Augmentation of Antitumour Function of Tumour-infiltrating Lymphocytes Against Triple-negative Breast Cancer by PD-1 Blockade

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Abstract

T-cell-based immunotherapy and immune checkpoint blockade have been successfully used to treat several human solid cancers. The present study attempted to investigate the feasibility and efficacy of the antitumour effect of adoptive cell therapy along with programmed cell death protein 1 (PD-1) inhibitor on triple-negative breast cancer (TNBC). We isolated and expanded tumour infiltration lymphocytes (TILs) from TNBC mouse tumour tissues and adoptive TIL transfusion (TILs-ACT) was applied in combination with a PD-1 inhibitor to the TNBC mouse model. The pre- and post-therapy antitumour efficacy, cytokine secretion, and pathological changes were assessed both in vitro and in vivo. TILs exhibited higher IFN-γ and TNF-α secretion than conventional T cells. The TILs-ACT combined with PD-1 inhibitor promoted active T-cell infiltration into the tumour tissue and exerted a strong antitumour effect in an in vivo model. Additionally, the strategy could downregulate the expression of inhibitory marker PD-1 on TILs. Our results suggested that PD-1 blockade regulated T-cell exhaustion which synergised with adoptive TIL transfer immunotherapy, leading to eradication of established TNBC tumours. These findings might be useful in developing a feasible and effective therapeutic approach for TNBC.

Introduction

Breast cancer is the most common cancer and the leading cause of cancer mortality in women worldwide. Triple-negative breast cancer (TNBC) is a heterogeneous breast cancer subtype that is defined by the lack of expressions of oestrogen receptors (ERs), progesterone receptors (PgRs), and human epidermal growth factor receptor 2 (HER2) and comprises 12–17% of all breast cancer cases[1]. It is associated with a poor prognosis because of a high rate of early relapse and limited therapeutic options[2]. Thus, targeted therapies been ineffective in improving patient survival in TNBC. Cytotoxic chemotherapy remains the primary established systemic treatment for all stages of TNBC. Despite its toxicity, several studies have demonstrated the significant benefit of chemotherapy in the neoadjuvant, adjuvant, and metastatic settings[3]. However, although patients with TNBC initially respond to chemotherapy, the disease frequently relapses, leading to a worse outcome than other breast cancer subtypes. Median overall survival of patients with metastatic TNBC with the current treatment options is 13–18 months[4]. Thus, exploring effective treatment strategies with low toxicity that may improve the survival rate and quality of life of patients remains challenging for physicians and researchers in daily clinical practice.

TNBC exhibits a remarkably heterogeneous tumour microenvironment (TME)[1]. The higher mutant burden and genomic instability of TNBC result in higher immunogenicity to produce new antigens. This can be identified by T cells, which excite specific antitumour immunity by the adaptive immune system[5]. Studies have exhibited higher enriched TILs and programmed cell death-ligand protein 1 (PD-L1) in TNBC compared with other breast cancer subtypes[6]. Additionally, higher tumour lymphocyte infiltration is related to a better prognosis and a reaction of neoadjuvant chemotherapy, which provides a promising research potential for studying TNBC immunology[7]. However, the efficacy of PD-1 in metastatic TNBC is low, with response rates of approximately 5%[6]. Studies have demonstrated that PD-1 checkpoint

blockades in TNBC are more effective with combination treatment than with a single agent. Several therapeutic regimens with various drugs are being tested[7]. The majority of patients with TNBC do not benefit from PD-1/PD-L1 blockade. Thus, strategies that can alter the heterogeneous TME and increase sensitivity to PD-1/PD-L1 blockade are required.

T cells play a major role in cell-mediated immunity. Studies on genetically modify T-cell therapies, such as chimeric antigen receptor (CAR) T-cell therapy and T-cell receptor (TCR) T-cell therapy, have indicated that the T-cell antitumour responses can be stimulated by recognising mutated neoantigens[8, 9], which has lead to substantial advances in the treatment of malignant tumours. Tumour neoantigens are peptides expressed on the surface of mammalian tumour cells. Because these antigens are not expressed on normal tissues, they can be recognised by antigen-specific TCRs through the integration of major histocompatibility complex (MHC) molecules[10]. High tumour specificity and immunogenicity of neoantigens make TNBC an ideal candidate for adoptive cell therapy (ACT)[11]. In 2014, Rosenberg et al. reported a case of a patient with metastatic cholangiocarcinoma who received ACT of highly selected TILs, which resulted in long-term disease stability[9]. The adoptive transfer of tumour-specific TILs in patients with TNBC appears promising and is being researched in a clinical trial on ‘Autologous Tumor Infiltrating Lymphocytes in Patients With Pretreated Metastatic Triple-Negative Breast Cancer’, sponsored by Yale University (NCT04111510). Genetically engineered T cell formed by fusing a synthetic construct called CAR can be rapidly expanded to the clinical dose, and this technology is ideal for the treatment of TNBC and lymphoma[12, 13]. However, T cells that have been persistently exposed to antigen stimulation may become “exhausted”. These exhausted T cells manifest numerous features such as reduced cytokine secretion, impaired cytotoxicity, and overexpressed inhibitory receptors such as PD-1, with corresponding upregulation of PD-L1 and PD-L2 on the tumour cells[14, 15]. Thus, the adaptive resistance and T-cell exhaustion affect tumour eradication and prolong disease stabilisation in ACT therapy.

Studies have exhibited that the PD-1/PD-L1 pathway, a central regulator of T-cell exhaustion, and the PD-1/PD-L1 blockade can reverse the exhausted T cells and restore antitumour T-cell immunity[16]. The present study attempted to investigate the antitumour effect of adoptive infusion TILs combined with the treatment of immune checkpoint blockade (PD-1 inhibitor) in the TNBC mouse model.

Materials And Methods

Materials

Reagents and antibodies used in the present study are listed in Supplementary Table 1.

Cell culture

The 4T1 cells, a murine TNBC cell line, which is derived from a spontaneous mammary carcinoma in a BALB/c mouse, were obtained from the American type culture collection (ATCC) and cultured according to the recommended protocols. The cells were cultured in RPMI 1640 medium, supplemented with 10%

foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ in the presence of 5% CO_2.

Mice

The study was conducted in 6–8-week-old female BALB/c mice purchased from Vital River Laboratory Animal Technology Co. Ltd. Beijing, China. All animal experiments were performed under specific pathogen-free conditions. The animal experiments were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University, Qingdao, China.

Tumour-bearing mouse model

To prepare TILs, 1×10^6 4T1 cells were injected into the mammary fat pad of female BALB/c mice. For the TIL-ACT experiment, 1×10^5 4T1 cells were injected directly into the mammary fat pad. The mice were monitored daily, and the tumour volume was measured every 2–3 days by using a caliper and determined using the following formula: $\pi/6 \times \text{length} \times \text{width}^2$, where length is the longest diameter and width is the shortest diameter.

Preparation of TILs and conventional T cells

Tumours could be palpated subcutaneously 7–9 days after inoculation of 4T1 cells. The fresh tumours were isolated, broken down into smaller fragments, and digested using the tumour dissociation Kit for the enrichment of single tumour cell suspensions. TILs were sorted using the CD45 (TIL) MicroBeads on a clean platform. TILs were incubated in complete RPMI 1640 medium supplemented with 10% inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1 × non-essential amino acid, and 55 μM 2-mercaptoethanol at 37℃ in the presence of 5% CO_2. Approximately 30 ng/mL OKT3 antibody was added to the fresh complete RPMI medium containing 6000 U/mL of IL-2 and 20 ng/mL of IL-7. Depending on TIL growth, OKT3 antibody, IL-2, and IL-7 were supplemented twice for re-stimulation. TILs were harvested on days 12–14, resuspended to a final concentration of 5×10^7 cells/mL. The conventional T cells were isolated from the fresh spleen of tumour-bearing mice by using the Pan T Cell Isolation Kit II and were prepared using the same method.

IFN-γ and TNF-α release assays

The TIL antitumour reactivity was determined using the IFN-γ and TNF-α release assay in vitro. TILs or conventional T cells were co-cultured overnight with the 4T1 cells in different ratios, as indicated in a capture antibody-coated 96-well plate. Then, the cells were removed, supernatant was collected, and secreted IFN-γ and TNF-α levels were determined through ELISA.

Flow cytometry

The anti-mouse monoclonal antibodies used for T cells were: CD3-APC, CD4-BV421, CD8-FITC, and CD279-PerCP-eFluor 710 (PD-1). CD45-FITC, CD11b-Percpcy5.5, and F4/80-PE were used for the surface

staining of tumour-associated macrophages (TAMs). The T-cell or tumour cell pellet was washed with the FACS buffer and then incubated with the anti-mouse CD16/32 for 10 min at room temperature to block Fc receptors. The surface antibodies were stained in the dark for approximately 30 min.

To determine the intracellular IFN-γ levels of T cells, $1–2 \times 10^6$ TILs/T cells were stimulated using the cell activation cocktail for 2 h. After stimulation, Fixable Viability Stain 780 or 520 (APC-CY7 or FITC) dye was used to exclude dead cells before Fc receptor blocking and cell surface staining. Then, the cells were fixed and permeabilised using a Fixation/Permeabilisation kit, followed by staining with IFN-γ (FITC) antibody. The cells were washed twice with the FACS staining buffer or Perm/Wash™ buffer prior to acquisition on an Arial II-Optics flow cytometer. All data were gated on live and single cells. The data were analysed using the FlowJo software.

Antitumour effects in vivo

A total of 1×10^5 4T1 cells were subcutaneously injected into the mammary fat pad of female BALB/c mice. When the average tumour volume reached approximately 50 mm^3, all the mice were pretreated with cyclophosphamide (CTX, 100 mg/kg) for lymphodepletion (day 1). A total of 1×10^7 TILs or conventional T cells were injected into the tumour-bearing mice through the tail vein (day 0), and rhIL-2 (100000 units) was injected intraperitoneally for 3 consecutive days (days 1–3). The anti-PD-1 (10 mg/kg) or an equal volume of PBS was injected intraperitoneally 4 times (days 5, 8, 11, and 14). The tumour growth was monitored through caliper measurements. The tumour-bearing mice were sacrificed on day 27. The tumour tissue, lung, liver, kidney, and intestine of each mice were harvested for pathological analysis.

Immunofluorescence analysis and histopathological evaluation

Tumour tissues were perfused with 0.1 M PBS, embedded into an optimal cutting temperature compound, and frozen for cryostat section. Cryostat sections were fixed with 4% paraformaldehyde for 15 min at and cryostat sections were incubated in the blocking solution for 30 min at room temperature. CD3 and CD137 expressions in tumour tissues were assessed immunohistochemically by using anti-mouse CD3 and anti-mouse CD137 antibodies as the primary antibodies, whereas Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-rabbit IgG were used as the secondary antibodies. The nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI). ECLIPSE C1 orthofluorescent microscopy (Nikon, Japan) was used to view and acquire immunofluorescent microscopic images.

For histopathological examination, the samples were fixed with neutrally buffered 3.5% formaldehyde and subjected to haematoxylin and eosin (H&E) staining. The microscopic evaluation of H&E-stained images was performed using DS-U3 (Nikon, Japan).

Statistical analysis

A completely randomised, balanced design was used for all experiments. Statistical analyses were performed using the paired Student’s t-test in Prism software (GraphPad). Data are represented as mean

± standard error of mean. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 were considered statistically significant.

Results

TIL sorting, expansion, and assessment

TILs-ACT has garnered attention in tumour immunology research because of its antitumour effect, which is exerted by both the infusion of cytotoxic T lymphocytes and stimulation of endogenous T-cell antitumour immunity. This may be due to the numerous neoantigen-reactive T cells and immunoreaction to the unique mutations by TILs[17, 18]. TNBCs have a high amount of TILs[5]. Dieci et al. reported that TILs in residual disease following neoadjuvant chemotherapy were associated with better clinical outcome in TNBCs[19]. Another study demonstrated better response rates with pembrolizumab monotherapy in tumours with high TIL levels in metastatic TNBC [20]. The association of high TIL infiltration with a low relapse risk, superior prognosis, and chemotherapy response makes TNBC an ideal candidate for TILs-ACT.

According to the scheme of the study as illustrated in Fig. 1a, TNBC tumours were prepared in and harvested from female BALB/c mice. TILs were sorted using the CD45 (TIL) MicroBeads, and the proportion of T cells ($CD3^+$ T) was more than 35% (Fig. 1b). TILs were amplified using a 10–14-day rapid expansion protocol (REP) to produce several TILs for ACT, which resulted in the final drug product for transfusion.

Immune response assessment of TILs in vitro

Because the secretion of effector cytokines is a major function of $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T cells (T-helper 1, TH1) in tumour immunity, particularly IFN-γ and TNF-α, the present study attempted to evaluate them through flow cytometry and ELISA assay after expansion[21]. To accurately examine the ability of T cells to secrete IFN-γ after expansion, T cells were purified using the Pan T Cell Isolation Kit II (Miltenyi Biotec). As expected, the flow cytometric staining exhibited a significant increase in the IFN-γ levels from 20.4% in conventional T cells to 31.9% in TILs (Fig. 2a). The increasing IFN-γ production suggested the enhanced cytotoxic effect of T cells and a higher percentage of T cells producing IFN-γ. Furthermore, the IFN-γ and TNF-α levels detected in the supernatant were significantly elevated after co-culturing with 4T1 cells (Fig. 2b and c). Thus, TILs are proliferative and able to stimulate an antitumour immune response in vitro.

Antitumour effect of TILs-ACT in combination with PD-1 inhibitor in vivo

T-cell exhaustion presents one of the major hurdles to cancer immunotherapy because these cells eventually become incapable of controlling tumour progression[22], which may be due to the PD-1 upregulation on T cells and the rising PD-L1 expression on cancer cells[14, 15]. The successfully revitalised exhausted T cells enhance the response to cancer immunotherapy by the blockade of PD-1 or

its ligand PD-L1, highlighting the significance of the PD-1/PD-L1 axis in T-cell dysfunction[23]. The present study established the TNBC mouse model and produced TILs for transfusion to verify the antitumour immunity conferred by TILs-ACT in combination with PD-1 blockade in vivo. After tumours became palpable, 1×10^7 TILs or conventional T cells were adoptively transferred to the tumour-bearing mice with or without anti-PD1 administration. The adoptive transfer of TILs or immune checkpoint inhibitors (ICIs) was associated with a similar decreasing tumour growth, whereas the combination strategy exhibited a stronger therapeutic effect than the other treatment groups (Fig. 3a and b). The present study indicated the therapeutic potential of a combination of TILs and ICIs against 4T1 tumours in vivo.

Then, the immunofluorescence analysis of the tumour tissue were performed. The immunofluorescence analysis revealed that the treatment with TILs induced more $CD3^+$ T-cell infiltration into the tumour tissue, which was the highest among the mice treated with the combination strategy(Fig. 3c). This phenomenon is concurrent with the increasing $CD4^+$ and $CD8^+$ T cells infiltrating into tumour tissues (Fig. 3d).

CD137 (4-1BB) is a surface glycoprotein that is expressed on primed T cells and natural killer (NK) cells, representing the specific T-cell interaction with their target cells[24]. Similarly, the clinical effects of ACT rely on the presence of antigen-specific cytotoxic T lymphocytes [25]. As shown in Fig. 3C, the number of active T cells ($CD3^+$ $CD37^+$ T cell) infiltrating into the tumour tissues after treatment with TILs alone or in combination with ICIs was more than that in single ICIs-treated mice. Additionally, the expression of inhibitory marker PD-1 of TILs was downregulated from 54.8% in the TILs group to 27.37% in the TIL + ICIs group. A certain degree of downregulation was observed in the T + ICIs group (Fig. 3e). These findings indicate that PD-1 blockade may revitalise exhausted T cells and enhance the response to cancer immunotherapy. Furthermore, ACT combined with PD-1 blockade may potentially induce endogenous tumour-reactive T cells and their differentiation into pleiotropic effector T lymphocytes.

TAMs analyzed and toxicity study

Current studies focus on engineering T cells for immunotherapies, However, a large number of TAMs recognised to promote tumors progression are infiltrated in the TME. These heterogeneous TAMs are often associated with an increased drug resistance in cancer treatments [26, 27]. In the present study, the TAM population of the single tumour cell suspensions was assessed to determine the influence of this combination therapy on the TME. No significant difference was observed in the infilitation of TAMs from the flow cytometry of TNBC tumour model in the TIL or ICIs combination group (Fig. 4a), indicating that the immunosuppressor cells may not influence the antitumour effect of the TILs-ACT therapy.

Finally, toxicity studies were performed to assess the immune-related adverse events (irAE) in other organs such as lungs, intestine, liver, and kidney during a limited observation period (Fig. 4B). The low toxicity of our treatment strategy was demonstrated by the nonsignificant morphological changes in the pathological results of any of the four groups.

Taken together, these results confirmed the feasibility, safety, efficacy, and antitumour effect of a combination of TILs-ACT and ICIs against TNBC in both in vitro and in vivo experiments.

Discussion

The immune system plays a crucial role in the antitumour process, and immunotherapy is the major therapeutic strategy for several tumours. The complex interplay between cancer and the immune system has been classified into the elimination, equilibrium, and escape phases[28]. The ideology of a cancer vaccine for effective and specific tumour recognition is on account of the idea that cancer develops as a result of failure of 'immune surveillance.' However, the clinical trial results based on cancer vaccines have been consistently unsatisfactory. Thus, it is believed that immune regulation and killing mechanisms are commonly coordinated and enhance the host antitumour immunity[29]. Thus, therapeutic strategies, such as targeting the immune checkpoints to PD-1/PD-L1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and ACT, have gradually gained attention to elicit, reinvigorate, and potentially expand the endogenous response of pre-existing anticancer immune responses[9, 10, 30]. TNBC is a heterogeneous complex disease and is also the most immunogenic breast cancer subtype, which make it an ideal candidate for immunotherapy. Immunotherapy with the checkpoint inhibitor PD-L1 atezolizumab, has exhibited promising results, and its use along with nabpaclitaxel was approved by FDA for treating patients with locally advanced or metastatic TNBC expressing PD-L1[31]. Immunotherapy based on TILs-ACT has demonstrated encouraging results in metastatic melanoma, cholangiocarcinoma, and colorectal cancer[9, 32, 33]. Additionally, CAR T cell-based therapy for TNBC exhibited tremendous potential. For CAR T therapy, the most vital objectives are target selection, CAR construction, antitumour response enhancement, and safety enhancement[12]. The present study successfully isolated TILs from 4T1 mouse tumour tissue and demonstrated their antitumour activity in vitro. The IFN-γ and TNF-α production was remarkably elevated through flow cytometry and ELISA assay before T-cell transfusion. In the in vivo experiment, TILs-ACT was applied in combination with PD-1 blockade to the TNBC mouse model. The animal experiments in the present study demonstrated that this treatment strategy can prevent tumour progression and exert a strong antitumour effect, which was confirmed by pathological results of more T cells ($CD3^+$ T) and neoantigen-specific T cells ($CD3^+/CD137^+$ T) infiltrating into the tumour tissue.

Although the emerging immunotherapy strategies have provided encouraging results in TNBC research and clinical trials, several unsatisfactory aspects also exist. The efficacy of a single PD-1 agent in metastatic TNBC is low, and the exhausted T cells in TILs-ACT or CAR T therapy impair cytotoxicity, weakening the antitumour effect of the immunotherapy. This may be due to the failed immune control that impairs the effector functions of infiltrating T cells by a broad spectrum of immunosuppressive mechanisms present in the TME[29]. The PD-1 upregulation on T cells was widely recognised as the hallmark of T-cell dysfunction, termed as T-cell exhaustion. In addition to PD-1, other inhibitory receptors such as CTLA-4, T-cell immunoglobulin and ITIM domain (TIGIT), and T-cell immunoglobulin and mucin domain-3 protein (Tim-3) are overexpressed on exhausted T cells[34]. However, studies on cancer co-expression of inhibitory receptors on T cells have revealed a dominant PD-1 expression[35]. This theory

establishes the core status of PD-1 in immunotherapy. The persistent antigen stimulation of T cells in TME may result in a state of dysfunction, which is characterised by a loss of effector functions and proliferation and distinct transcriptional and metabolic changes[14]. The progressively reduced secretion of cytokines, including IL-2, TNF-α, IFN-γ, and β-chemokines[36], impaired T-cell cytotoxicity and resulted in overexpression of inhibitory receptors such as PD-1/PD-L1. These mechanisms contribute to the adaptive resistance and thus affect the antitumour effect in ACT therapy. However, current efforts are focused on developing strategies that can revitalise exhausted T cells and enhance cancer immunotherapy response, including the combination of PD-1/PD-L1 blockade, CTLA-4 blockade, radiation therapy, and chemotherapy[23, 37, 38]. The TONIC trial demonstrated that low-dose chemotherapy or irradiation followed by nivolumab is safe and leads to clinical benefit with high response rates and durable responses in patients with TNBC[7] through mechanisms such as stimulation of tumour neoantigen release, induction of type I interferons[39], depletion of regulatory T cells[40], upregulation of MHC-I expression[41], suppression of myeloid-derived suppressor cell letion[42], and enhancing the antitumour effect of cytotoxic T lymphocytes. The present study used a combination of TILs-ACT with PD-1 blockade in the TNBC mouse model and demonstrated that this treatment strategy can exert a strong antitumour effect. The PD-1 expression of T cells isolated from the tumours was downregulated after the combination therapy, suggesting that the PD-1 blockade may reverse the exhausted state of T cells and restore antitumour T-cell immunity. Furthermore, no irAE was observed in the pathological staining of the lung, liver, intestine, and kidney after the treatment. Hence, the combination strategy involving TILs-ACT and PD-1 blockade is feasible and effective. In clinical practice, TILs can be acquired from TNBC tumours and tumour-adjacent tissues. According to the good manufacturing practice, the final drug product of clinical TILs were ready for transfusion after REP. This novel therapeutic strategy provides an opportunity to treat patients with TNBC who exhibit a low response to a single PD-1 agent or trapped in CAR T therapy because of T-cell exhaustion. Thus, the present study provided a rigorous scientific foundation for the clinical application of TILs-ACT combined with PD-1 blockade.

The present study has certain limitations. Although our in vivo experiments confirmed that the combined therapy enhances the antitumour effect and reduces PD-1 expression on TILs, the specific mechanism reversing the exhausted state of T cells and restoring antitumour T-cell immunity remains unclear. Two studies have reported that ‘metabolic reprogramming of terminally exhausted CD8 + T cells by IL-10 enhances antitumour immunity’ and ‘4-1BB co-stimulation ameliorates T-cell exhaustion induced by tonic signalling of chimeric antigen receptors,’ which provided new insights to improve T-cell exhaustion in ACT[22, 43]. Additionally, the general biological traits of tumour and T cells such as enhanced trafficking of T cells to solid tumour sites, overcoming the suppressive TME, and promoting proliferation and survival of T cells also affect the antitumour effect[44, 45]. Further studies are required to overcome these limitations and facilitate the development of an effective ACT.

In summary, the present study demonstrated that the combination strategy involving TILs-ACT and PD-1 blockade can elicit a strong antitumour reaction against mouse TNBC. Additionally, this combination therapy may reverse T-cell exhaustion and reinvigorate antitumour immunity in ACT. These results further

highlight the significance of ACT in combination with immune checkpoint blockade, which may serve as a feasible therapeutic approach for TNBC in the clinical setting.

Declarations

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Conflicts of interest/Competing interests:

Not applicable.

Availability of data and material:

The data presented in this study are available in the article or supplementary files.

Code availability:

Not applicable.

Author Contributions:

Hongming Song, Haibo Wang and Haiyan Hu designed the study. Hongming Song, Haibo Wang, Mingkai Gong, Li Wu, Weihong Cao, Xueqiang Gao conducted experiments and analyzed data. Xueqiang Gao, Rongrong Dou and Qiaoyu Chen performed and analyzed the histomorphology. Haiyan Hu, Hongming Song, Haibo Wang and Mingkai Gong perfomed data curation. Haiyan Hu, Hongming Song and Haibo Wang wrote the paper with input from all authors. All authors have read and agreed to the published version of the manuscript.

Ethics approval:

This study was conducted with the approval of the Ethics Committee of the Affiliated Hospital of Qingdao University. All experimental methods and clinical treatment were carried out in accordance with the approved guidelines.

Consent to participate:

Not applicable.

Consent for publication:

Not applicable.

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Conflicts of Interest:

The authors declare no conflict of interest.

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Figures

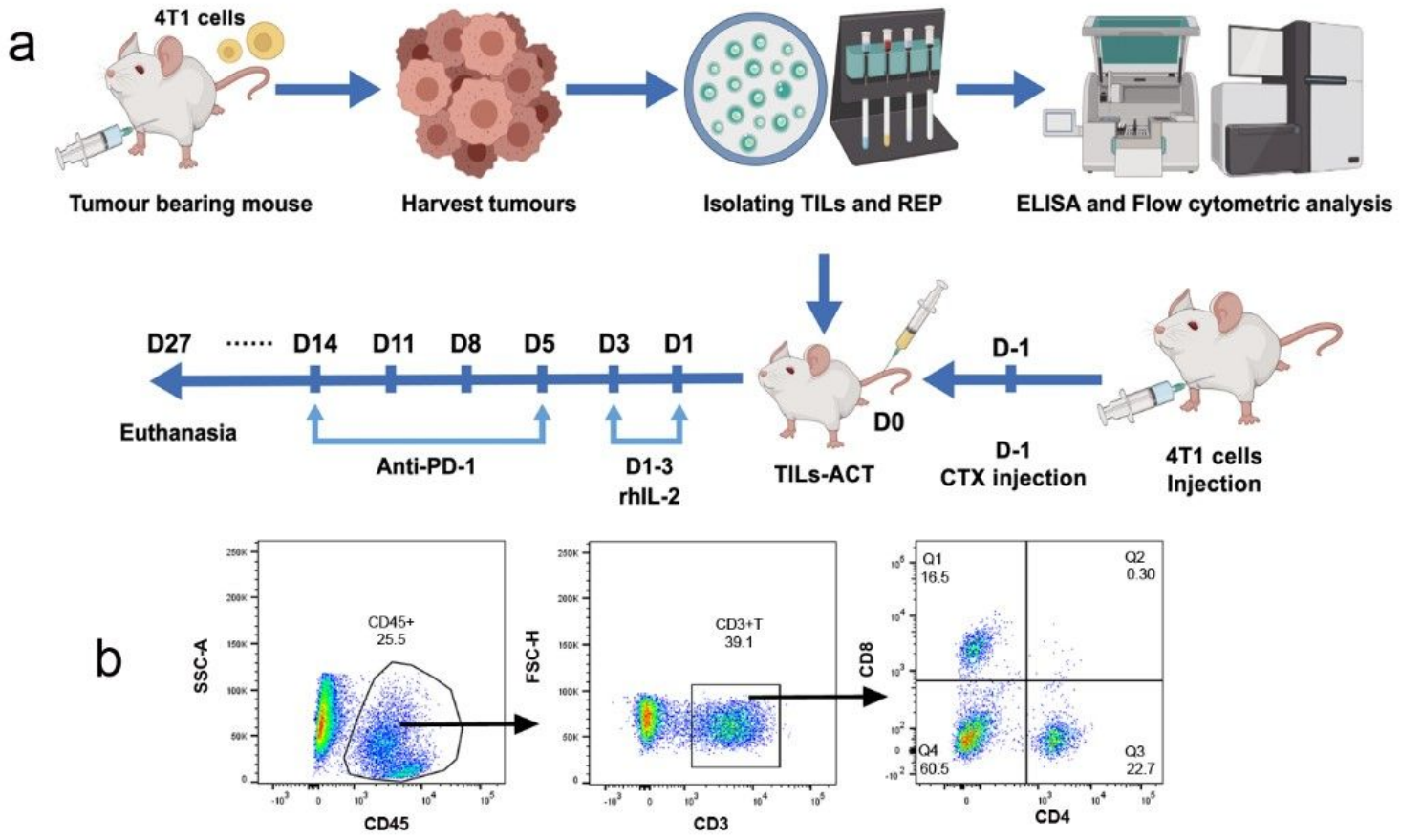

Figure 1

Diagram of TILs-ACT workflow and analysis of TILs subgroup a The scheme of the study. b Proportion of TILs isolated from TNBC tumour tissue. The size of the events (FSC-A and SSC-A) was used to exclude cellular debris and define all cells. Single cells were identified using FSC-A and FSC-H. Live cells were negative for the FVS-780 stain. CD45+ population was defined as tumour-infiltrated leukocyte. TILs were defined as CD3+ cells and were further divided into CD4+ and CD8+ cells. Presented data are representative of one of three independent experiments.

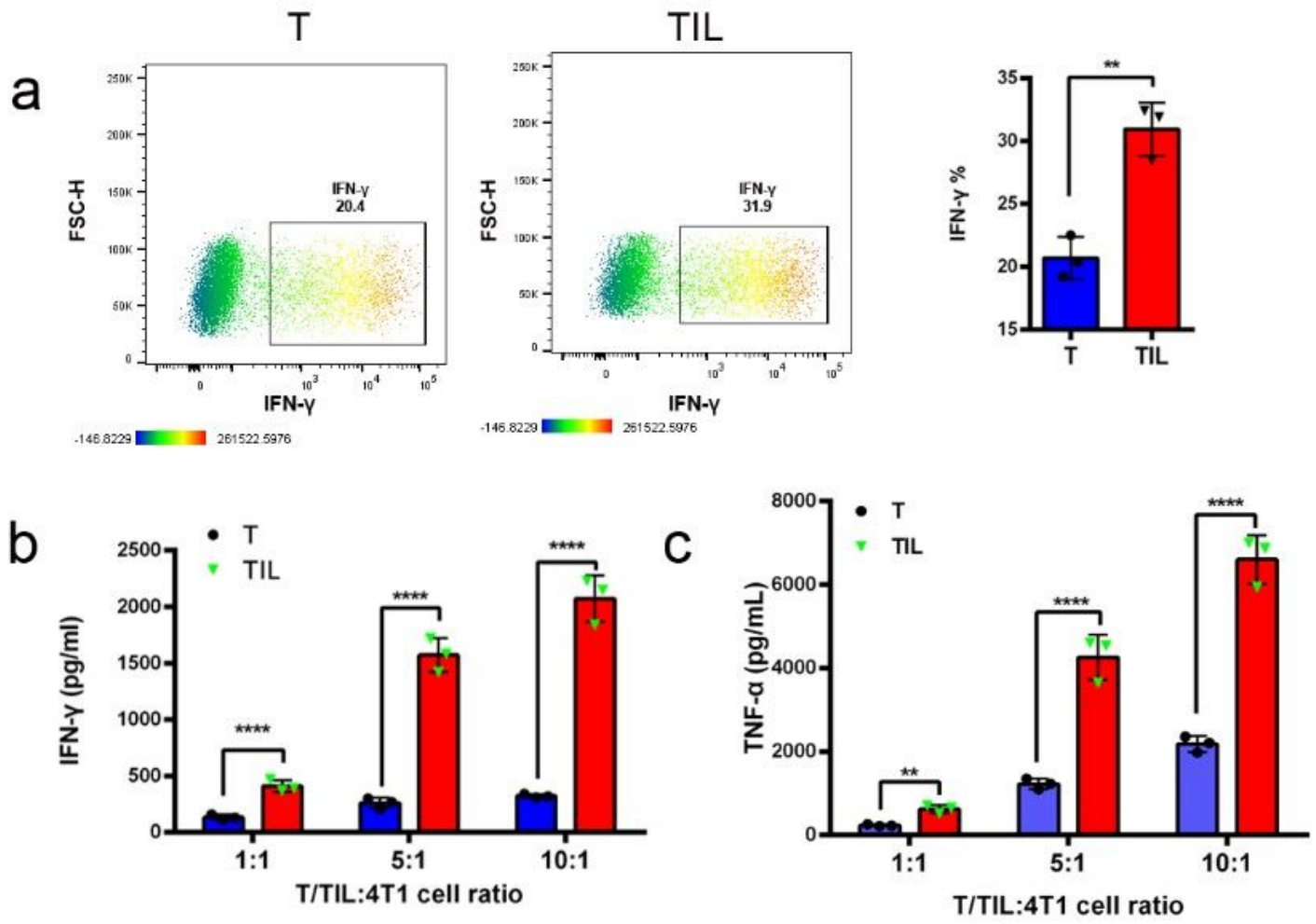

Figure 2

Functionality and immune response assessment of TILs T cells were acquired after REP and purified using the Pan T Cell Isolation Kit II. a Differences between IFN-γ secretion of T cells and TILs following intracellular IFN-γ flow cytometry staining. Cells were gated on CD3+T, and percentages were calculated from the total number of live T cells (CD3+ population). b and c T cells or TILs were co-cultured overnight with 4T1 and the secreted IFN-γ and TNF-α levels were determined through ELISA. T: conventional T-cell; TIL: tumour infiltration lymphocytes; Presented data are representative of three independent experiments.

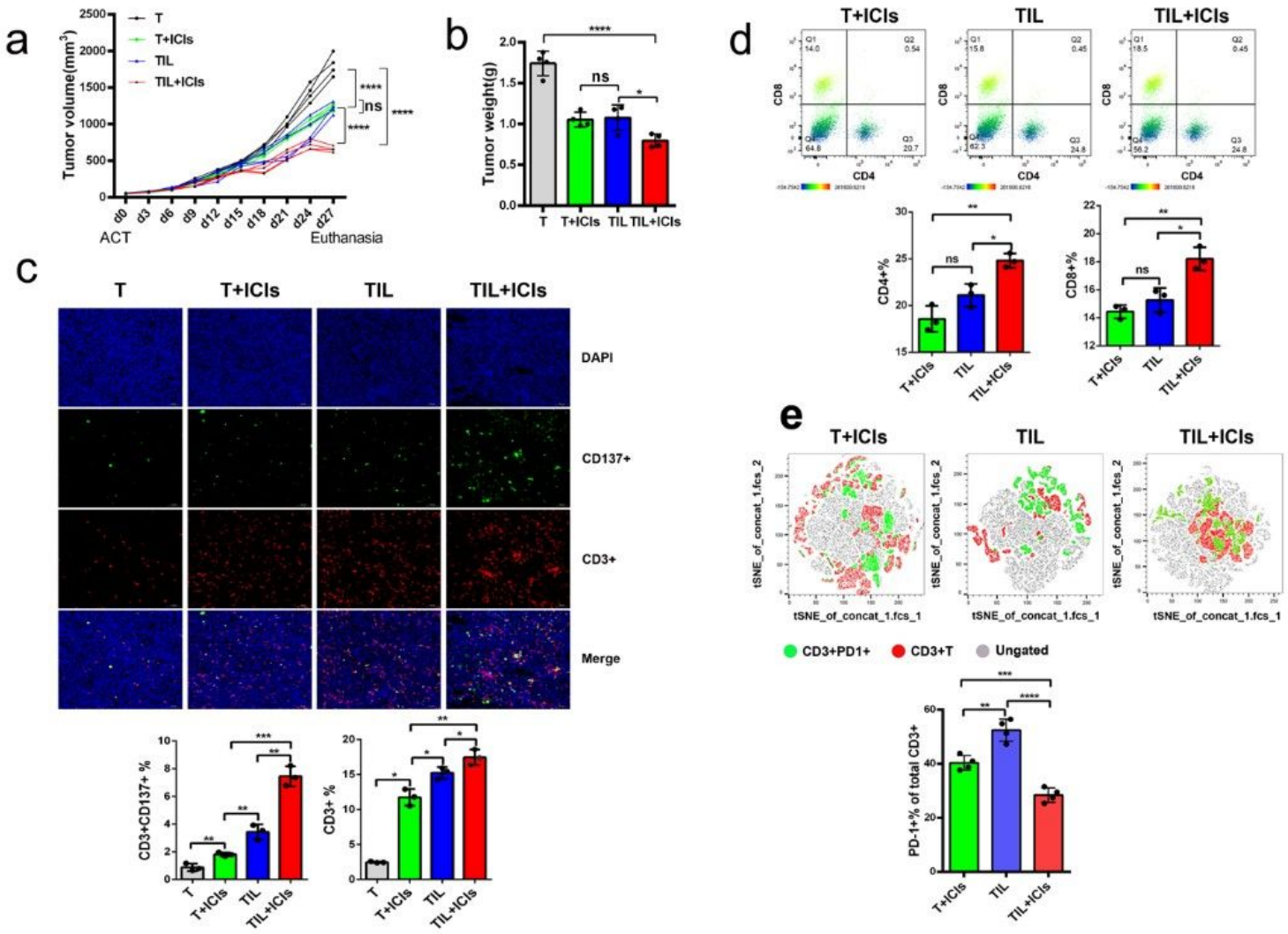

Figure 3

Antitumour immunity of TILs-ACT in combination with PD-1 blockade in vivo 1× 105 4T1 cells were injected into the mammary fat pads of BALB/c mice. a and b The tumour volume and weight of each group were depicted over time after ACT with or without anti-PD1 administration. c IF analysis of tumour tissues. The IF analysis used a combination of anti-CD3 antibody (red) and anti-CD137 antibody (green) for primary staining. The nuclei were stained with DAPI (blue). Microscopic examination of the IF samples was conducted at 200× magnification. Scale bar, 50um. d The proportion of TILs isolated from TNBC tumour tissue after therapy. TILs were isolated from the TNBC tumour cell suspensions by the CD45 (TIL) MicroBeads and further divided into CD4+ and CD8+ cells. e PD-1 expression on T cells isolated from TNBC tumour tissue after therapy. T cells were gated on CD3+ and further gated for PD-1+ and PD-1- cells. tSNE map of total CD3+ cells (red and green dots) and CD3+PD1+cells (green dots) in live cells (grey dots). In the T + ICIs, TIL, and TIL + ICIs groups, PD-1 expression on T cells was 40.94%, 54.8%, and 27.37%, respectively. T: adoptive transfer of conventional T-cell; T+ICIs: adoptive transfer of conventional T-cell with PD-1 blockade; TIL: adoptive transfer of TIL; TIL+ICIs: adoptive transfer of TIL with PD-1 blockade. n = 4 mice per group. Presented data are representative of three independent experiments.

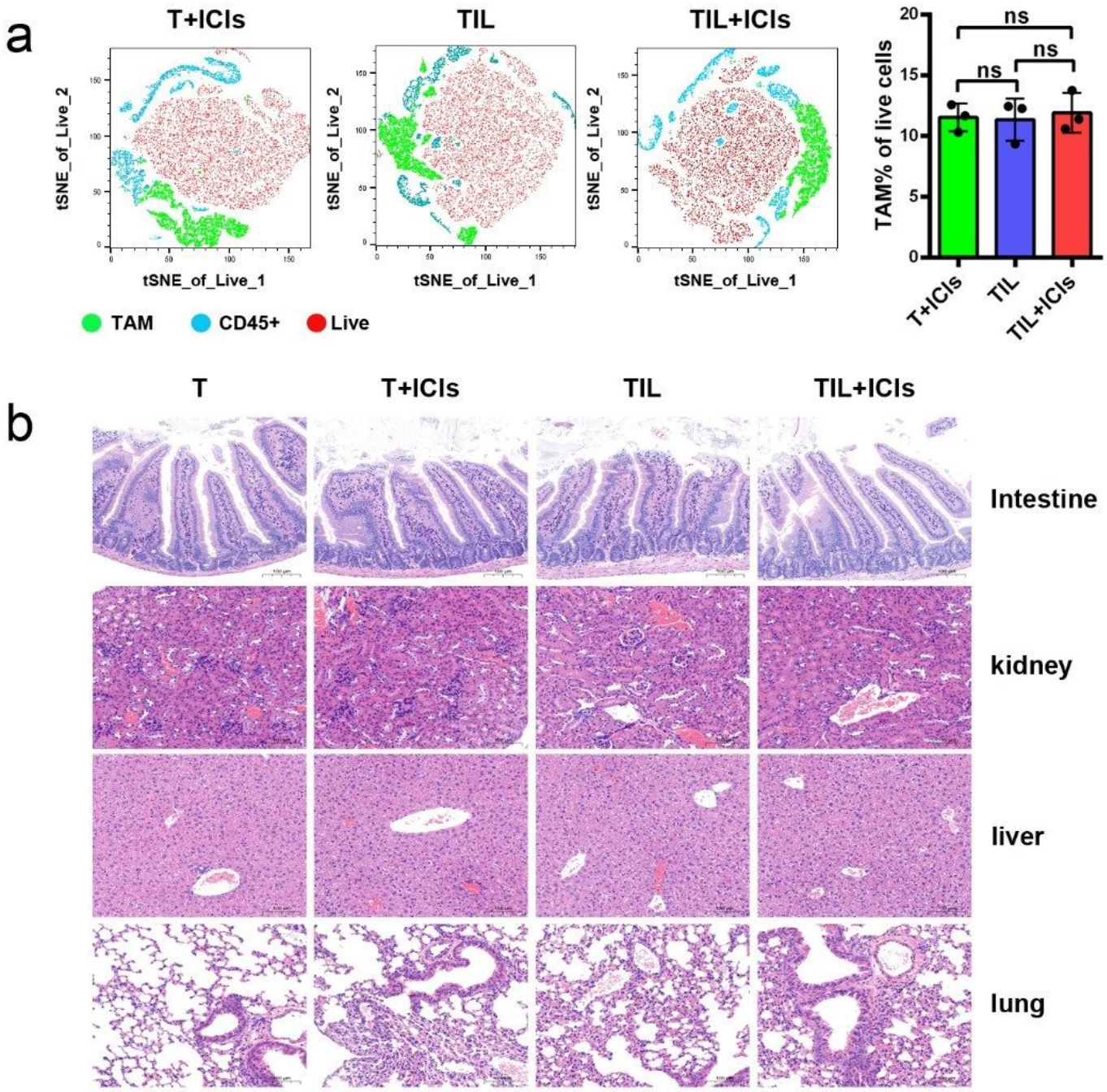

Figure 4

TAM population isolated from TNBC tumour tissue after therapy a TAMs were gated on CD45 + CD11b + F4/80 + cells. tSNE map of total CD45+ cells (blue and green dots) and TAMs (green dots) in live cells (red dots). In the T + ICIs, TILs, and TILs + ICIs groups, the TAM density in live cells was 11.67%, 12.39%, and 12.44%, respectively. b Mice were sacrificed at the end of treatment and organs were dissected. Morphologies of the lung, intestine, liver and kidney after H&E staining. Microscopic examination of the H&E samples was conducted at 100× magnification. Scale bar, 100um. Presented data are representative of three independent experiments

Supplementary Files

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- SupplementaryTable1.docx