumors at day 14 (400× magnification, H&E staining). More lymphocytes infiltrated in the tumors in the tumor with skin transplant group than that in the tumor alone group. The yellow arrows indicated the infiltrated lymphocytes.

Skin Transplantation Increased the Percentage of Mature DCs

To explore the population of mature DCs in different groups, the DC population in splenocytes was identified by double staining with anti-mouse CD11c antibody, together with either anti-MHC class II antibody or anti-CD86 antibody. The DC maturation were determined by expressing high levels of both CD11c and antigen presenting molecule MHC class II or co-stimulatory molecule CD86 on DC surface through FACS analysis. As shown in Figure 2, the expression of these two markers in skin transplant alone group were higher than those of normal control group (MHC class II, $P = .002$; CD86, $P = .11$), and were further increased in the tumor with skin transplant group (MHC class II, $P < .001$; CD86, $P < .001$). At the same time, the population of mature DCs in the tumor with skin transplant group was significantly increased as compared with that in the tumor alone group (MHC class II, $P < .001$; CD86, $P < .001$), which indicate that the allogenic skin transplantation increases the splenic mature DCs that could capture and present tumor/allogenic antigens.

Skin Transplantation Increased the Population of T Cells but Decreased the Population of Tregs

Generally, the mature DCs could present antigens to both $CD3e^{+-}$ $CD4^+$ T-helper (Th) cells and $CD3e^+CD8a^+$ cytotoxic T lymphocytes (CTLs), both of them are essential for adaptive immune system and play a key role in cellular immunity. In contrast, the $CD4^{+-}$ $CD25^+Foxp3^+$ T cells, known as Tregs, can induce tolerance to the tumors. Thus, in this study we have measured the populations of these three types of T cells in these experimental groups. The $CD3e^+$

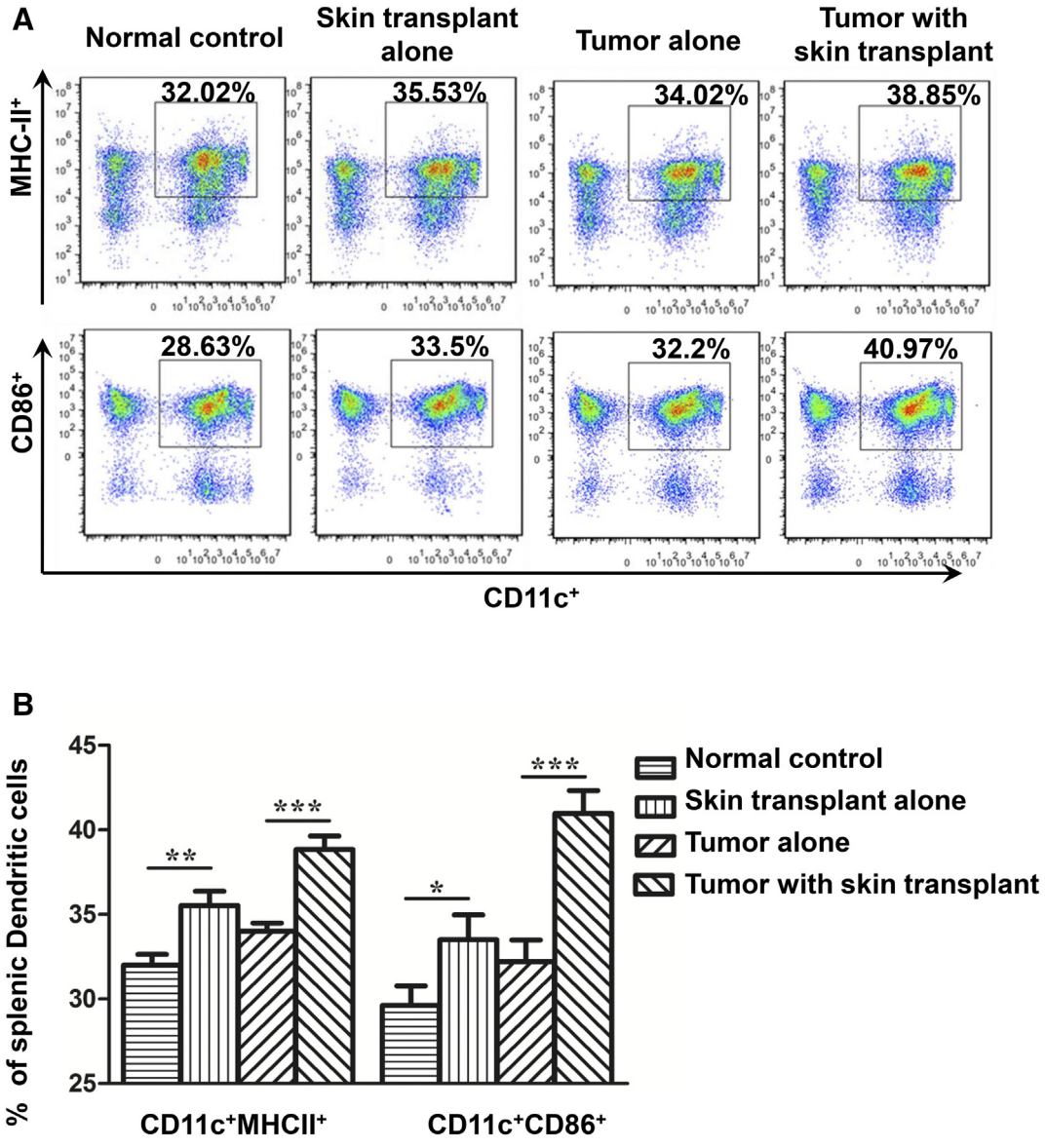

Figure 2. Skin transplantation increased the percentage of mature DCs. FACS analysis of mature DCs was performed in the splenocytes. (a) Dot plots of $CD11c^+$MHC class II^+ DC and $CD11c^+CD86^+$ DC in each group. (b) Percentage of $CD11c^+$MHC class II^+ DC and $CD11c^+CD86^+$ DC in each group. Values were presented as mean ± SEM, statistical analysis was done by one-way ANOVA followed by the LSD test, n = 6. * indicated $P < .05$, ** indicated $P < .01$, *** indicated $P < .001$.

T cells were isolated from the splenocytes of BALB/c mice, and combination with either anti-mouse CD4 antibody or ant-mouse CD8*a* antibody was used to measure Th cells and CTLs, respectively. As expected (Figure 3), the percentages of $CD3e^+CD4^+$ and $CD3e^+CD8a^+$ T cells in normal control group were lower than those in skin transplant alone group ($CD3e^+CD4^+$, $P = .001$; $CD3e^+CD8a^+$, $P = .009$), and further lower than those in tumor with skin transplant group ($CD3e^+CD4^+$, $P < .001$; $CD3e^+CD8a^+$, $P < .001$). At the same time, we found that the populations of both $CD3e^+CD4^+$ and $CD3e^+CD8a^+$ T cells in the tumor with skin transplant group were extremely higher than those in the tumor alone group ($CD3e^+CD4^+$, $P = .002$; $CD3e^+CD8a^+$, $P < .001$).

In the analysis of Tregs, the cells were firstly gated by anti-mouse CD4 antibody in the splenocytes, followed by the double positive staining of anti-mouse CD25 and Foxp3 antibodies. As shown in Figure 3, the percentage of Tregs was lower in the skin transplant

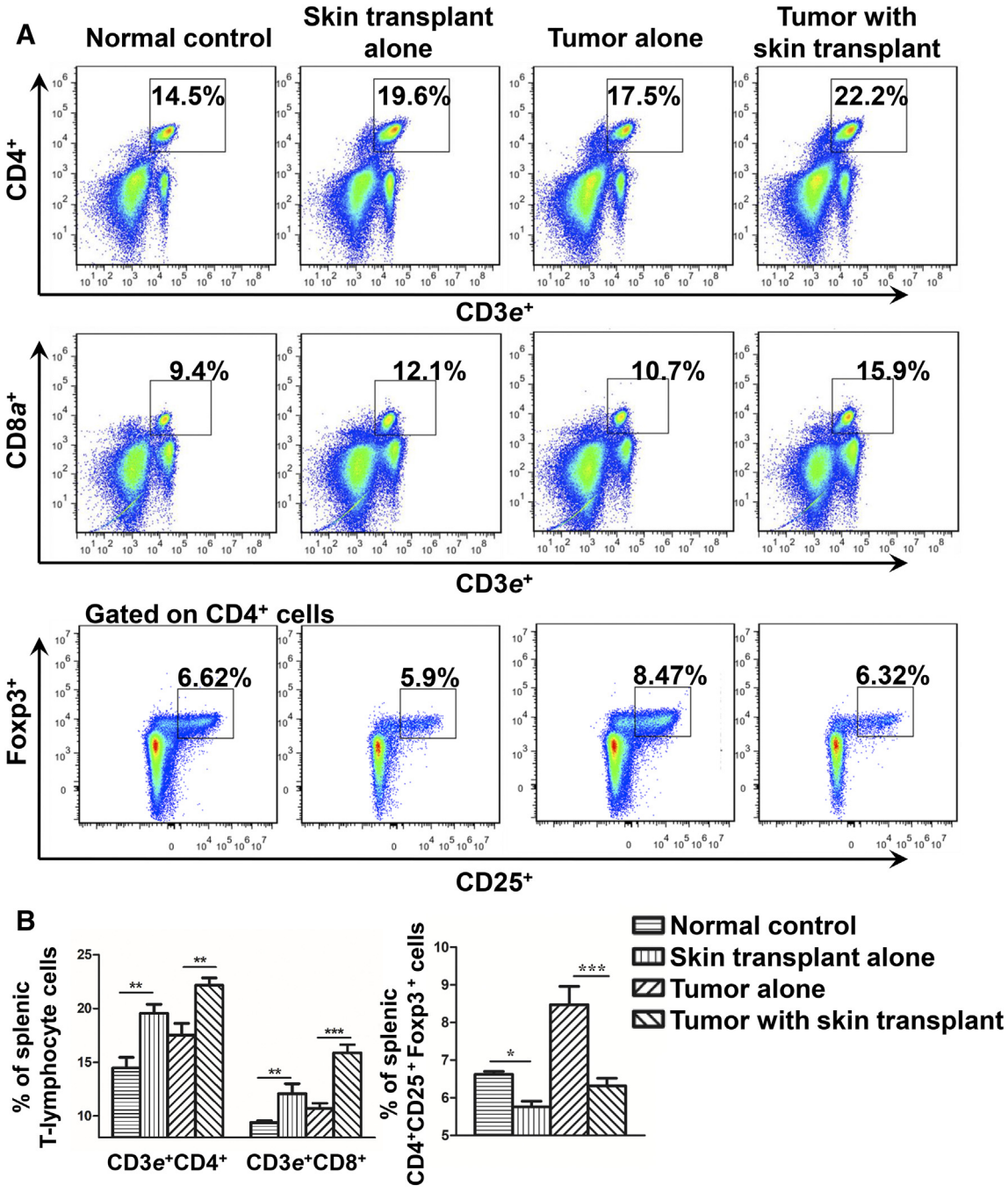

Figure 3. Skin transplantation increased the percentage of T cells and decreased the percentage of Tregs. FACS analysis of T cells and Tregs was performed in the splenocytes. (a) Dot plots of $CD3e^+CD4^+$ T cells, $CD3e^+CD8a^+$ T cells, and $CD4^+CD25^+Foxp3^+$ T cells in each group. (b) Percentage of $CD3e^+CD4^+$ T cells, $CD3e^+CD8a^+$ T cells, and $CD4^+CD25^+Foxp3^+$ T cells in each group. Values were presented as mean ± SEM, statistical analysis was done by one-way ANOVA followed by the LSD test, n = 6. * indicated $P < .05$, ** indicated $P < .01$, *** indicated $P < .001$.

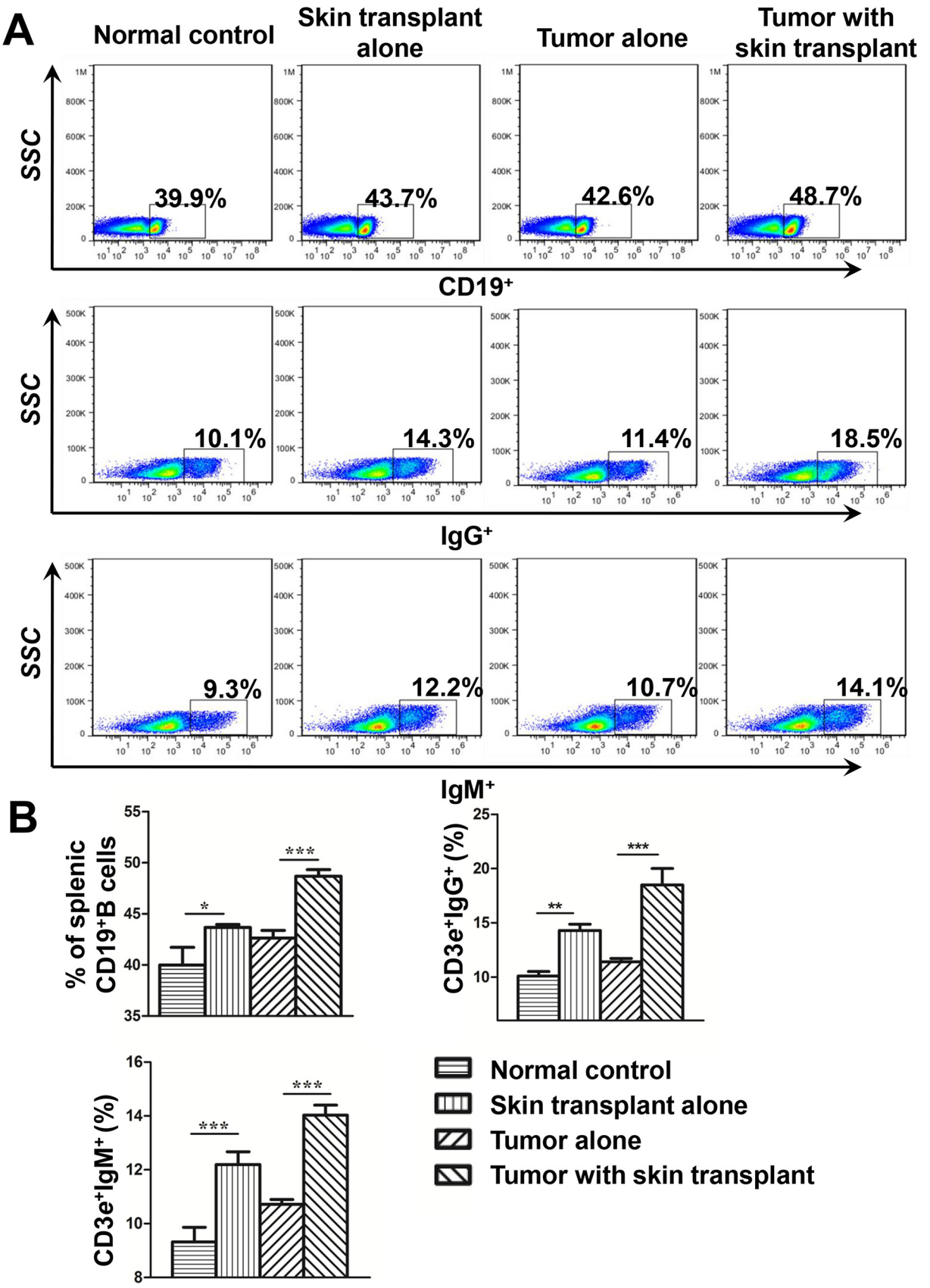

Figure 4. Skin transplantation increased the percentage of B cells, CD3e^+IgM$^+$ and CD3e^+IgG$^+$ antibodies. FACS analysis of B cells, CD3e^+IgM$^+$ antibodies and CD3e^+IgG$^+$ antibodies was performed in the splenocytes. (a) Dot plots of CD19$^+$ B cells, CD3e^+IgM$^+$ antibodies and CD3e^+IgG$^+$ antibodies in each group. (b) Percentage of CD19$^+$ B cells, CD3e^+IgM$^+$ antibodies and CD3e^+IgG$^+$ antibodies in each group. Values were presented as mean ± SEM, statistical analysis was done by one-way ANOVA followed by the LSD test, n = 6. * indicated $P < .05$, ** indicated $P < .01$, *** indicated $P < .001$.

alone group than that in the normal control group ($P < .05$), but higher in the tumor alone group than that in the normal control group ($P < .001$). However, as compared to the tumor alone group, skin grafting could significantly decrease the percentage of tumor-specific Tregs ($P < .001$). Taken together, these data indicate that allogenic skin transplantation could active cellular immune system and suppress tumor-specific Tregs, which is associated with its anti-tumor effect.

Skin Transplantation Increased B Cell Population, and CD3e$^+$IgM$^+$ and CD3e$^+$IgG$^+$ Antibody Levels

B cells mainly play humoral immunity by secreting antibodies such as CD3e^+IgM$^+$ and CD3e^+IgG$^+$ in the cancer treatment [25,26]. Thus, we have measured the percentage of CD19$^+$ B cells in the splenocytes, as well as CD3e^+IgM$^+$ and CD3e^+IgG$^+$ antibodies in the sera. The results in Figure 4 showed that the percentage of B cells, CD3e^+IgM$^+$ and CD3e^+IgG$^+$ antibodies in both skin transplant alone group and tumor with skin transplant group were all higher than that in normal control group (skin transplant alone vs. normal control: B cells, $P = .018$; CD3e^+IgM$^+$, $P < .001$; CD3e^+IgG$^+$, $P = .002$; tumor with skin transplant group vs. normal control: B cells, $P < .001$; CD3e^+IgM$^+$, $P < .001$; CD3e^+IgG$^+$, $P < .001$). Further, the treatment effect of skin transplantation was also associated with an increase in the percentage of B cells, CD3e^+IgM$^+$ and CD3e^+IgG$^+$ antibodies in the tumor with skin transplant group compared to the tumor alone group (B cells, $P < .001$; CD3e^+IgM$^+$, $P < .001$; CD3e^+IgG$^+$, $P < .001$). These data may suggest that allogenic skin transplantation can also active humoral immune system in its anti-tumor activity.

Skin Transplantation Increased Serum Levels of TNF-α and IFN-γ

The serum concentrations of TNF-α and IFN-γ were measured by using ELISA in the experimental groups. As shown in Figure 5, the lowest concentrations of both TNF-α and IFN-γ were found in the normal control group, and the highest levels of these cytokines were detected in the tumor with skin transplant group, when compared with skin transplant alone group (skin transplant alone vs. normal control: TNF-α, $P = .005$; IFN-γ, $P = .042$; tumor with skin transplant vs. normal control: TNF-α, $P < .001$; IFN-γ, $P = .001$). In addition, the differences of the levels of TNF-α and IFN-γ between the tumor with skin transplant group and the tumor alone group were significant (TNF-α, $P = .001$; IFN-γ, $P = .012$). Taken together, allogenic skin transplantation can promote the secretion of anti-tumor cytokines (TNF-α and IFN-γ) that may be part of anti-tumor mechanisms induced by allogenic skin transplantation.

Discussion

During solid tumor development, tumor cells escape from the immune clearance, expand and metastasize gradually with the immunoediting mechanisms. For the cancer treatment, besides surgery, the more frequently used methods in clinic are chemotherapy, radiotherapy and targeted drugs. However, their side-effects cannot be ignored and limit their applications [27–29]. Therefore, in order to maximally inhibit the tumor development, much more treatment methods are needed. Immunotherapy is making an amazing progression recently, and some of them have been used in clinic, such as IL-2 [30] and IFN-α [31]. In this study, we have

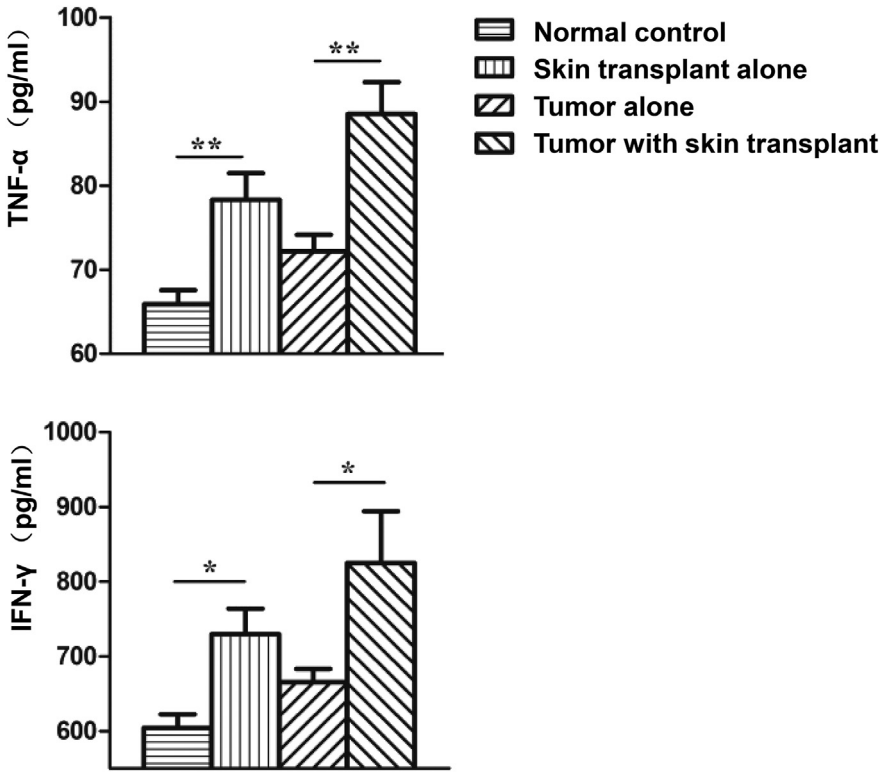

Figure 5. Skin transplantation increased the levels of serum TNF-α and IFN-γ. Serum samples were collected from mice of each group at day 14. The levels of TNF-α and IFN-γ were measured by Elisa. Values were presented as mean ± SEM, statistical analysis was done by one-way ANOVA followed by the LSD test, n = 6. * indicated $P < .05$, ** indicated $P < .01$.

demonstrated that activation of allogenic immune response by skin allografting could inhibit tumor development by affecting immune cell populations, antibody levels and cytokine profiles in a murine cancer model.

DCs, T cells, B cells are essential immunosurveillance cells in the immune system, together with the production of antibodies and cytokines, all of them present powerful immunoregulatory effect on the cells with heterologous antigens generally. DCs are well-characterized as the strongest antigen presenting cells (APCs) in immune system [32], and play a key role in phagocytosing, processing and presenting allogenic antigens, resulting in forming a bridge between innate and adaptive immune system [33,34]. The mature DCs crosstalk with and activate T cells, as well as contribute to B cell-mediated immunity, by which both of the T cells and B cells further response to the allogenic cells and tissues to achieve immune defense function [35]. However, the tumor cells can induce the generation of tumor-toleragenic DCs through influencing and suppressing the maturation of DCs mainly by secreting soluble mediators, such as VEGF and TGF-β [32]. VEGF is important for tumor neovasculature, and it can also inhibit DCs maturation, induce them apoptosis and recruit immature DCs to the tumor site [36,37]. TGF-β secreted by the tumors can downregulate the expression of DC surface markers, such as MHC class II, CD86 and CD80 [10,32]. In this study, the percentage of mature DCs in the tumor with skin transplant group was much higher than that of the tumor alone group, which was consistent with the smaller tumor size in the tumor with skin transplant group. These results may suggest that allogenic skin grafting could inhibit the growth of tumor cells by inducing the differentiation of mature DCs.

T cells play a central role in cellular immunity in adaptive immune system. Both $CD4^+$ and $CD8^+$ T cells can be activated by DCs through antigen presentation. However, as the results showed in our present study, in tumor microenvironment, the tumor-toleragenic DCs suppress the activity of T cells [38,39], and they can also induce the generation of tumor-tolerogenic Tregs [40]. Tregs, as immuno-regulatory cells, not only induce immune tolerance to the tumors, but also inhibit the activation and differentiation of $CD4^+$ and $CD8^+$ T cells [41,42]. At the same time, the tumors could also induce antigen-specific T cell tolerance [43]. However, when it comes to the field of transplantation, the situation is inverted. The allografts could induce the generation of both $CD4^+$ and $CD8^+$ T cells [44]. Thus, as shown in this study, allogenic skin transplantation could increase the percentage of both $CD4^+$ and $CD8^+$ T cells, as well as decrease the percentage of Tregs, and there together by facilitating the anti-tumor effect of the allogenic skin transplantation.

B cells, as another kind of important adaptive immune cells, play a major role in humoral immunity, by secreting antibodies such as IgM and IgG, which could come into an anti-tumor action [25,26]. Unfortunately, like T cells, the tumors can also attract naïve B cells into their microenvironment and promote their differentiation into Bregs that are tolerogenic to the tumors, and these Bregs can further suppress other effector immune cells by secreting anti-inflammatory factors such as IL-10 [45]. As both allograft and tumors have new antigens, the antibodies induced by allogenic skin transplantation can also target the tumors. Then these antibodies would directly target the tumor cells, activate the complement system and make a bridge between immune effector cells and tumor cells, finally achieve a tumor killing effect [46]. In this study, the levels of both IgM and IgG were elevated in the tumor with skin transplant group compared to those of the normal control group and the tumor alone group. This finding may indicate that humoral immunity enhanced by the skin transplantation is an important part of anti-tumor activity.

Cytokines play important roles in both tumor immunity and transplantation immunity. In this study, we measured the levels of TNF-α and IFN-γ to investigate the anti-tumor mechanisms of the skin transplantation. TNF-α is a pleiotropic cytokine that plays important roles in host defense, inflammation and apoptosis [47]. It was first described in 1975 for causing different transplanted tumors hemorrhagic necrosis in vivo and a mouse fibrosarcoma cell line cytolysis in vitro [47]. IFN-γ can be produced by both innate and adaptive immune cells and plays a role of preventing the development of tumors [47,48]. During the process of allograft rejection, the levels of TNF-α and IFN-γ are increasing [49,50]. In this study, the results have shown that both the serum levels of TNF-α and IFN-γ were much higher in the tumor with skin transplant group than those in both the normal control and the tumor alone groups, suggesting that skin allografting has a therapeutic effect on tumor suppression.

In fact, the idea of using allogenic skin transplantation for cancer treatment had been tested previously. Yolcuoğlu [18] and colleagues showed that the rejection caused by allogenic skin graft, but not autograft, could help the rejection of tumor cells. However, they came into this conclusion only by measuring survival time of mouse, without the further mechanism research. Muir [19] and colleagues carried out a clinical study to observe the treatment effect of full-thickness skin transplantation from different, unrelated donors on the hormone-refractory prostate cancer by measuring the changes of serum prostate-specific antigen (PSA). For each patient, the transplant procedure was repeated about every two weeks and six skins from different donors were received. Some of the patients who participated and completed the study had a declined or stable serum PSA levels for 1 to 2 years. Similarly, this research showed the treatment effect mainly by the indicators of PSA, but the deeper principles did not present. In our study, compared to the previous studies, we not only demonstrated the anti-tumor effects of the skin allografting, but also explored and discussed the anti-tumor mechanisms of this strategy - mainly by activating the allogenic immune response. More concretely, the anti-tumor effects were related to the increased numbers of intra-tumor infiltrating lymphocytes, increased populations of $CD11c^+$MHC-classII^{+-} $CD86^+$ DCs, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD19^+$ B cells, as well as decreased percentage of $CD4^+CD25^{+-}$ $Foxp3^+$ T cells in the splenocyte, and further increased the levels of serum IgM and IgG donor-reactive antibodies, TNF-α and IFN-γ. Our study not only provides evidence for the further research but also theoretical basis for the clinical development of this concept.

Although the current study is very inspiring, some limitations were acknowledged, such as the mechanism of how the immune cells activated by the transplanted tissues recognize and clear the tumor cells, whether the transplanted tissues would bring some other problems to the recipients such as graft versus host disease (GVHD), need to be further elucidated in the future study.

Conclusions

In summary, in this study, we demonstrated that allogenic skin transplantation inhibited the development of the tumors through activating the immune system mainly by increasing the tumor-infiltrating lymphocytes, the percentage of DCs, T cells and B cells, and decreasing the percentage of Tregs in splenocytes, as well

as increasing the serum levels of IgM, IgG, TNF-α and IFN-γ. Although more in-depth understanding of its mechanism is needed, we provide a new idea for the immunotherapy for the tumor treatment, and it may offer alternative choice for the clinical treatment of refractory tumors.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for Publication

All authors have read the manuscript and approve of its submission.

Availability of Data and Materials

The dataset supporting the conclusions of this article is included within the article.

Authors' Contributions

X. Li, X. Lan and G. Wang are co-first authors on this paper.

X.Li designed and carried out the research, analyzed the data and drafted the manuscript; X. Lan designed and carried out the research, and drafted the manuscript; G. Wang and Y. Liu participated in research design and paper revision; K. Zhao, S. Lu, X. Xu, G. Shi, K. Ye, B. Zhang, Y. Zhao performed the research and analyzed the data; H. Han designed the research, C. Du helped to review the data and the manuscript; T. Ichim helped to review the data; H. Wang conceived of the study, participated in research design and coordination, and helped to draft and edit the manuscript. All authors read and approved the final manuscript.

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References

[1] Kendal WS and Frost P (1986). Genetic instability and tumor progression. *Pathol Immunopathol Res* **5**, 455–467.

[2] Seremet T, Brasseur F, and Coulie PG (2011). Tumor-specific antigens and immunologic adjuvants in cancer immunotherapy. *Cancer J* **17**, 325–330.

[3] Saeterdal I, Bjorheim J, Lislerud K, Gjertsen MK, Bukholm IK, Olsen OC, Nesland JM, Eriksen JA, Moller M, and Lindblom A, et al (2001). Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer. *Proc Natl Acad Sci U S A* **98**, 13255–13260.

[4] Vermeij R, Leffers N, Melief CJ, Daemen T, and Nijman HW (2012). Antigen-specific immunotherapy in ovarian cancer and p53 as tumor antigen. *Curr Pharm Des* **18**, 3804–3811.

[5] Gajewski TF, Schreiber H, and Fu YX (2013). Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* **14**, 1014–1022.

[6] Vesely MD, Kershaw MH, Schreiber RD, and Smyth MJ (2011). Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* **29**, 235–271.

[7] Quail DF and Joyce JA (2013). Microenvironmental regulation of tumor progression and metastasis. *Nat Med* **19**, 1423–1437.

[8] Urosevic M and Dummer R (2003). HLA-G and IL-10 expression in human cancer–different stories with the same message. *Semin Cancer Biol* **13**, 337–342.

[9] Platten M, Wick W, and Weller M (2001). Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech* **52**, 401–410.

[10] Liu CZ, Zhang L, Chang XH, Cheng YX, Cheng HY, Ye X, Fu TY, Chen J, and Cui H (2012). Overexpression and immunosuppressive functions of transforming growth factor 1, vascular endothelial growth factor and interleukin-10 in epithelial ovarian cancer. *Chin J Cancer Res* **24**, 130–137.

[11] Kim R, Emi M, and Tanabe K (2007). Cancer immunoediting from immune surveillance to immune escape. *Immunology* **121**, 1–14.

[12] Nauts HC, Fowler GA, and Bogatko FH (1953). A review of the influence of bacterial infection and of bacterial products (Coley's toxins) on malignant tumors in man; a critical analysis of 30 inoperable cases treated by Coley's mixed toxins, in which diagnosis was confirmed by microscopic examination selected for special study. *Acta Med Scand Suppl* **276**, 1–103.

[13] Coley WB (1991). The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin Orthop Relat Res* , 3–11.

[14] Jiang T and Zhou C (2015). The past, present and future of immunotherapy against tumor. *Transl Lung Cancer Res* **4**, 253–264.

[15] Ali JM, Bolton EM, Bradley JA, and Pettigrew GJ (2013). Allorecognition pathways in transplant rejection and tolerance. *Transplantation* **96**, 681–688.

[16] Ponticelli C (2012). The mechanisms of acute transplant rejection revisited. *J Nephrol* **25**, 150–158.

[17] Fabre JW (2001). The allogeneic response and tumor immunity. *Nat Med* **7**, 649–652.

[18] Yolcuoğlu B, Önal MA, Sipahi O, Temiz A, Pekçetin C, Açıkgöz O, Kar H, and Güre A (1999). The effect of skin transplantation on tumor growth in mice. *Am J Surg* **178**, 329–331.

[19] Muir G, Rajbabu K, Callen C, and Fabre JW (2006). Preliminary evidence that the allogeneic response might trigger antitumour immunity in patients with advanced prostate cancer. *BJU Int* **98**, 989–995.

[20] Murray JE (1971). Organ transplantation (skin, kidney, heart) and the plastic surgeon. *Plast Reconstr Surg* **47**, 425–431.

[21] Lee SY, Yang CY, Peng CL, Wei MF, Chen KC, Yao CJ, and Shieh MJ (2016). A theranostic micelleplex co-delivering SN-38 and VEGF siRNA for colorectal cancer therapy. *Biomaterials* **86**, 92–105.

[22] Cheng CH, Lee CF, Fryer M, Furtmuller GJ, Oh B, Powell JD, and Brandacher G (2017). Murine Full-thickness Skin Transplantation. *J Vis Exp* **119**e55105.

[23] Sun P, Liu J, Li WW, Xu XX, Gu XY, Li HY, Han HQ, Du CG, and Wang H (2016). Human endometrial regenerative cells attenuate renal ischemia reperfusion injury in mice. *J Transl Med* **14**, 28.

[24] Wang H, Qi F, Dai XC, Tian WJ, Liu T, Han HQ, Zhang B, Li HY, Zhang ZX, and Du CG (2014). Requirement of B7-H1 in mesenchymal stem cells for immune tolerance to cardiac allografts in combination therapy with rapamycin. *Transpl Immunol* **31**, 65–74.

[25] Moav N and Witz IP (1978). Characterization of immunoglobulins eluted from murine tumor cells: binding patterns of cytotoxic anti-tumor IgG. *J Immunol Methods* **22**, 51–62.

[26] Bellelli L, Nista A, and Sezzi ML (1977). IgM participation in tumor rejection by immunized mice. *Tumori* **63**, 15–24.

[27] Schneider BP, Hershman DL, and Loprinzi C (2015). Symptoms: Chemotherapy-Induced Peripheral Neuropathy. *Adv Exp Med Biol* **862**, 77–87.

[28] Yock TI and Caruso PA (2012). Risk of second cancers after photon and proton radiotherapy: a review of the data. *Health Phys* **103**, 577–585.

[29] Le DL, Cao H, and Yang LX (2014). Cardiotoxicity of molecular-targeted drug therapy. *Anticancer Res* **34**, 3243–3249.

[30] Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, Abrams J, Sznol M, Parkinson D, and Hawkins M, et al (1999). High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol* **17**, 2105–2116.

[31] Mocellin S, Pasquali S, Rossi CR, and Nitti D (2010). Interferon alpha adjuvant therapy in patients with high-risk melanoma: a systematic review and meta-analysis. *J Natl Cancer Inst* **102**, 493–501.

[32] Zong J, Keskinov AA, Shurin GV, and Shurin MR (2016). Tumor-derived factors modulating dendritic cell function. *Cancer Immunol Immunother* **65**, 821–833.
[33] Bhargava A, Mishra D, Banerjee S, and Mishra PK (2012). Dendritic cell engineering for tumor immunotherapy: from biology to clinical translation. *Immunotherapy* **4**, 703–718.
[34] Hanke N, Alizadeh D, Katsanis E, and Larmonier N (2013). Dendritic cell tumor killing activity and its potential applications in cancer immunotherapy. *Crit Rev Immunol* **33**, 1–21.
[35] Banchereau J and Steinman RM (1998). Dendritic cells and the control of immunity. *Nature* **392**, 245–252.
[36] Ohm JE and Carbone DP (2001). VEGF as a mediator of tumor-associated immunodeficiency. *Immunol Res* **23**, 263–272.
[37] Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, Kavanaugh D, and Carbone DP (1996). Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med* **2**, 1096–1103.
[38] Hurwitz AA and Watkins SK (2012). Immune suppression in the tumor microenvironment: a role for dendritic cell-mediated tolerization of T cells. *Cancer Immunol Immunother* **61**, 289–293.
[39] Shurin GV, Ma Y, and Shurin MR (2013). Immunosuppressive mechanisms of regulatory dendritic cells in cancer. *Cancer Microenviron* **6**, 159–167.
[40] Janikashvili N, Bonnotte B, Katsanis E, and Larmonier N (2011). The dendritic cell-regulatory T lymphocyte crosstalk contributes to tumor-induced tolerance. *Clin Dev Immunol* **2011**, 430394.
[41] Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, and Bandeira A (2001). CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol* **166**, 3008–3018.
[42] Khazaie K and von Boehmer H (2006). The impact of CD4+CD25+ Treg on tumor specific CD8+ T cell cytotoxicity and cancer. *Semin Cancer Biol* **16**, 124–136.
[43] Adler AJ (2007). Mechanisms of T cell tolerance and suppression in cancer mediated by tumor-associated antigens and hormones. *Curr Cancer Drug Targets* **7**, 3–14.
[44] Jiang S, Herrera O, and Lechler RI (2004). New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance. *Curr Opin Immunol* **16**, 550–557.
[45] Inoue S, Leitner WW, Golding B, and Scott D (2006). Inhibitory effects of B cells on antitumor immunity. *Cancer Res* **66**, 7741–7747.
[46] Derer S, Beurskens FJ, Rosner T, Peipp M, and Valerius T (2014). Complement in antibody-based tumor therapy. *Crit Rev Immunol* **34**, 199–214.
[47] Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, Duan S, Eiwegger T, Eljaszewicz A, and Ferstl R, et al (2016). Interleukins (from IL-1 to IL-38), interferons, transforming growth factor beta, and TNF-alpha: Receptors, functions, and roles in diseases. *J Allergy Clin Immunol* **138**, 984–1010.
[48] Dighe AS, Richards E, Old LJ, and Schreiber RD (1994). Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* **1**, 447–456.
[49] Kutukculer N, Shenton BK, Clark K, Rigg KM, Forsythe JL, Kirby JA, Proud G, and Taylor RM (1995). Renal allograft rejection: the temporal relationship and predictive value of plasma TNF (alpha and beta), IFN-gamma and soluble ICAM-1. *Transpl Int* **8**, 45–50.
[50] Azzawi M, Hasleton PS, and Hutchinson IV (1999). TNF-alpha in acute cardiac transplant rejection. *Cytokines Cell Mol Ther* **5**, 41–49.