One signal activated TIL enable potent anti-tumor activity across diverse solid tumor types

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Abstract:

Adoptive T cell therapy (ACT) with autologous tumor-infiltrating lymphocytes (TIL) has shown compelling anti-tumor activity across various solid tumors. The function and status of TIL significantly influence its anti-tumor potential. In this study, we present the development of One-Signal-Activated TIL (OSAT), highlighting its robust anti-tumor activity in various syngeneic tumor models. We designed 10 unique signals by integrating CD3Z with a secondary signaling molecule. From these, we identified a specific signal, denoted as OSAT, which efficiently induces T cell activation when paired with a sole CD3 antibody. Subsequently, we benchmarked delivery approaches for OSAT, such as genome editing, lentivirus integration, and plasmid electroporation. We found that electroporation yielded the most potent expression effect among these methods. Further, we demonstrated that OSAT exhibited robust antitumor activity both *in vitro* and *in vivo* across diverse solid tumors. Finally, we synergized OSAT with PD-

1 blockade therapy to achieve a highly effective and enduring anti-tumor impact in cancer immunotherapy, facilitating sustained enhancement of anti-tumor activity.

Introduction:

The immune system plays a major role in fighting tumor development. Immunotherapies, especially adoptive cell therapy (ACT)[1-3] and immune checkpoint inhibitors (ICI)[4], have shown great potential for cancer treatment. Specifically, CAR-T technology, characterized by its targeting of CD19 and CD20, has demonstrated remarkable effectiveness in treating hematological malignancies[5]. However, the application of CAR-T technology in treating solid tumors still encounters significant obstacles[6]. This is primarily attributed to the heterogeneity of solid tumors, which poses challenges for CAR-T targets to achieve complete tumor elimination. Tumor-infiltrating T lymphocytes (TIL) therapy involves isolating and purifying specific antitumor lymphocytes from solid tumor tissue, amplifying and activating them, and subsequently administering a transfusion. TIL can naturally target the tumor-specific antigen of the patient and infiltrate into the tumor tissue to kill tumor cells through direct contact or release of cytokines. This capability positions TIL as a promising solution to overcome the challenge of recognizing tumor antigens in the context of solid tumor therapy[7,8]. However, TIL therapy still faces challenges in the treatment of solid tumors due to various microenvironments and immune cell states of solid tumors.

Checkpoint blocking antibodies have been widely used in the treatment of a variety of solid tumors, especially in melanoma[9]. The overexpression of PD-L1 molecules in tumor cells and their stromal cells is a major immune escape mechanism, through the binding of PD-1/PD-L1, effector T cells lose their killing function to tumor cells[10]. Therefore, blocking the PD-L1/PD-1 pathway by PD-L1 or PD-1-specific monoclonal antibodies prevents T cell dysfunction, thereby restoring the immune response in the tumor immune microenvironment. TIL therapy combined with immune checkpoint blocking therapy can play a better synergistic antitumor effect, but the current effective rate is still low, which may be related to the intrinsic characteristics of TIL[7,8,11].

TIL therapy has a strong congenital advantage in the treatment of solid tumors, but

there are still some technical bottles to be broken through. T cell activation and cytokine secretion require T cells to simultaneously recognize tumor cells that express MHC class I molecules/antigenic peptides and antigen-presenting cells (APCs) that provide stimulatory signals. In the tumor microenvironment, native T cells may not be activated due to the lack of co-stimulatory signals provided by APCs, leading to immune tolerance[12]. Additionally, immune checkpoints, especially PD-1/PDL-1, negatively regulate TIL activity, hindering its antitumor effects[13].

In this study, we introduce the One-Signal-Activated approach, aimed at optimizing the T cell activation process to augment the antitumor effectiveness of TIL. Our innovation involves the creation of a single-step activation receptor that integrates crucial intracellular co-stimulatory signals within TIL cells. We conducted a comprehensive assessment of various delivery methods for OSAT, including genome editing, lentivirus, and plasmid electroporation. Our findings demonstrate that the modified TIL cells possess the ability to directly identify and eliminate tumor cells, circumventing the necessity for co-stimulatory signals from APCs. Crucially, these modified TILs maintained their specific tumor recognition and tissue invasion capabilities. Furthermore, we substantiated the efficacy and safety of the modified TILs in conjunction with an immune checkpoint inhibitor (ICI) through experimentation in mouse tumor models, resulting in robust anti-tumor activity. Our study showcases a compelling therapeutic strategy harnessing TIL and its synergistic interplay with immune checkpoint inhibitors for effectively combating malignant solid tumors.

Results

Overview of OSA-design.

T cell activation is a complex series of continuous processes, involving stimulus reception, signal transduction, transcriptional expression of related genes, and activation of intracellular enzymes. The activation of naïve T cells necessitates the recognition of both major histocompatibility complex (MHC) molecules/antigenic peptides and the provision of costimulatory signals by APCs (Fig.1a).

T cell anergy can occur when there is only antigenic stimulation without concomitant costimulatory signal stimulation. The tumor microenvironment is characterized by global hypoxia, acidification, interstitial hypertension, vascular hyperpermeability, inflammatory response, and immunosuppression. In some conditions, the hostile tumor microenvironment and the infiltration of inhibitory immune cells can seriously affect the function of APCs, the only cells that directly activate the initial T cells[14]. Additionally, persistent TCR activation and inadequate costimulation of CD28 lead to TIL depletion in the tumor microenvironment[12]. These conditions can lead to T cell dysfunction, severely affecting anti-tumor immunity (Fig.1b).

To address this issue, we have designed a One Signal Activated molecule that fuses CD3Z and second signaling molecules, which we hypothesize can directly activate T cells strongly in response to tumor cells (Fig.1c). The C terminal of the first signaling molecule CD3 is fused with the intracellular activation domain of the second signaling molecule. Therefore, even in the presence of tumor cells only, if the TCR of T cells can recognize the antigen presented by tumor cells, it can be stimulated by fusion signals similar to both the antigen signal and co-stimulatory signal, leading to direct activation and effective killing of the tumor cells. We hypothesize that this design could vitalize TILs and enhance their anti-tumor ability in a more simple and stronger manner.

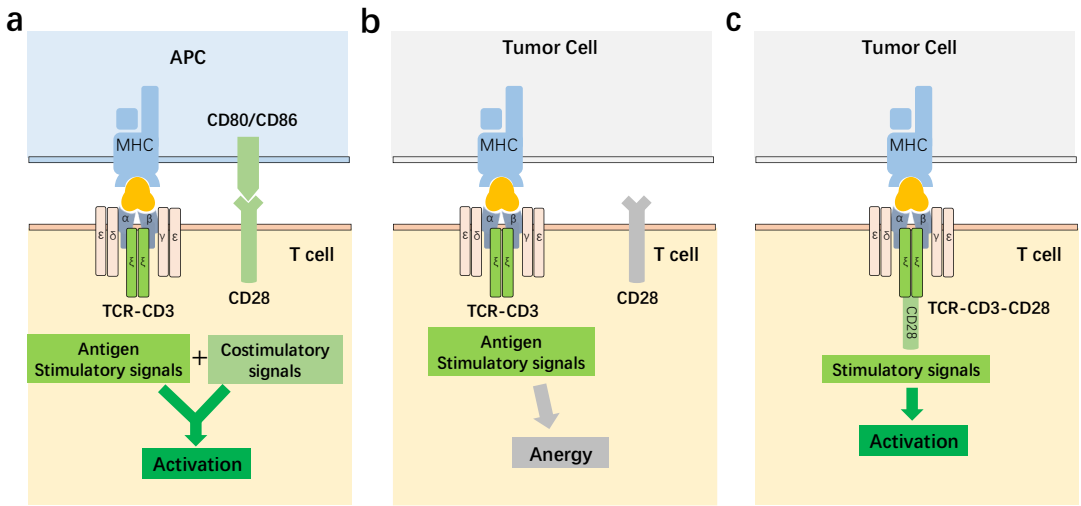

Fig.1: Schematic of T cell activation model and hypothesized OSA model.

(a) T cell activation requires both antigen stimulatory signals and co-stimulatory signals. Lack of co-stimulation can lead to T cell anergy and apoptosis.

(b) Stimulation of TCR alone can cause T cell anergy. Tumor-reactivated T cells may become anergic and undergo apoptosis.

(c) Hypothesized working model of OSA-TIL, which includes CD3Z and CD28 ATAM domain, allowing T cell activation in the presence of tumor cells alone.

Design and Screening of OSA Molecules

The αβ T cell receptor (TCR), in association with the CD3γε-CD3δε-CD3ζζ signaling hexamer, plays a pivotal role in T cell activation and the initiation of killing[15]. The CD3-Zeta dimer, detected only in the intact TCR-CD3 complex, is linked by disulfide bonds and serves as the primary mediator of antigen-stimulated signals. Chimeric antigen receptor (CAR)-modified T cells, primarily mediated by Immunoreceptor tyrosine-based activation motif (ITAM) of CD3Z and CD28/CD137, have demonstrated significant success in treating B-cell malignancies. In this study, we designed OSA molecules by fusing the intracellular domains of CD28 and CD137 with whole CD3Z in different configurations for testing and screening effective OSA molecules (Fig.2a) (supplementary file1).

To enable high sensitivity screening, we utilized the EF1a promoter to construct CD3Z-CD28/CD137 fusion proteins in the lentiviral vector PLVX, allowing for efficient lentivirus packaging and the establishment of stable T cell lines. we transduced various OSA lentiviruses into Jurkat cells and employed puromycin selection to generate stable cell lines expressing OSA molecules (Fig2.b). Subsequently, we stimulated the Jurkat cells with the anti-CD3 antibody (OKT3) to activate them and analyzed the secretion of IL-2 via ELISA to assess their activation under single signal stimulation. The results indicated that two OSA molecules, CD3Z-CD28 and CD3Z-CD137-CD28, exhibited strong activation effects (Fig.2c). Interestingly, although the latter included the additional activation domain of CD137, its effect was not significantly enhanced, suggesting that CD28 remains the primary mediator of co-stimulatory signals.

Consequently, we selected CD3Z-CD137-CD28 as the OSA molecule for further testing.

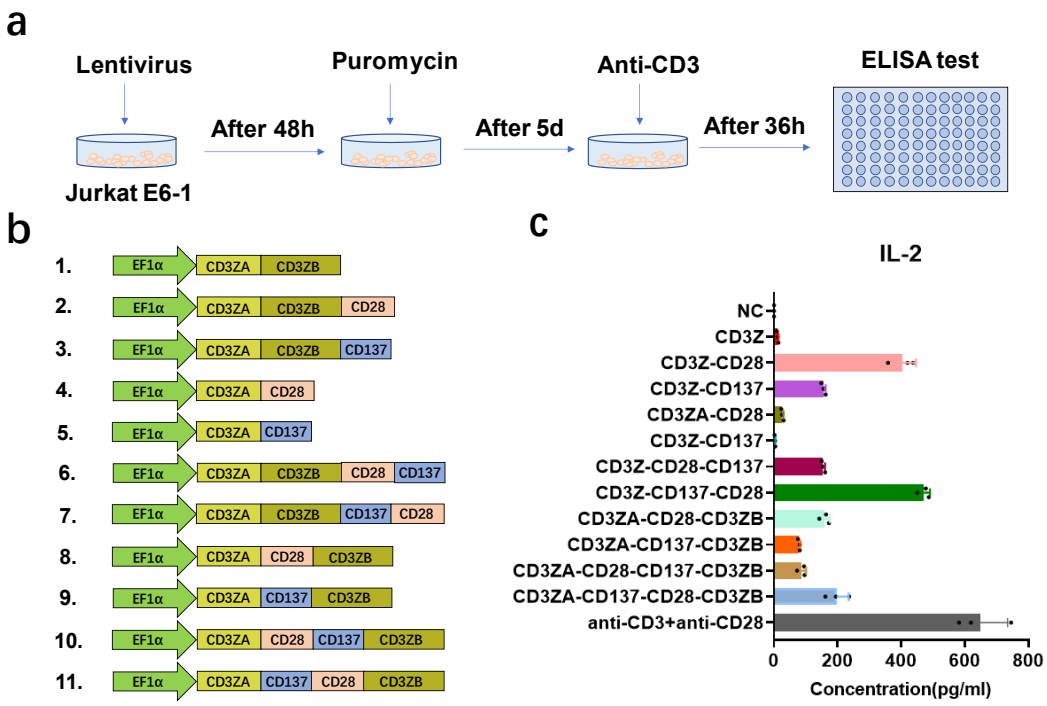

Fig.2: Design and screening of OSA molecules through combining CD3Z and CD28/CD137

(a) Overview of the screening strategy for OSA molecules. Jurkat cells were infected by lentivirus with virous OSA molecules, and express OSA molecules stably. Then Jurkat cells were simulated by anti-CD3 antibody alone and ELISA assay was performed to analysis the activation.

(b) Schematics of the constructs with different co-stimulatory molecular fused to CD3Z. CD3Z was split into two parts. CD3ZA is the N-terminal of CD3Z (1-51aa) and contains both extracellular and transmembrane domains. CD3ZB is the C-terminal of CD3Z (52-163aa), which is the intracellular ITAM domain. Both CD28 and CD137 are intracellular ITAM domains.(1.CD3Z; 2.CD3Z-CD28 ; 3.CD3Z-CD137; 4.CD3ZA-CD28; 5.CD3ZA-CD137; 6.CD3Z-CD28-CD137; 7.CD3Z-CD137-CD28; 8.CD3ZA-CD28-CD3ZB ; 9.CD3ZA-CD137-CD3ZB ; 10.CD3ZA-CD28-CD137-CD3ZB ; 11.CD3ZA-CD137-CD28-CD3ZB.)

(c) IL-2 secretion of variant OSA molecules. JurkatE6-1 cells expressed the indicated

constructs stably and the concentration of IL-2 was determined by ELISA. Data are means ± s.d. (n = 3 independent experiments)

Comparison of Gene Introduction Methods

In order to utilize OSA to modify tumor-infiltrating lymphocytes (TILs) for tumor eradication, a safe, efficient, and rapid gene introduction method is essential. Currently, mature gene introduction methods include CRISPR-Cas9-based gene editing, lentiviral transduction, and electroplasmid transfer[16,17].

The gene editing method based on Cas9-HITI[18] can precisely insert the OSA gene sequence into the CD3Z gene region and simultaneously knock out the wild-type CD3Z gene, minimizing competition between wild-type CD3Z and OSA molecules. (Fig.3a). We initially tested the cutting effects of several sgRNAs in the CD3Z region and identified the most effective sgRNA through T7E1 analysis (supplementary figure1). Based on the selected sgRNA, we designed the corresponding OSA-donor vector, and then transferred Cas9-sgRNA and OSA-Donor into Jurkat cells using electroporation. However, after 72 hours, the gene editing efficiency, even with the most efficient sgRNA（Guide1）, was found to be only 16.7% (Fig.3b).

In contrast, for the lentivirus transduction test, we also inserted the 2A-GFP gene sequence into the C-terminal of the original lentiviral vector OSA to facilitate flow detection of transduction efficiency (Fig.3c). Subsequently, the lentivirus was packaged and infected with Jurkat cells. After 72 hours, the results of flow cytometry indicated that the transduction efficiency of the lentiviral method was 60.1% (Fig.3d), with the expression rate of OSA significantly higher than that achieved through the gene editing method.

Considering that both gene editing and lentiviral transduction can enable cells to stably express OSA molecules for an extended period. Therefore, we further explored the plasmid transfection method using a self-replicating plasmid with S/MAR ori[19]. We also used EF1a promoter to facilitate the expression of OSA-2A-GFP gene and added S/MAR ori sequence to the plasmid (Fig.3e). Following plasmid transfection, we

detected the expression rate of OSA molecules after 72 hours, which was as high as 80%. Additionally, we re-examined the OSA expression rate of two week later after plasmid transfection and obtained similar results (supplementary figure2).

Finally, we compared the effect of T cell activation under different gene introduction methods. After 72 hours of gene transduction, Jurkat-OSA cells were stimulated by anti-CD3 for 36 hours, and the IL-2 concentration was detected by ELISA. Our findings indicated that the electroplasmid method had the best effect in activating T cells (Fig.3g).

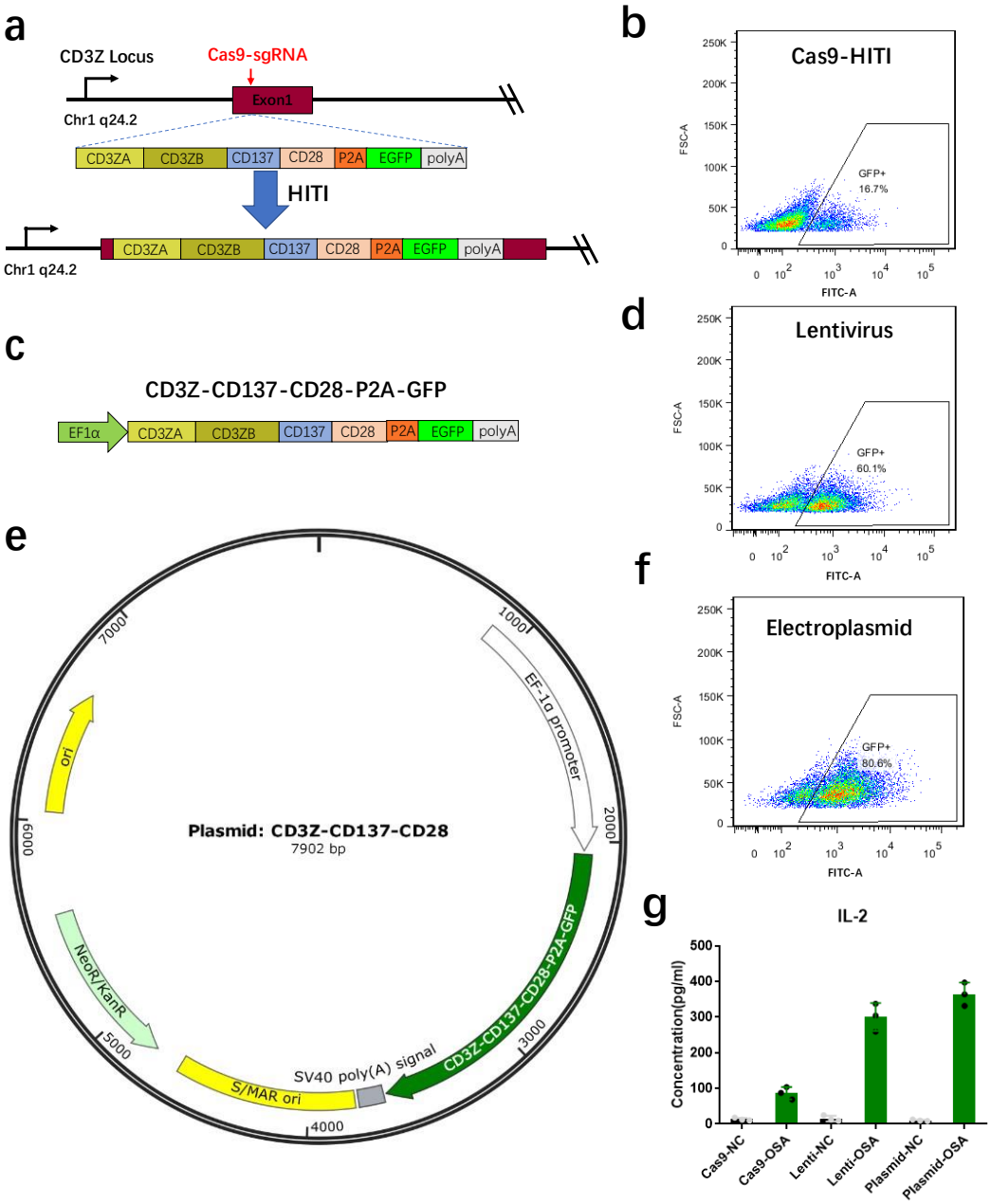

Fig.3: T cell activation efficiency varies with different gene introduction methods

(a) Cas9-HITI integration of the OSA gene and reporter gene (GFP) into the CD3Z locus.

(b) Evaluation of OSA gene knock-in efficiency at the CD3Z locus using FACS.

(c) Schematic of OSA gene and reporter gene structure in the lentiviral vector.

(d) Evaluation of OSA gene transduction efficiency using FACS.

(e) Schematic of the self-replicating plasmid with OSA-2A-GFP gene.

(f) FACS analysis of the expression rate of OSA molecules after 72 hours of plasmid electroporation.

(g) ELISA analysis of IL-2 concentration after gene transfer and stimulation by anti-CD3 antibody.

***In vitro* Antitumor activity of OSAT**

In order to further validate the antitumor effect of the modified TIL in vivo, we aimed to test it in a mouse subcutaneous tumor model. To ensure the effectiveness of OSA molecules in mouse T cells, we replaced the human-specific sequences of OSA with mouse-related sequences and tested their efficacy in the mouse T cell line EL-4. Approximately 72 hours after electric transfer of OSA-plasmid or NC-plasmid, we stimulated the cells with anti-mouse CD3 monoclonal antibody. The results showed a significant increase in IL-2 secretion in OSA-EL4 cells, while the IL-2 concentration in the wild-type EL-4 cell cultural supernatant remained unchanged (Fig.4a). We also conducted parallel comparisons with double stimulation of anti-CD3 and anti-CD28. The results indicated that the stimulation intensity of OSA molecules was slightly lower than that of double antibody stimulation, approximately two-thirds of its strength.

To evaluate the antitumor function of OSA-TIL, mouse subcutaneous tumor models of B16, MC38, and TC-1 were constructed. When the tumor reached approximately $0.5cm^3$, the tumor tissue was separated, and $CD3^+$TIL was extracted. Following transfection with OSA molecules, the TIL were co-incubated with corresponding tumor cells in vitro to assess the killing efficiency at different target ratios and the secretion

of IL2 and IFN-γ at a ratio of 20:1. The results demonstrated that with an increase in effector ratio, the clearance of tumor cells by TILs significantly improved, and the clearance efficiency of TIL-OSA on target cells was significantly higher than that of n-TIL cells (Fig.4b-d). Furthermore, to demonstrate the specificity and reduced off-target effects of TIL, we compared TILs with $CD3^+$ T cells in PBMC. The results showed that only TILs exhibited killing activity when in contact with corresponding tumors, further indicating the good targeting specificity of TIL(Fig.4b-d). ELISA analysis of related cytokines showed that the secretion of IL2 and IFN-γ by TIL-OSA was significantly higher than that of others during co-incubation. These results suggest that OSA molecules can significantly enhance the killing efficiency of mouse TIL cells.

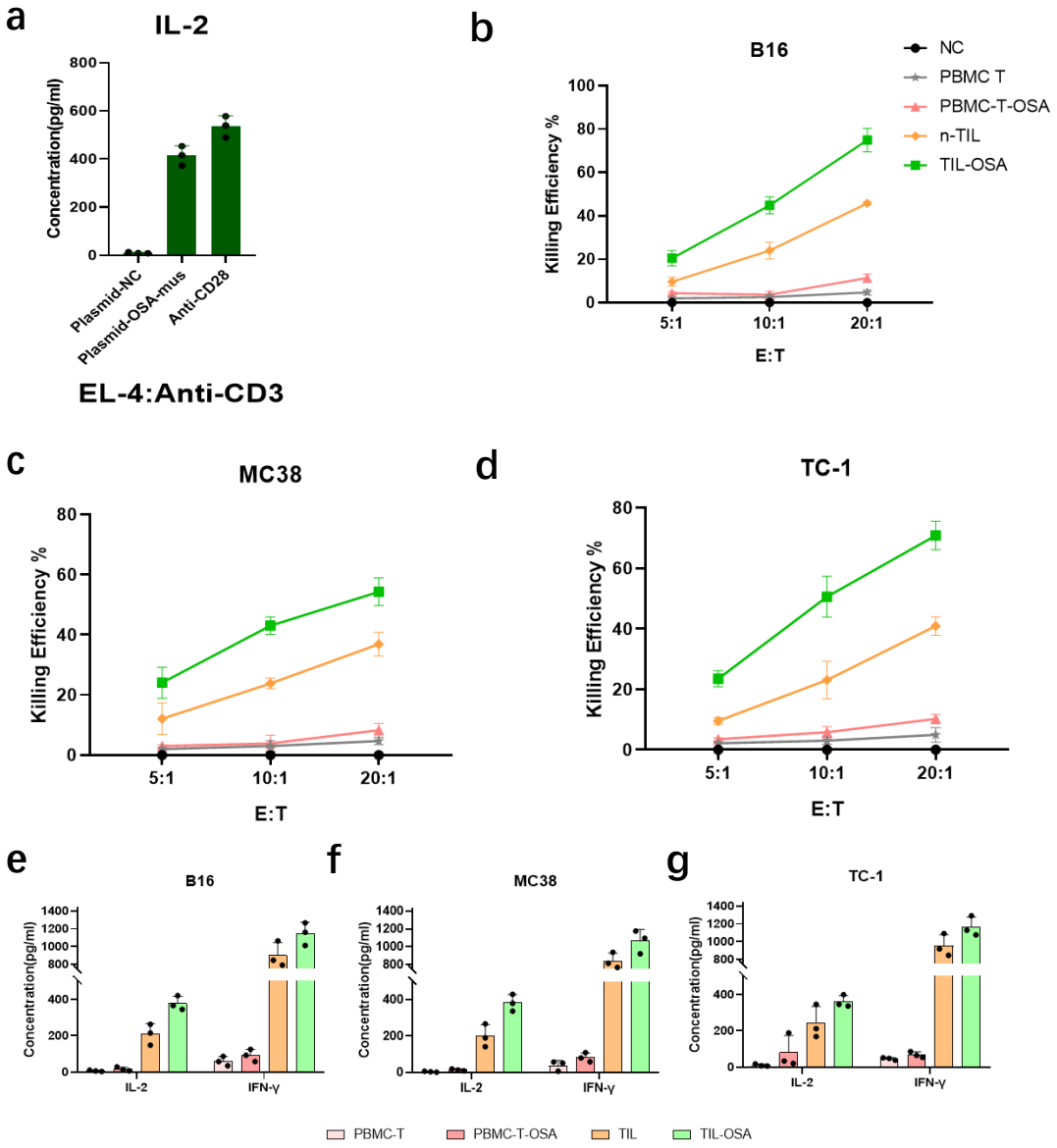

Fig.4: Functional evaluation of OSAT in vitro

(a) Functional evaluation of OSA molecular in mouse T cell line (EL-4).

(b) OSA-TIL specific cytotoxicity against target cells at multiple effector-to target cell ratio (5:1,10:1,20:1) of B16 tumor model.

(c) OSA-TIL specific cytotoxicity against target cells at multiple effector-to target cell ratio (5:1,10:1,20:1) of MC38 tumor model.

(d) OSA-TIL specific cytotoxicity against target cells at multiple effector-to target cell ratio (5:1,10:1,20:1) of TC-1 tumor model.

(e) ELISA analysis of cytokine production of IL-2 and IFN-γ in target cell ratio20:1 of

B16 tumor model.

(f) ELISA analysis of cytokine production of IL-2 and IFN-γ in target cell ratio20:1 of MC38 tumor model.

(g) ELISA analysis of cytokine production of IL-2 and IFN-γ in target cell ratio20:1 of TC-1 tumor model.

***In vivo* Anti-tumor activity of OSAT**

After observing the impact on TIL function in vitro, we sought to investigate the anti-tumor activity of OSA-TIL in vivo compared with traditional TIL. Overall, OSA-TIL demonstrated the most potent anti-tumor activity in all three tumor models, with particularly strong efficacy observed in the MC38 model, where OSA-TIL exhibited superior anti-tumor function compared to n-TIL (Fig.5b-d). Furthermore, the survival rate of the OSA-TIL group was significantly improved compared to traditional TIL therapy (Fig.5e-g). In summary, OSA-TIL displayed enhanced tumor inhibition effects and improved survival rates compared to traditional TIL therapy.

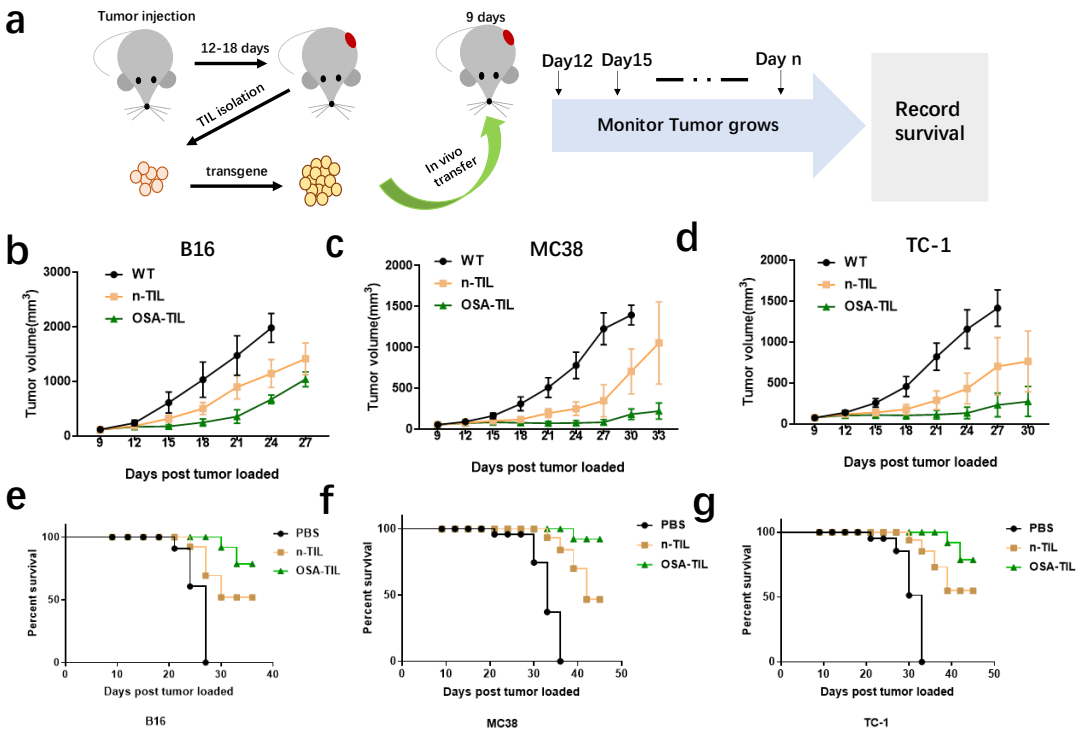

Fig.5: *in vivo* Anti-tumor function evaluation of OSAT in multiple tumor models

(a) Schematics of experimental design for adoptive transfer treatment in mouse models.

(b) Tumor growth curves of B16 that implanted subcutaneously and subjected to the indicated treatments.

(c) Tumor growth curves of MC38 that implanted subcutaneously and subjected to the indicated treatments.

(d) Tumor growth curves of TC-1 that implanted subcutaneously and subjected to the indicated treatments.

(e) Survival curve of mice bearing B16 treated with PBS, natural-TIL and OSA-TIL.

(f) Survival curve of mice bearing MC38 treated with PBS, natural-TIL and OSA-TIL.

(g) Survival curve of mice bearing TC-1 treated with PBS, natural-TIL and OSA-TIL.

OSA-TIL combined with PD-1 blockade therapy can significantly enhance the antitumor activity in vivo

After 12 days of OSAT treatment, the tumor volume in all three tumor models started to increase rapidly again, particularly in the B16 model. (Fig.5b). We hypothesized that TIL may gradually express immune suppressive molecules over time, thereby weakening its anti-tumor activity. Using flow cytometry, we detected the expression levels of PD-1, TIM-3, and LAG-3 in wild-type TIL cells isolated from the B16 tumor model at the onset of tumor formation (day 9) and after 12 days of treatment (day 21) (supplementary figure3). We found that the expression of PD-1 significantly increased after injection, especially in the OSA-TIL group, from less than 5% at the beginning to about 50% (Fig.6a). The expression of TIM-3 showed a slight increase, and the rate of change was minimal (Fig.6b). Furthermore, although the expression rate of LAG-3 differed significantly from that of initial TIL, the highest expression rate of LAG-3 was only about 15% (Fig.6c). Based on the expression tests of the three immune checkpoint molecules, we hypothesized that the substantial increase in PD-1 expression may be the main reason for the rapid growth of tumor volume after 12 days of treatment.

Subsequently, we tested the *in vivo* anti-tumor activity of OSA-TIL in combination with PD-1 blockade. We observed that the OSA-TIL group exhibited sustained inhibitory effects on the B16 tumor model (Fig.6d). All groups showed improved survival rates compared to those without PD-1. OSA-TIL demonstrated the greatest improvement in

survival, with no deaths occurring at day 42(Fig.6e). In conclusion, OSA-TIL cell therapy combined with PD-1 immune checkpoint inhibitor demonstrated enhanced anti-tumor effects.

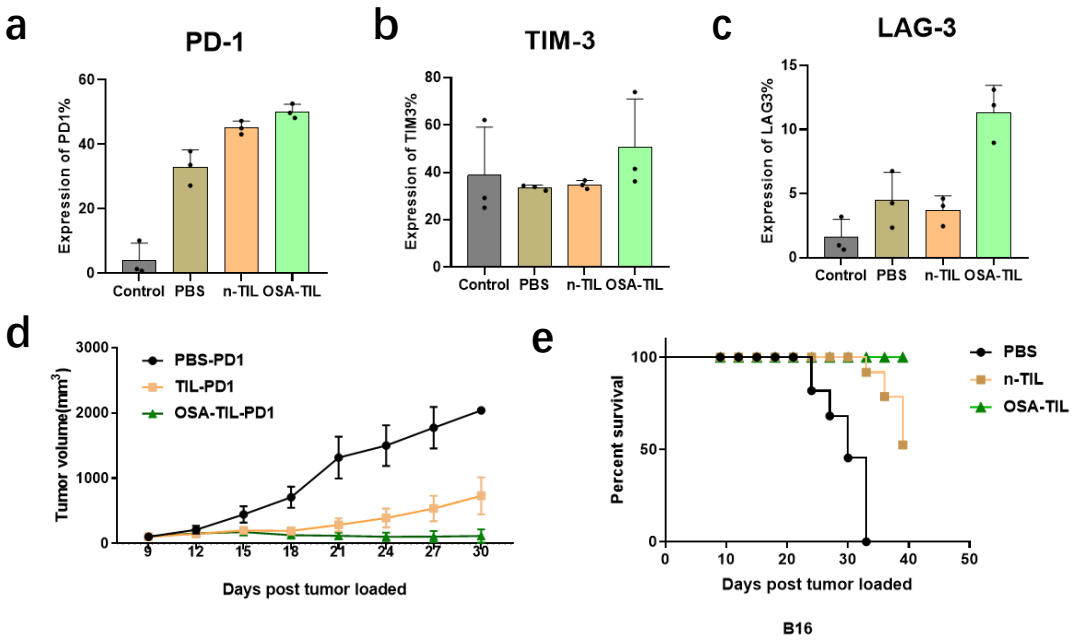

Fig.6: PD-1 blockade can further enhance in vivo anti-tumor activity of OSA-TIL

(a-c) Comparison of the expression levels of PD-1, TIM-3 and LAG-3 before and after ACT treatment on the 12th day. Control (grey) is the expression levels of TIL before ACT treatment and PBS(brown), n-TIL(orange) and OSA-TIL(green) are the expression level of TIL cells with indicated treatment at 12 days after adoptive transfer.

(d)Tumor growth curves of B16 that combined with PD-1 blockage and subjected to the indicated treatment.

(e)Survival curve of mice bearing B16 and treated with PD-1 blockage and indicated treatment.

Discussion

Monoclonal antibodies, ADC drugs, CAR-T, and TCR-T all recognize a single target, meaning that these drugs can only eliminate the target cells expressing the corresponding antigen[20-22]. For heterogeneous solid tumor tissue, it is obviously difficult for a single therapy to completely eliminate all tumor cells, despite the bystander effect has been observed in preclinical studies with some ADC drugs, which may have a feeble killing effect on target cells that do not express the corresponding

target antigen[23]. In addition, due to the lack of tumor-specific targets (TSA) in solid tumors, tumor antigens expressed in solid tumor tissues are expressed to different degrees in normal tissues, which may lead to "on-target, off-tumor" effects[24]. As living drugs, CAR T and TCR T need to infiltrate into tumor tissues to exert anti-tumor effects, while cytokines, Treg cells, macrophages, and other factors in the microenvironment of solid tumors limit the role of effector cells in solid tumors. Therefore, to overcome the treatment challenges of solid tumors, it is necessary to identify as many safe targets as possible to eliminate heterogeneous tumor cells. Additionally, it is also crucial to overcome the limitations of effector cells by negative regulatory factors in the solid tumor microenvironment.

Compared to CAR-T or TCR-T therapy, TIL therapy offers the advantage of innate recognition of multiple tumor targets for solid tumors characterized by heterogeneity[2,7,8,25]. Additionally, TIL possesses unique advantages: TIL demonstrates better infiltration after reinfusion as it originates from tumor tissue, and TIL exhibits stronger tumor specificity and has less impact on normal cells. However, traditional TIL therapy has limitations, particularly in cases where isolated TIL is not fully activated or has become exhausted, resulting in weak killing ability or an inability to maintain killing activity. Furthermore, the quality levels of TILs separated from different tumor patients are not uniform. Previous studies have attempted to address these deficiencies by using genetic engineering methods to introduce IL-2/IL-12 genes, or by combining PD-1 antibodies or knocking out PD-1 genes[1,3,16]. However, these approaches have not significantly improved the anti-tumor activity of TIL and have led to increased side effects. Therefore, the optimal modification of TIL to achieve the best therapeutic effect remains to be explored.

Based on the analysis of T cell activation pathways, we proposed a single signal activation strategy described in this study, drawing on the potent killing effect of CAR-T cells. As parts of the T cells in TIL are in an unactivated state, and the proportion of T cells in the early stage of activation and the late stage of activation is uncertain,

traditional TIL therapy involves simple amplification and then transfusions, leading to varying therapeutic effects. In this study, the single-signal activation strategy could theoretically activate inactivated T cells directly upon contact with tumor cells, initiating killing and avoiding side effects. For the activated T cells, continuous exposure to activation signal stimulation could enhance their sustained killing effect. The results of in vitro and in vivo experiments confirmed this conjecture. Furthermore, we combined the single-signal activation strategy with PD-1 antibody therapy, significantly enhancing the anti-tumor effect of OSA-TIL. Due to the complexity of TIL itself, although CD3+T cells have been isolated and modified, they are still divided into multiple subtypes, each with a variety of anti-tumor periods. Further research is needed to explore which period of T cell is more suitable for the OSA strategy, and whether OSA can significantly reduce the differentiated response of different patients.

The efficiency and safety of gene editing are crucial aspects of genetically modified cell therapy. Currently, the gene delivery tools widely used in preclinical or clinical studies include lentivirus, AAV vectors, and plasmids[17,26,27]. The FDA approved CAR-T products are currently produced using viral vectors, as lentivirus can achieve persistent gene expression. However, it can also cause mutagenesis through the random genomic integration of CAR-coding genes. Additionally, the manufacturing of viral vectors is time-consuming and expensive. Plasmid vectors can achieve rapid delivery and expression of target genes, but their duration is short and they are not suitable for clinical therapeutic applications[28]. Nonintegrating DNA vectors may be a better option for gene delivery, as they can dissociate from chromosomes without activating potentially carcinogenic genes[17]. Self-replicating nonintegrating DNA vectors can lead to consistent gene expression by replicating and distributing evenly in offspring with cell division[19]. Furthermore, their lack of a bacterial backbone structure prevents them from being easily cleared by cells[17].

In summary, our study proposed a new engineering transformation strategy for TIL to

manufacture single-signal activated TIL. Preliminary studies have shown that this strategy has significantly improved anti-tumor activity in vitro and in vivo. This may open up a new avenue for the transformation of TIL, offering the potential to address some of the deficiencies of TIL therapy and facilitate its broader application in the treatment of various solid tumors.

Methods

Mice

Animal experiments were conducted in accordance with the guidelines and regulations set by the Hebei University of Engineering Animal Care and Use Committee in Hebei, China. Female C57BL/6 mice aged 6 to 9 weeks were procured from Charles River Laboratory (Beijing, China) and randomly assigned to either the experimental or control group.

Plasmid construction

DNA fragments encoding human/mouse CD3Z, CD28, and CD137 were synthesized and cloned into the Plvx-puro plasmid for Lentivirus Packaging (supplementary file). Additionally, portions of these DNA fragments were also cloned into the T easy plasmid for gene editing donor purposes and into PMC, which includes the APOB3' MAR sequence for self-replication (supplementary file). Furthermore, we replaced the CMV promoter with the human EF1α promoter in the respective plasmids mentioned above. The DNA fragments of sgRNA for CRISPR-Cas9 were synthesized and cloned into PX330.

Cell lines and reagents

The human/mouse T cell cancer cell lines JurkatE6-1, EL-4, MC38 and TC-1 were procured from the American Type Culture Collection (ATCC) and cultured in RPMI-

1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were incubated at 37℃ with 5% CO_2. HEK293T and B16 cell lines were also obtained from the ATCC and cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, and incubated at 37℃ with 5% CO_2.

Lentivirus packaging and transduction

The HEK293T packaging cells were transfected with OSA-expressing plasmids with lentiviral packaging plasmids using Lipofectamine Reagent 2000(Thermo Fisher Scientific). After 48 hours, the culture supernatants containing the lentiviral vectors were harvested and utilized for gene transduction.

Tumor models

C57BL/6 mice were shaved and subsequently subjected to subcutaneous injection of B16 cells ($2x10^5$ cells/ 0.2ml/mice), MC38 ($5x10^5$ cells/0.2ml/mice), or TC-1 ($5x10^5$ cells/0.2ml/mice). The tumor volumes were assessed by measuring the size of the tumor in two dimensions using a caliper. The tumor volume was then calculated using the formula: Volume = (length x $width^2$)/2.

Mouse CD3+ T-cell isolation

Tumors were excised 12-18 days after tumor engraftment. Fresh tumor tissues were washed and cut into 1 mm^3 pieces. The samples were then digested using DNase, collagenase, and hyaluronidase, and filtered. Subsequently, density gradient centrifugation was performed to separate lymphocytes, and the white cell layer enriched with TIL between 75% and 100% separation liquid layers was collected. CD3+ T cells were isolated using positive selection with mouse CD4/CD8 (TIL) MicroBeads (Miltenyi Biotec), following the manufacturer's instructions. For CD3+ T-cell isolation from PBMCs, density gradient centrifugation was performed to separate lymphocytes.

CD3+ T cells were then separated from the white cells using mouse CD4/CD8 (TIL) MicroBeads (Miltenyi Biotec), according to the manufacturer’s protocol. Purified CD3+ T cells were amplified in RPMI-1640 medium supplemented with 10% FBS and a high dose of mouse IL2 (100 units/mL; eBioscience).

Generation of OSA-T cells

To generate OSA-T cells, JurkatE6-1 cells and CD3+ T cells isolated from tumors or PBMCs were transfected with the OSA plasmid using the preset program DS104 of the 4D-Nucleofector™ system (Lonza). Electroporation was performed following the manufacturer’s instructions. After electroporation, cells were resuspended in pre-warmed RPMI-1640 medium containing 10% FBS.

In vivo murine tumor experiments

To assess the in vivo anti-tumor activity of OSA-TIL compared to traditional TIL, we conducted experiments using three tumor models over two time periods. In the first period, tumor tissue was removed, and CD3+ TIL cells were extracted 12-18 days after tumor formation. After amplification with IL2, half of the TIL cells were introduced to the OSA plasmid through electroporation, referred to as TIL-OSA, while the remaining half remained as traditional TIL (n-TIL). These cells were then injected back into the tumor model mice via tail vein injection ($2x10^7$ cells/mice). Anti-PD-1 antibody (clone RMP1-14, BioXcell) was injected intraperitoneally in the corresponding groups at day 6 and day 18 after tumor cell inoculation. Tumor volume was measured every three days using a Vernier caliper, and survival time was recorded. Tumor volume was calculated using the formula: volume = (length $\times$ width^2)/2.

Flow cytometry analysis

Flow cytometry was used to analyze the gene editing or gene introduction efficiency of the OSA molecule. In addition, CD3+ T cells from different time periods were stained

with the following fluorescently conjugated antibodies: anti-CD3 (FITC, Miltenyi), anti-PD-1 (VioBlue, Miltenyi), anti-TIM-3 (PE, Miltenyi), anti-LAG-3 (APC, Miltenyi), and analyzed by flow cytometry. All flow cytometry data were analyzed using FlowJo software.

Cytotoxicity Assay

The effector cells include n-TILs and OSA-TILs, and the corresponding tumor cell lines were used as target cells. A 96-well plate was prepared with a control well containing RPIM-1640 complete medium supplemented with 10% FBS, and experimental and control wells. Subsequently, 100 μl of target cells (2×10^4/ml) and 100 μl of effector cells (1×10^5/ml, 2×10^5/ml, 4×10^5/ml) were added to the experimental wells. The plate was then incubated at 37℃ with 5% CO_2 for 24 hours. Afterward, 20 μl of CCK8 solution was added to each well. Following a 4-hour incubation, the anti-tumor activity of TILs in vitro was assessed using the CCK8 method according to manufacturer's instruction(MCE).

ELISA Analysis

The secretion of IL-2 by T cell lines and interferon gamma (IFN-γ) by oligoclonal TIL cultures from different treatment groups was measured using an enzyme-linked immunosorbent assay (ELISA) method. The obtained experimental data were subsequently subjected to statistical analysis.

Statistical Analysis

Statistical analysis was performed using Prism 9.3.1 (GraphPad Software). Two-tailed parametric or nonparametric t-tests (Mann－Whitney U-test) were employed to compare two groups of unpaired data, while two-tailed paired Student's t-tests were used for matched samples obtained from the same donor. Statistical significance was set at $P < 0.05$.

Author Contributions:

Yongqiang Wu and Xueshuai Ye conceived the project, designed all experiments. Yongqiang Wu, Xueshuai Ye and Yufeng Zhu performed the experiments. Yongqiang Wu, Xueshuai Ye, Elijah Q. Yuan, Dejing Kong and Lianmei Zhao analyzed the data. Yongqiang Wu and Lianmei Zhao wrote the manuscript with help from all authors.

Declaration of competing interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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