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Research Article
Unraveling the Effect of B16 Reprogrammed by *Tremella fuciformis*-Derived Polysaccharide on Lung Metabolism and Immunity

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Abstract

Background and Objective: Therapeutics-activated tumor microenvironment immune have a vast potential for establishing effective cancer interventions. The aim of the study was to investigate the role and mechanisms of melanoma cell line B16 cells cultured by TFP (*Tremella fuciformis*-derived polysaccharide) upon the immune response in the lung through intravenous injection. **Materials and Methods:** The B16 cells were exposed to a series of dosages of TFP, melanin deposition was observed, *TYR* (Tyrosinase), *TRP1* (Tyrosinase-related protein 1) mRNA and protein were detected by RT-PCR and WB, the relationship between *TYR* and *Srebf1* (sterol regulatory element-binding factor 1) was explored and the effect of *Srebf1* on immune checkpoints were investigated, the function of TFP on B16 and Raw264.7 cells on immune-related proteins were compared. After B16 cells cultured by TFP were injected into the vein, immunofluorescence of lipid and immune-related proteins was examined. **Results:** The TFP blocked melanogenesis *in vitro* and decrease *TYR* mRNA and protein *in vivo*. The relationship between *TYR* mRNA and protein with *Srebf1* mRNA was positive and *si-Srebf1* also down-regulated ACLY (ATP-citrate lyase) and PDL1 protein expression. TFP reduced PDL1 in B16 cells and Sirpα in Raw264.7 cells significantly, TFP increased iNOS but reduced ARG1 expression in Raw264.7 cells. The B16 reprogrammed by TFP downregulated ACLY and CD47 upregulated PDL1 and CD86 and promote the lung immune response. **Conclusion:** These findings suggested that B16 reprogrammed by TFP might stimulate the immune system through metabolism regulation, a low number of B16 reprogrammed by TFP injections by the tail vein may be a way to enhance immunity against melanoma.

Key words: B16 melanoma cells, *Tremella fuciformis* polysaccharide, *Srebf1*, *TYR*, *TRP1*, immunity response

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Melanoma is the result of the malignant transformation of melanocytes[1], metabolically heterogeneous[2] and a malignant tumor with rapid disease development, few therapeutic drugs and poor efficacy[3]. Metabolism disorder is a complex symbolic feature in the pathogenesis of melanoma[4], there is an increasing interest in studying the metabolic interactions, involving multiple metabolic modes of lipid metabolism and melanin biosynthesis[5].

Lipid metabolism in melanoma cells is relatively high plasticity to ensure sufficient energy and precursors for tumor behavior and survival. Melanin biosynthesis is an important characteristic of melanoma and was modulated by various cytokines. The expression of *TYR* (Tyrosinase) and *TRP* (Tyrosinase-related protein) affects the melanin formation process[6,7]. *TYR* was thought to be the most restrictive enzyme during melanin biosynthesis[8], most skin-whitening agents are *TYR* inhibitors[9]. Therefore, it is necessary to deeply understand the process of metabolic dysregulation in melanoma.

Metabolic reprogramming of tumor cells has significant implications for immunotherapy[10]. Immunotherapies targeting tumor immune enhancement such as immune checkpoint blockade have shown considerable efficacy on multiple cancers[11]. Anti-tumor immunology has been extensively studied, including the use of tumor vaccines, adoptive lymphocyte therapy and tumor cryotherapy. They have the same mechanism of activating immune cells to kill tumor cells[12]. Adoptive cell therapy is a promising alternative approach, using *ex vivo*-expanded tumor-infiltrating immune cells such as lymphocytes and natural killer cells in tumor patients and infused into patients to attack tumor[13]. Compared with traditional chemotherapy or immunotherapy, this therapy has shown improved clinical results. However, they are limited by tumor immune escape and cytokine release syndrome[14].

The causes of immunosuppression concluded the metabolic changes of tumor cells[15]. If the metabolism of tumor cells is changed *in vitro*, then infused these reprogrammed tumor cells *in vivo*, can they achieve immune enhancement? It is necessary to study the reprogrammed metabolic statuses of melanoma *in vitro* and the impact of melanoma metabolism on the immune system *in vivo*.

In this study, *Tremella fuciformis* polysaccharide (TFP) was used to reprogramme B16 cells. The TFP is closely associated with skin whitening and immune regulation. The effect of TFP on melanogenesis and immunity was studied *in vitro* and then the function of reprogrammed B16 cells on lung immunity was explored *in vivo*. These methods will provide a new anti-tumor immune mechanism and potential immunotherapy pathways.

MATERIALS AND METHODS

Study area: The study was carried out at the Engineering Technological Center of Mushroom Industry, Minnan Normal University (April to July, 2020).

Cells and animals: The B16 mouse melanoma cells line, purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China), was grown in DMEM (Dulbecco's modification of Eagle's medium Dulbecco) media, supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C in a 5% CO_2 humidified atmosphere. The cells were exposed to a series of dosages of TFP (0, 312.5, 625, 1250, 2500 and 5000 μg mL^{-1}) for 24 hrs.

Male ICR mice (6 weeks old) were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The total 20 experimental mice were housed in a temperature and humidity-controlled laboratory environment, kept on a 12 hrs light/dark cycle and provided standard food and water. The experiment was approved by the Animal Care and Use Committee of Minnan Normal University to study the effects of reprogrammed B16 cells on immunity, B16 cells treated by TFP (2500 and 5000 μg mL^{-1}) were preincubated *in vitro* for 24 hrs, after being washed 3 times and resuspended 1×10^6 B16 cells in 100 μL of PBS, then the cells were inoculated intravenously. After 3 weeks, the mice were killed by spinal dislocation, lungs were removed.

RNA isolation and qPCR analysis: Total RNA was isolated using an RNeasy kit according to the manufacturer's protocol. The isolated RNA was reversed with M-MLV reverse transcriptase transcribed (Promega, USA) and cDNA was stored at -20°C. Real-time PCR was performed using ABI QuantStudio™ 6 Flex Sequence Detection System (Thermo Fisher Scientific). The mean concentration of the gene β-*actin* was used as an internal control for the standardization of the expression levels and analyzed using the $2^{-\Delta\Delta Ct}$ method[16]. The gene-specific primers were listed in Table S1.

RNA interference (siRNA): The B16 cells were inoculated in a 6-cell plate overnight and 1 mL Opti-MEM was used to replace the culture media before transfection. Cells were transfected with *Srebf1* siRNA or nonspecific control by using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Briefly, a 20 nM final concentration of siRNA was used to transfect B16 cells at 60-70% confluency. The B16 cells were placed in full serum after 24 hrs of transfection and harvested after 48 hrs of transfection.

Table S1: Target gene and their PCR primers

Target gene	Primer	Sequence	Product size
TYR	Forward	5'TCTTCAGCAGATGTGGAATT 3'(20)	484 bp
	Reverse	5'AGATACGACTGGCTTGTTC 3'(19)	
TRP 1	Forward	5'TACAGTGGAAGGTTACAGTG 3'(20)	499 bp
	Reverse	5'AGCATAGCGTTGATAGTGAT 3'(20)	
β-*actin*	Forward	5'GAGACCTTCAACACCCCGC 3'(19)	446 bp
	Reverse	5'CCACAGGATTCCATACCCAA 3'(20)	

Western blotting (WB): The protein levels were examined by Western blotting. Wash cells twice with cold PBS and lysed in lysis buffer. The lysates were dissolved in 8-10% SDS polyacrylamide gel and transferred to NC membrane, blocked in 5% nonfat milk and blotted with antibodies: Anti-TYR and anti-*TRP1* (1:1000, Abnova). After incubation with peroxidase-conjugated secondary antibodies (1:8000, R&D system), the immune reactivity was detected using an ECL solution. Densitometric analyses of bands were performed by ImageJ software (National Institutes of Health, Bethesda, USA).

Immunohistochemistry: For immunohistochemistry, tissue sections were deparaffinized with xylene and gradually rehydrated with a series of diluted ethanol. After epitope retrieval and endogenous horseradish peroxidase activity quenched, the tissue sections were then blocked in goat serum and incubated with rabbit anti-mouse (ACLY, CD47) and mouse anti-mouse antibody (PDL1, CD86) at 4°C overnight. Immunofluorescent-stained sections by an anti-rabbit Alexa Fluor 647 and an anti-mouse FITC conjugated antibody were visualized using a confocal laser scanning microscope (Leica TCS SP8, German).

Statistical analysis: Pearson's correlation coefficient (r) (for *Srebf1* mRNA with *TYR*) was analyzed by GraphPad Prim 5. The level of significance was set to $p<0.05$.

RESULTS

TFP decreased melanogenesis through decreasing *TRP1* and *TYR* levels *in vitro*: In this study, B16 cells showed a marked increase in pigmentation as compared to B16 treated with TFP of various dosages (Fig. 1a).

Moreover, to investigate the expression of melanogenesis enzymes after B16 exposure to various dosages of TFP, the levels of *TYR*and *TRP1* were detected by western blotting and RT-PCR analysis (Fig. 1b-c), the results showed that the protein and mRNA levels of *TYR* treated by TFP were significantly decreased compared with vehicle, while that of *TRP1* was not in that way by TFP, however, protein levels decreased according to increased TFP dosage.

Relationship between *TYR* mRNA and protein with *Srebf1* mRNA: Then the association of *TYR* with *Srebf1* using real-time PCR was detected (Fig. 2a). There seemed to be a positive relationship between *TYR* and *Srebf1* ($r = 0.2832$, $p = 0.0411<0.05$). Then *Srebf1* in B16 cells was inhibited with si-RNA, especially the mature SREBP1 (68kDa) expression was significantly inhibited (Fig. 2b). How was the correlation between *Srebf1* mRNA and *TYR*, *TRP1*, ACLY (Fig. 2c)? After *Srebf1* inhibition, a marked decrease in *TYR* and ACLY expression were observed, *TRP1* was slightly declined.

These data showed that TFP can synergistically regulate *TYR* and ACLY protein expression dependent in *Srebf1* mRNA manner.

TFP-regulated PDL1 and CD47 in dependent on *Srebf1* mRNA or not: Whether there was a correlation between SREBP1 and CD47, PDL1? CD47 expression in si-*Srebf*1 B16 cells not decreased, but PDL1 expressions were declined (Fig. 2d). These data indicated that targeted therapies against metabolic checkpoints by TFP work in synergy with PDL1 immune checkpoint therapy.

The TFP decreased PDL1 of B16 cells slightly, but did not decrease CD47 levels (Fig. 3a, c), while TFP reduced sirpα of Raw264.7 cells significantly, but not decreased PDL1 obviously (Fig. 3b, c). The TFP upregulated iNOS and downregulated ARG1 of Raw264.7 cells (Fig. 3d, e) and the inhibition of ARG1 was more effective than the enhancement of iNOS. This data indicated that TFP had different effects on PDL1 expression in different cells and the influence of TFP on sirpα in Raw264.7 cells is stronger than that of TFP on CD47 in B16, which seems to suggest that TFP has a stronger effect on the immune system than on tumor cells.

B16 reprogrammed by TFP-induced lung lipid metabolism and immunity changes: As immunohistochemistry shows (Fig. 4), ACLY and CD47 expression of TFP treatment were decreased more or less, while, the expression of PDL1 and CD86 was increased. More importantly, in the model group, there was no co-localization of PDL1 and ACLY expression, as well as CD86 and CD47 expression, while, in 2500 and 5000 μg mL^{-1} TFP group, PDL1 and ACLY expression was almost completely co-located, as are CD86 and CD47 expression. This data suggested that B16 cells treated by 2500 and 5000 μg mL^{-1} TFP in the vein inhibited B16 cell metastasis in the lung and recruited more macrophages in the lung.

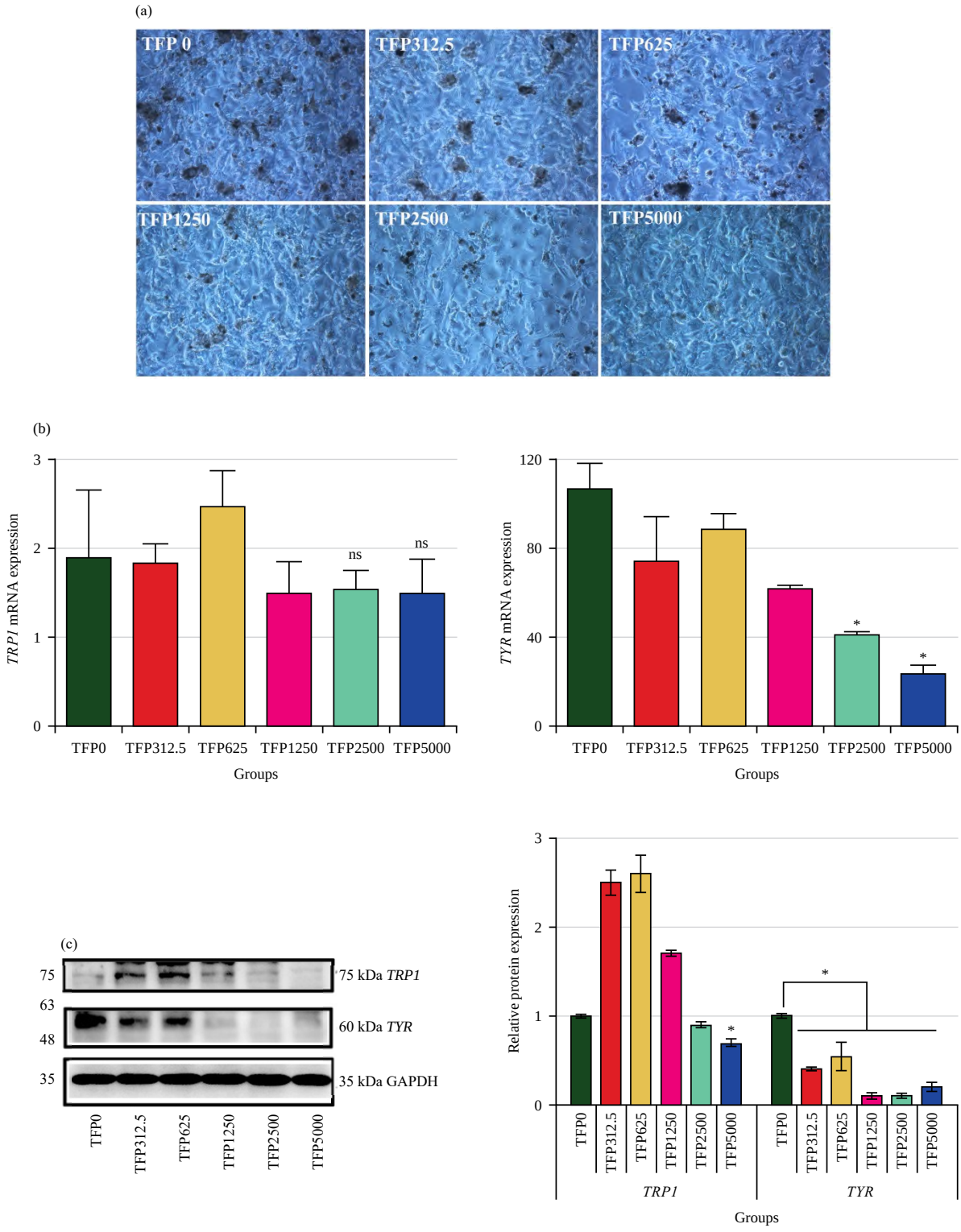

Fig. 1(a-c): TFP decreased melanogenesis through decreasing *TYR* and *TRP1* levels *in vitro*, (a) B16 cells showed a marked increase in pigmentation as compared to B16 treated with TFP of various dosages observed at bright fields, (b) Levels of *TYR* and *TRP1* were detected by RT-PCR analysis and (c) Western blotting

*p<0.05 vs TFP0 and ns: No significance

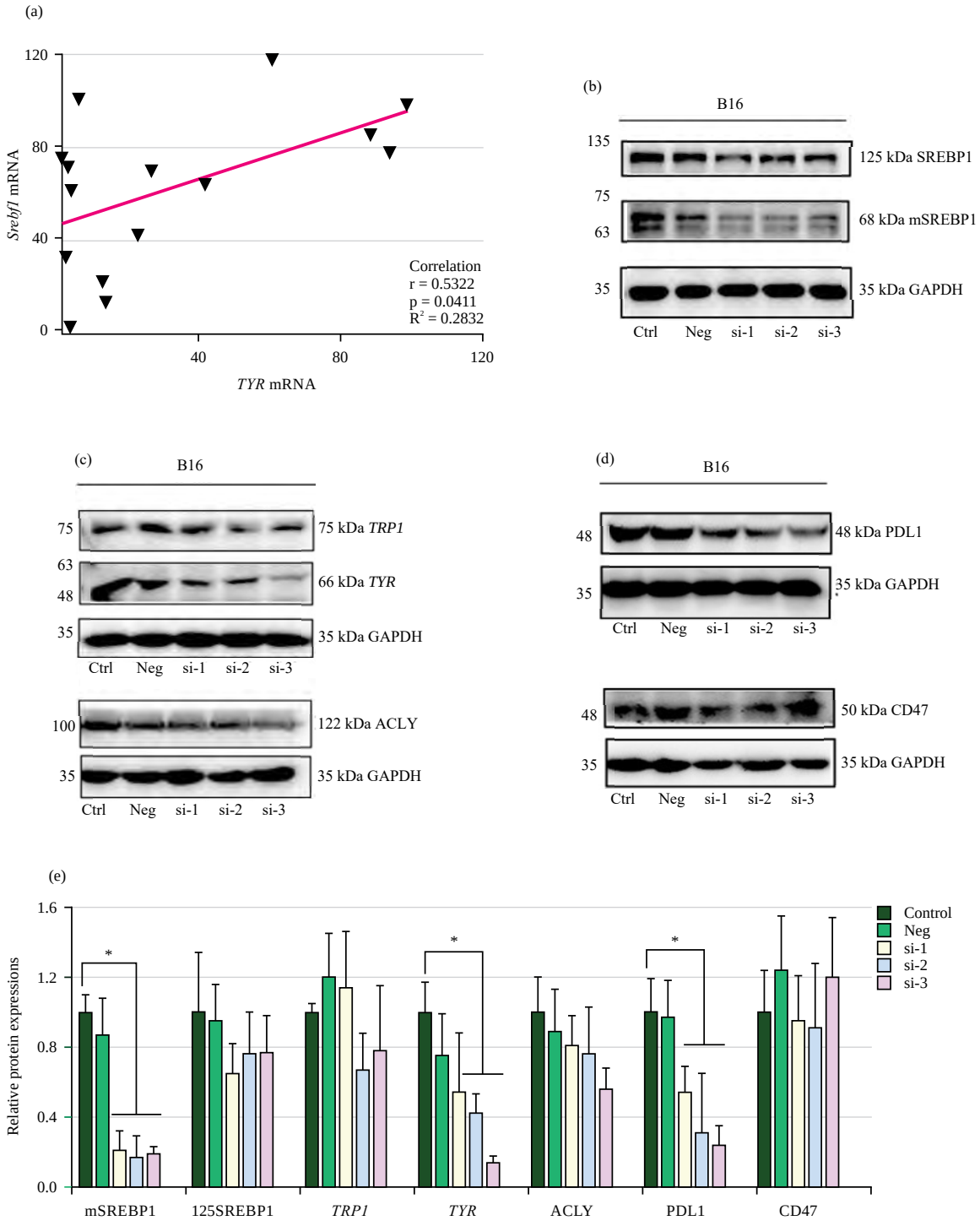

Fig. 2(a-e): TFP regulated *TYR* in SREBP1 mRNA and protein manner, (a) There was a positive relationship between *TYR* and *Srebf1*, (b) Then *Srebf1* in B16 cells was inhibited with si-RNA, especially the mature SREBP1 (68 kDa) expression was significantly inhibited, (c, e) After *Srebf1* inhibition, a marked decrease in *TYR* and ACLY expression were observed and *TRP1* slightly declined and (d, e) CD47 expression in si-*Srebf1* B16 cells was not decreased, but PDL1 expressions were markedly declined

*p<0.05 vs Ctrl

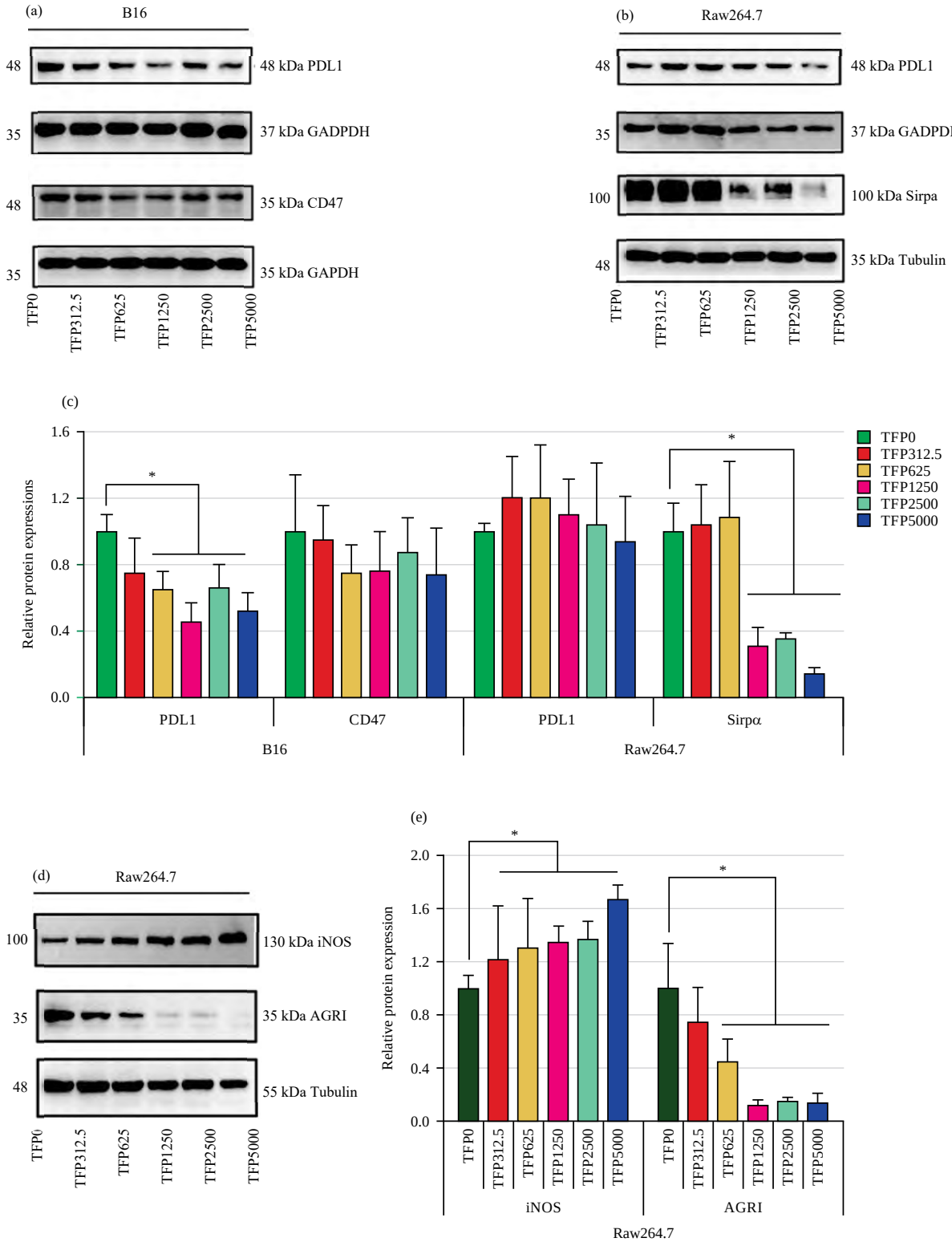

Fig. 3(a-e): TFP regulated immune checkpoints in B16 and Raw264.7 cells, (a,c) TFP decreased PDL1 of B16 cells slightly, but not obviously decrease CD47 levels, while (b, c) TFP reduced sirpα of Raw264.7 cells significantly, but not decreased PDL1 obviously and (d, e) TFP upregulated iNOS and downregulated ARG1 of Raw264.7 cells

*p<0.05 vs TFP0

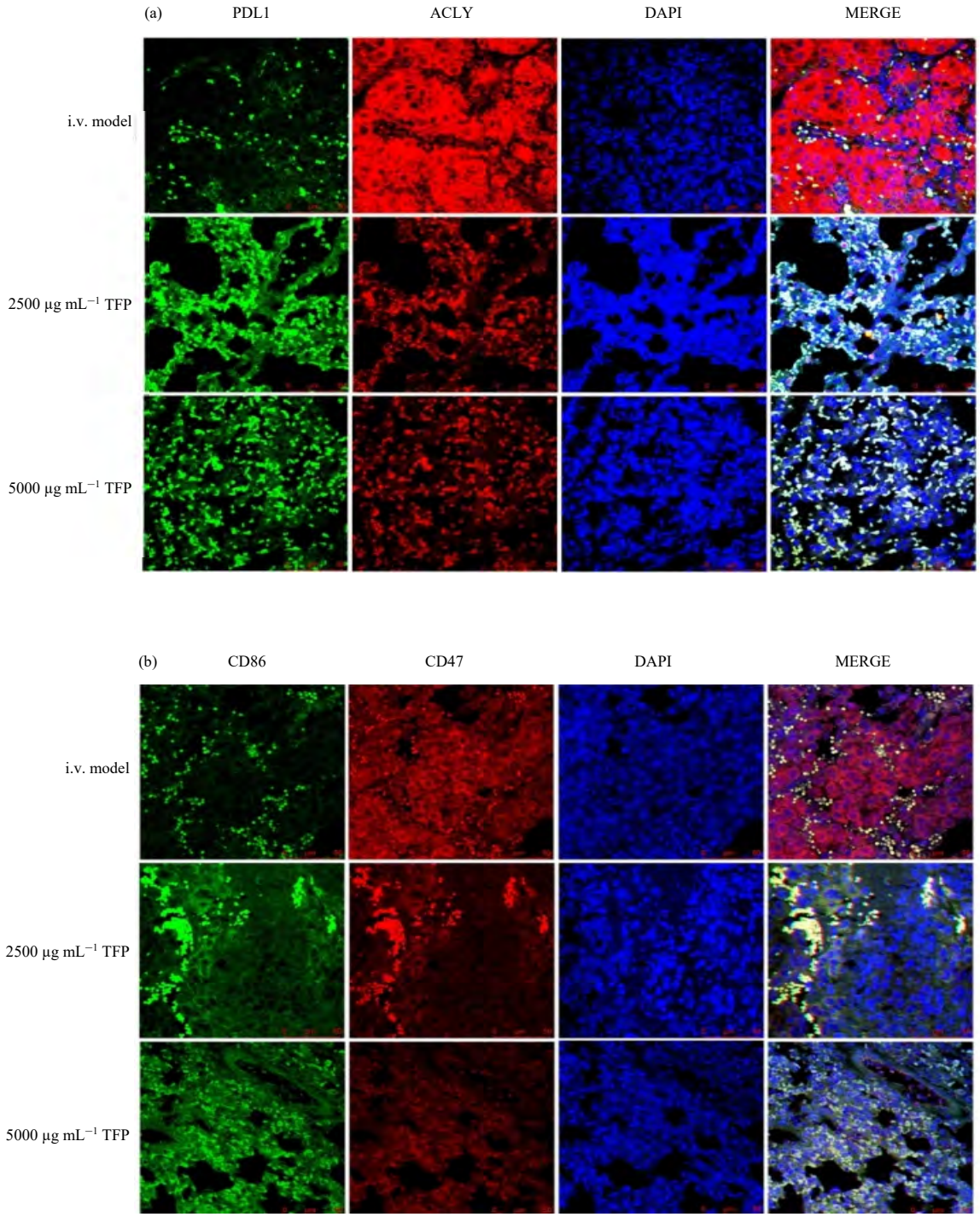

Fig. 4(a-b): TFP regulated ACLY expression, promoted macrophages infiltration. Representative images of immunofluorescent staining against (a) ACLY, PDL1 and (b) CD47 and CD86. The ACLY and CD47 expression of TFP treatment were decreased more or less, but the expression of PDL1 and CD86 was increased. In the model group, there was no co-localization of PDL1 and ACLY expression, as well as CD86 and CD47 expression, in TFP group, PDL1 and ACLY expression was almost completely co-located, as are CD86 and CD47 expression. The B16 reprogrammed by TFP raised PDL1 expression in the space between tumor cells or alveolar structure, but not on the tumor cells in the lung

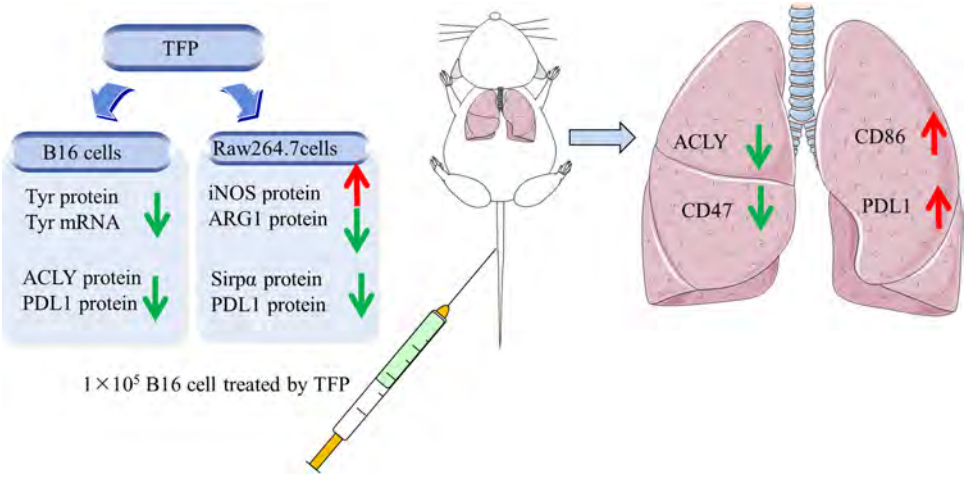

Fig. 5: Mechanism of TFP reprogrammed B16 cells on lipid metabolism and immune system regulation *in vitro* and *in vivo*. The TFP inhibited Try protein and mRNA in SREBP1 dependent manner and cooperatively regulate metabolism and PDL1 immune checkpoint. The TFP reconstituted B16 cells entered in the tail vein making a large number of immune cells (PDL1 upregulation) recruitment in the lung including M1 macrophages (CD86 upregulation) and the expression of ACLY decreases significantly

DISCUSSION

In the present study, TFP decreased melanogenesis through decreasing *TYR* in *Srebf1* mRNA dependent manner *in vitro*. The TFP has received high attention in skin whitening, lipids regulation and immune function[17]. According to the effect of TFP on melanogenesis and lipogenesis, we speculated that the expression level of SREBP1 might have a close relationship with immune regulation. The present study showed that the protein expression of ACLY and PDL1 was also positively correlated with the decline of the mSREBP1 protein. The results suggested that TFP inhibited melanogenesis by decreasing *TYR*[18] mRNA and protein, which was positively correlated with mSREBP1 protein expression, additionally, mSREBP1decreased stimulated immune activation. Furthermore, such TFP reconstituted B16 cells entered the mice body with the tail vein made a large number of immune cells (PDL1 upregulation) recruitment in the lung including M1 macrophages (CD86 upregulation) and the expression of ACLY decreases significantly, which was consistent with downregulation of ACLY in B16 cells by TFP. The ACLY is closely related to energy metabolism and supports cell growth. Lower levels of ACLY in the tumor cells indicated that tumor cells may at least partially lead to reduced lipid production and impaired bioenergy, thereby reducing tumor growth and invasion and cell apoptosis promotion[19]. The PDL1 is widely expressed on the surface of tumor cells and immune cells and other cells in the tumor microenvironment[20]. Evidence showed that PDL1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for some cancers[21]. The TFP reprogramming B16 raised PDL1 expression in the space between tumor cells or alveolar structure, but not on the tumor cells, indicating the importance of immune cells against tumor.

Melanoma has well-proven immunogenicity, which is conducive to investigating different immunotherapeutic strategies based on melanoma antigen-specific and nonspecific immune stimulation or adoptive transfer of melanoma-specific activated T cells. However, the overall results of immunotherapy clinical studies were not satisfactory[22]. Metabolic dysregulation in cancer cells interferes with the immune system[15]. The metabolism of tumor cells is regulated by TFP, then infused these reprogrammed tumor cells *in vivo*, it did achieve immune enhancement.

Circulating tumor cells can colonize distant organs to form metastases or rein filtrate primary tumor[23]. Modified circulating tumor cells may serve as targeted vectors to deliver antitumor agents[24], such as cytokines and immune cells. It is rarely reported that tumor cells modified with polysaccharides are used to treat tumors. The B16 cells manipulated *ex vivo* by TFP may serve as a uniquely effective carrier for the therapeutic delivery of cytokines or immune cells to parental tumors. The following anti-tumor effect and mechanism of B16 cells reprogrammed by TFP in veins was urgently needed in B16 subcutaneously implanted mice.

CONCLUSION

The study showed TFP-regulated B16 cell's lipid metabolism profile and macrophage immune profile *in vitro*, the inhibition of lipid metabolism and activation of the immune system effect still can be exerted *in vivo*. Mice injected with the reprogrammed B16 cells showed marked inhibition of tumor growth and strong antitumor effects compared with the vehicle B16 cells and the possible mechanism of these effects is also discussed. Frequencies of activated macrophages cells with an M1 profile were increased in the transplanted tumor and the CD47 expression was also decreased. Taken together, these data indicated that the novel utilization of reprogrammed B16 cells by TFP can effectively retard tumor growth. The present study provides thoughts to explore antitumors through immunological enhancement.

SIGNIFICANCE STATEMENT

This study uncovered the role and mechanisms of B16 cells cultured by TFP on lipid regulation and immune response in the lung through intravenous injection. Firstly, TFP regulated B16 cell's lipid metabolism and macrophage immunity *in vitro*. Secondly, the inhibition of lipid metabolism of B16 cells incubated with TFP can be exerted an active immune system effect and marked inhibition of tumor growth *in vivo*. Hence, this study may provide important thoughts to explore antitumors through immunological enhancement.

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