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# The bispecific anti-CD3 × anti-CD155 antibody mediates T cell immunotherapy for human prostate cancer

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## Summary

Expression of CD155 differs between tumor and normal tissues, and high expression of this molecule can promote tumor metastasis. Here, we investigate whether CD155 can serve as a target for T cell-mediated immunotherapy of human prostate cancer. We first demonstrate that prostate cancer cells, including PC-3, PC-3 M, and LNCAP cells, express CD155 at high levels. Next, the specific cytotoxic activity of activated T cells (ATCs) armed with a novel anti-CD3 × anti-CD155 bispecific antibody (CD155Bi-Ab) against tumor cells was evaluated by flow cytometry, lactate dehydrogenase assay (LDH), and ELISA. In contrast to unarmed ATCs, an increase in the cytotoxic activity of CD155Bi-armed ATCs against tumor cells was observed at an effector/target (E/T) ratio of 5:1. Moreover, CD155Bi-armed ATCs secreted more IFN-$\gamma$, TNF-$\alpha$, and IL-2 and expressed higher levels of the activation marker CD69 than did unarmed ATCs. As CD155 Bi-Ab enhances the ability of ATCs to kill prostate cancer cells, CD155 is an effective target for cytotoxic T cells in human prostate cancer therapy.

**Keywords** Prostate cancer · Immunotherapy · CD155 · Bispecific antibody

## Introduction

Prostate cancer (PCa) is the most common cancer among males worldwide and the second leading cause of cancer-related death [1]. Although radical prostatectomy is an effective treatment, the disease is difficult to detect at early onset cancer, with vague symptoms, such as difficultly urinating, that are similar to the main symptoms of benign prostatic hyperplasia [2]. In most patients, the tumor has already excavated and metastasized by the time it is detected, and the opportunity for radical surgery has therefore passed. Indeed, clinical prostate cancer cases remain dominated by advanced cancer. Although endocrine therapy has a good initial effect, most cases eventually progress to castration-resistant prostate cancer (CRPC) and gradually develop into metastatic castration-resistant prostate cancer (mCRPC) [3, 4].

Tumor immunotherapy is now being applied for treating prostate cancer, and Sipuleucel-T has been used in patients with light-symptomatic castration-resistant prostate cancer [5]. Other therapies for prostate cancer include immunologic checkpoint inhibitors (Ipilimumab) [6], dendritic cell vaccines (DCVax) [7], and tumor vaccines (GVAX and PROSTVAC-VF) [8], which have shown potential in early clinical trials and have entered clinical III studies. Thus, immunotherapy for prostate cancer is feasible and effective and is worthy of further exploration and research.

In addition to developing novel therapeutic agents for new targets, another approach to improving current treatment is bispecific antibody generation. Immunotherapy for bispecific antibody-mediated cancer killing has been extensively studied, and since the first study of bispecific antibodies 20 years ago, different molecules directed against tumor cell surface antigens have been tested clinically [9, 10].

The human CD155 molecule was discovered in 1989 and named PVR (Poliovirus receptor) [11]. Membrane CD155 (mCD155) is expressed at a low level on a variety of normal cells and a high level on tumor cells, which may be closely related to tumor cell metastasis and immune surveillance [12, 13]. Here, we demonstrate high expression of CD155 by

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human prostate cancer cells. We then armed ATCs from both healthy donors and prostate cancer patients with CD155Bi-Ab, in which the anti-CD3 antibody is bound to the anti-CD155 antibody, and assessed the ability of these ATCs to kill prostate cancer cells. We found the activation marker CD69 to be overexpressed in CD155Bi-Ab-armed ATCs; these cells also secreted higher levels of IFN-$\gamma$ and TNF-$\alpha$ than did unarmed ATCs.

## Materials and methods

### Cell culture

The human prostate cancer cell lines LNCAP, PC-3, and PC-3 M were obtained from the National Infrastructure of Cell Line Resource (Beijing, China). All cells were cultured in RPMI-1640 medium with 10% FBS at 37 °C in a 5% carbon dioxide incubator. Cells were digested with 0.25% trypsin for subculturing.

### Flow cytometry

Anti-human CD69-PE, anti-human CD8-APC, anti-human CD3-FITC, anti-human CD4-PE, anti-CD56-APC, anti-human CD155-PE mAbs and mouse IgG1-PE isotype antibodies were purchased from eBioscience (San Diego, CA, USA). Cells were analyzed using a CytoFLEX (Beckman Coulter), and the data were processed using the manufacturer's software (CytExpert, Beckman Coulter).

### Preparation of PBMCs and activated T lymphocytes from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque. Blood was obtained from healthy donors, as provided by the Beijing Blood Bank. PBMCs were cultured at $10^6$ /ml in 10% FBS RPMI-1640 medium containing 5 μg/ml anti-CD3 mAb (eBioscience, San Diego, CA, USA). ATCs cells were stimulated with interleukin 2 (IL-2) every 3 days. Cells were cultured for 14 days using a previously described method [14]. On day 14, the ATC amplification products of healthy donors were 3.14% CD3+CD4+ cells and 93.28% CD3+CD8+ T cells. The cells were cryopreserved for later use.

### Synthesis of the anti-CD3 × anti-CD155 bispecific antibody (CD155Bi-Ab) and arming of ATCs

First, 1 mg/ml anti-CD155 mAb (R & D System, Minneapolis, MN, USA) was reacted with 10 times molar excess of sulfated-SMCC (2 mg/ml), and 1 mg/ml anti-

CD3 (OKT3, eBioscience, San Diego, CA, USA) was reacted with 10 times molar excess of Traut reagent (2 mg/ml USA). The two reaction mixtures were incubated at room temperature for 1 h, and excess crosslinking agent was removed using a PD-10 column. The anti-CD155-SMCC and anti-CD3-SH samples were immediately mixed at an equal molar ratio and mixed overnight at 4 °C. ATCs were armed with the CD155Bi-Ab at 150 ng/$10^6$ cells at room temperature for 30 min and washed to remove unconjugated antibodies. A mixture of OKT3 (150 ng/$10^6$ cells) and anti-CD155-mAb (150 ng/$10^6$ cells)-pre-incubated ATCs was used as unarmed control ATCs.

### In vitro cytotoxicity assay

PC-3, PC-3 M, and LNCAP cells were plated in triplicate in 96-well round-bottom plates at a concentration of $10^4$ cells per well and incubated overnight. The next day, the medium was removed, and fresh medium alone or with CD155Bi-armed or unarmed ATCs was added to the wells at an effector-to-target (E/T) ratio of 5:1. After the ATCs and tumor cells were allowed to interact in an incubator for 18 h, the cell-free supernatant was collected, and cytotoxicity was determined using the lactate dehydrogenase activity kit (Sigma-Aldrich, St. Louis, MO, USA).

### ELISA assay

PC-3, PC-3 M, and LNCAP cells were plated in triplicate in 96-well round-bottom microplates at a concentration of $10^4$ cells per well and incubated overnight. The original medium was removed, and fresh medium or medium containing CD155Bi-armed or unarmed ATCs was added to the wells at an E/T of 5:1. The ATCs and prostate cancer cells were incubated in an incubator for 18 h. ELISA was performed on the supernatants using a human cytokine ELISA kit (IFITROGN) to quantify IFN-$\gamma$, TNF-$\alpha$, and IL-2 secretion.

### Cytotoxicity of ATCs from prostate cancer patients

PBMCs from prostate cancer patients were isolated by density gradient centrifugation using Ficoll-Hypaque. T cells were cultured and armed with CD155Bi-Ab via the same method as described above. The killing effect was confirmed by the cytotoxicity assay and ELISA described above.

### Statistical analysis

Each experiment was repeated in three independent trials. All data from independent experiments are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 6.0 statistical

software (GraphPad Software Inc., La Jolla, USA). The unpaired Student's t test (two-tailed) was used for comparison of the two groups. A *P* value of <0.05 was considered statistically significant.

## Results

### High CD155 expression on human prostate cancer cells

CD155 expression by prostate cancer cells, including PC-3, PC-3 M, and LNCAP cells, was measured by FACS analysis. As shown in Fig. 1, high CD155 expression was detected in all of these cell lines.

### Preparation and characterization of CD155Bi-Ab and ATCs

To produce large numbers of T cells, PBMCs isolated from the buffy coat of healthy donors were stimulated by the combination of anti-CD3 mAb and IL-2 for 14 days and then subjected to FACS analysis. ATCs comprising CD3$^+$CD8$^+$ T cells and CD3$^+$CD4$^+$ T cells are shown in Fig. 2a. Most CD3 cells were positive for CD8, which typically indicates that the cell population is mainly composed of ATCs. ATCs were probed with CD155Bi-Ab, i.e., heteroconjugated anti-human CD155 mAb and OKT3, or a combination of OKT3 and anti-human CD155 mAb; anti-mouse IgG1-FITC was added to detect the CD155 part of CD155Bi-Ab, and anti-mouse IgG2a-PE was added to detect the CD3 portion (Fig. 2b). Only functional CD155Bi-Ab can bind to ATCs by CD3-identified OKT3 (isotype: mouse IgG2a) and be detected by the anti-mouse IgG1 antibody through anti-CD155 mAb (isotype: mouse IgG1).

### Cytotoxic effects of CD155Bi-armed ATCs on different prostate cancer cell lines

Cytotoxic effects of CD155Bi-armed ATCs on different prostate cancer cells were examined in vitro in assays that were performed at E/T ratios of 5:1. According to data provided, approximately 150 ng/10$^6$ cells were selected as the CD155Bi-Ab concentration for all subsequent experiments [15–17]. After 18 h of coculture with CD155Bi-armed or unarmed ATCs, photographs of each prostate cancer group were taken at 80× magnification (Fig. 3a). In addition, FACS analysis of the CD155Bi-armed ATCs showed an increase in CD69 expression over their unarmed ATCs counterpart after 18 h of incubation with prostate cancer cells (Fig. 3b). These results show that the ATCs were activated by CD155Bi-Ab.

### Cytotoxic effects of CD155Bi-armed ATCs with IFN-γ, TNF-α and IL-2 production on prostate cancer cells

After incubation with CD155Bi-armed or unarmed ATCs for 18 h, cell culture supernatants at an E/T of 5:1 were collected and analyzed for cytokine secretion and cytotoxicity using a specific ELISA kit. As shown in Fig. 4a-c, there was a significant increase in IFN-γ, TNF-α and IL-2 levels with armed effectors compared with unarmed effectors when cocultured with prostate cancer cells.

### Determination of the cytotoxicity of CD155Bi-armed ATCs

LDH activity assays were performed using a kit to evaluate damage of target prostate cancer cells at an E/T ratio of 5:1. After 18 h of incubation, there was a significant increase in the concentration of LDH with CD155Bi-armed ATCs compared with unarmed ATCs in PC-3, PC-3 M, and LNCAP cells, as shown in Fig. 4d.
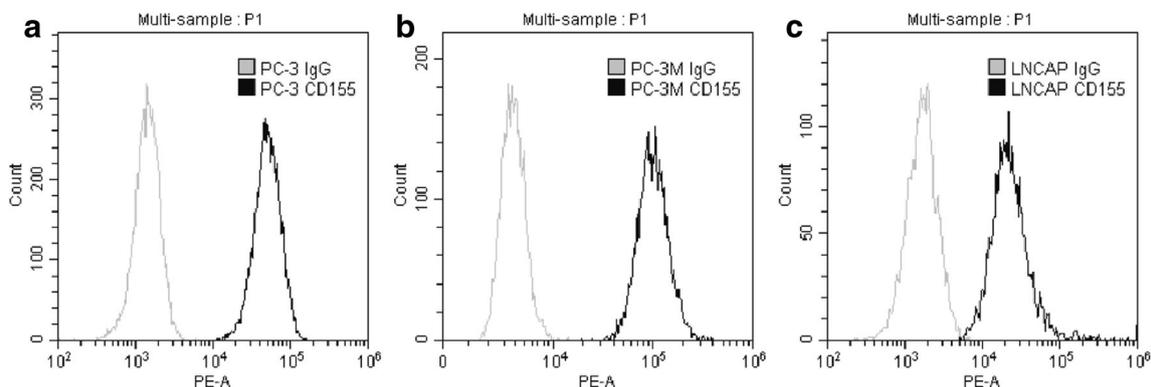


**Fig. 1** Expression of CD155 on different human prostate tumor cells. CD155 expression was evaluated by flow cytometry on (**a**) PC-3, (**b**) PC-3 M and (**c**) LNCAP cell lines. Black lines represent cells stained with the anti-CD155 mAb, and gray lines represent cells stained with the control mouse IgG. High CD155 expression was detected for all prostate cancer cells evaluated

**Fig. 2** General scheme for the generation of the anti-CD3 × anti-CD155 bispecific antibody and analysis of ATCs. **a** Flow cytometric analysis of cell-specific surface molecule expression on ATCs. **b** The binding assay for CD155Bi-Ab was assessed by flow cytometry. ATCs were probed with CD155BiAb (the black line) or a combination of OKT3 with anti-CD155 (the gray line). **a** FITC-anti-mouse-IgG1 was added to detect the CD155 part of CD155Bi-Ab. **b** FITC-anti-mouse IgG2a was added to detect the anti-CD3 part of CD155Bi-Ab
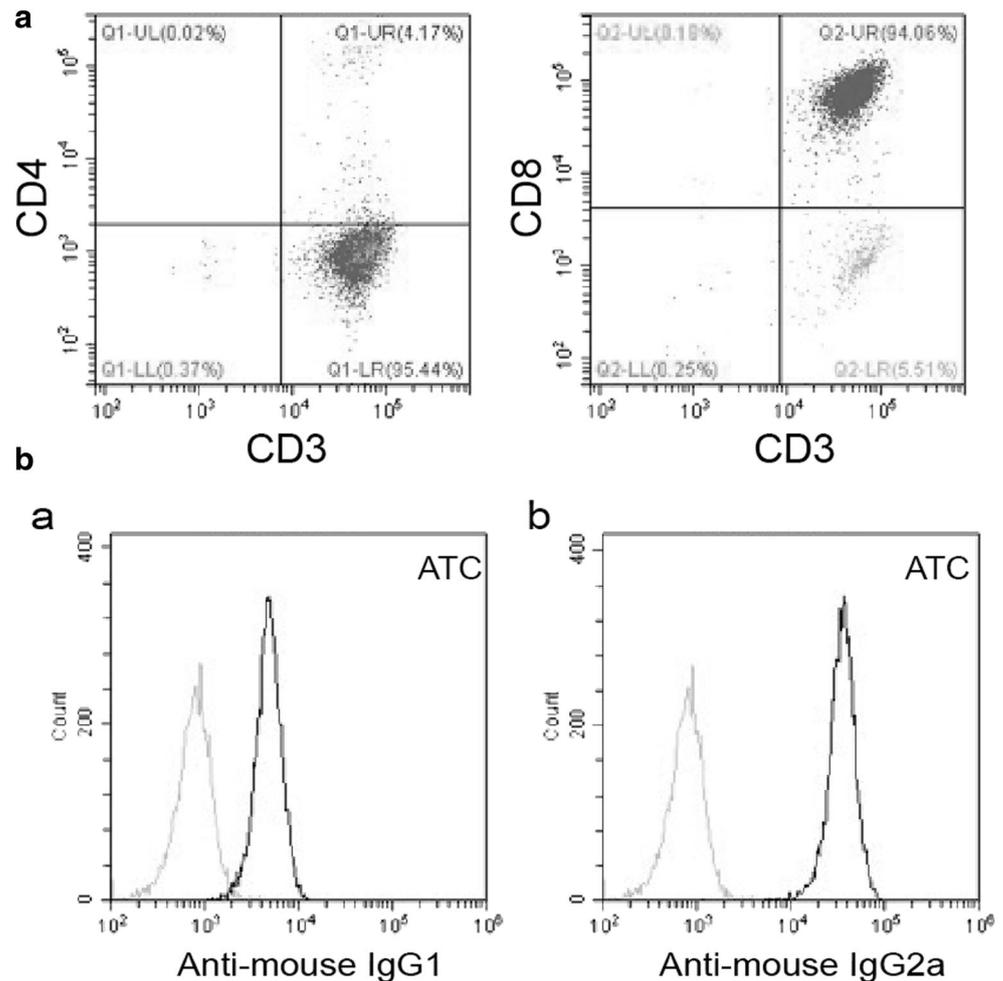


## Cytotoxic effects of CD155Bi-armed ATCs from prostate cancer patients

To validate the cytotoxic effects of CD155Bi-armed ATCs from prostate cancer patients, ATCs of peripheral blood were armed with CD155Bi-Ab; OKT3 and anti-CD155-mAb-pre-incubated ATCs were also used as unarmed ATCs. After 18 h of incubation with CD155Bi-armed or unarmed ATCs, cell culture supernatants were analyzed for cytotoxicity using specific ELISA and lactate dehydrogenase kits at an E/T of 5:1. As shown in Fig. 5, there was a significant increase in IFN-γ, TNF-α, IL-2 and LDH secretion in the CD155Bi-armed ATC group compared to the unarmed ATC group when cocultured with prostate cancer cells.

## Discussion

The incidence of prostate cancer is increasing, and in 20–30% of patients, early-stage prostate cancer will relapse after surgery and radiotherapy [3]. Endocrine therapy has a good initial effect, yet most of the final progression occurs in CRPC. However, immunotherapy has opened up new possibilities for the treatment of prostate cancer. A bispecific antibody or bifunctional antibody is a compound prepared by chemical coupling [18], cell engineering (hybridoma) [19] and genetic engineering [20] that can bind to two specific antigens in vitro and in vivo and produce biological effects. Bispecific antibodies are widely used for tumor drug-directed therapy, immunological detection, and mediating cytotoxicity [21].

The human CD155 molecule is a highly glycosylated type I transmembrane glycoprotein that belongs to the immunoglobulin superfamily (IgSF) [22]. Because the structure of CD155 is similar to Nectins, it is also classified as a member of the Nectin family. CD155 is expressed in a variety of tissues and cells, such as the small intestine, lung, liver, heart, spinal cord neurons, and skeletal muscle motor endplates, as well as the notochord, floor, neural tube, and visual system in the central nervous system. Initial studies found CD155 molecules to be
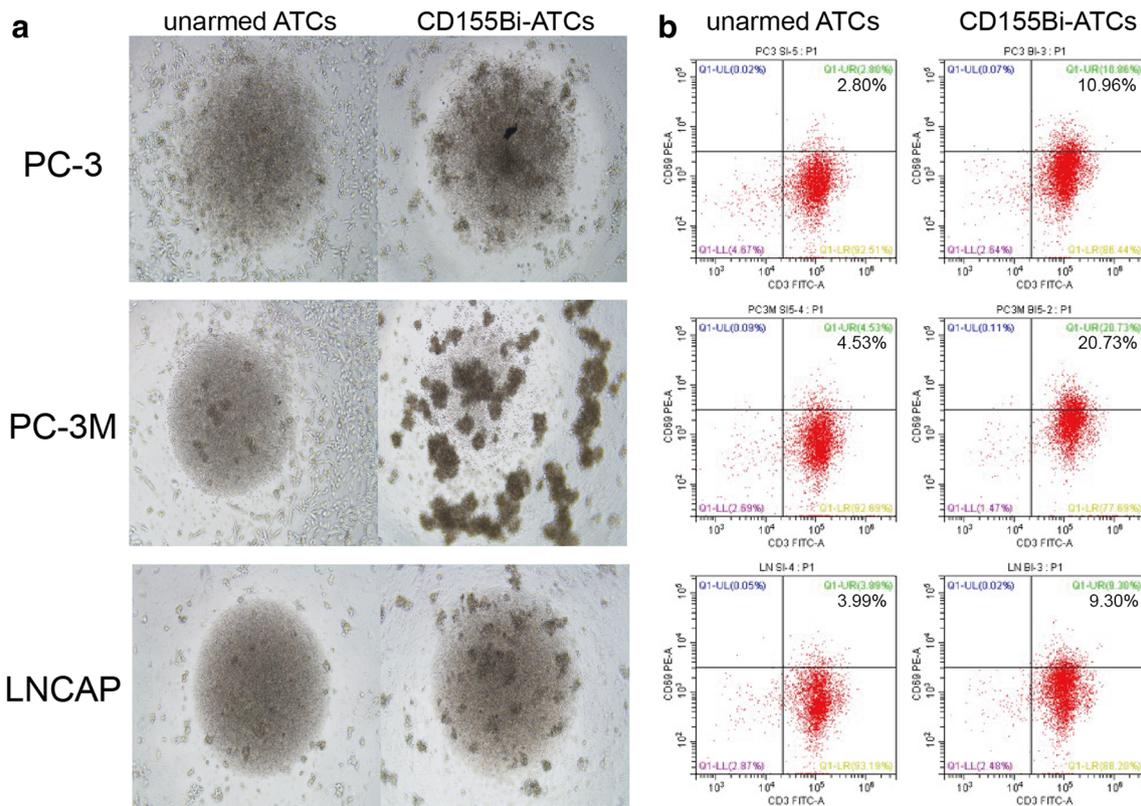
**Fig. 3** Cytotoxic effects of CD155Bi-armed ATCs on different prostate cancer cell lines. **a** Real-time photographs of each prostate cancer group were taken at 80× magnification, after 18 h of incubation with CD155Bi-armed ATCs or unarmed ATCs. **b** Expression of CD69 on CD155Bi-armed ATCs or unarmed ATCs was detected by flow cytometry after 18 h of coculture

highly expressed in colon cancer cells [12] and malignant glioma [13], and further analyses revealed expression in more types of tumors, such as prostate, kidney, pancreas, lung, ovary, thymus and brain tumors. The results show that CD155 molecules are highly expressed on various tumor cells and play a role in tumor development.

**Fig. 4** IFN-$\gamma$, TNF-$\alpha$ and IL-2 production by CD155Bi-armed ATCs and the lactate dehydrogenase (LDH) activity assay. **a** IFN-$\gamma$, **b** TNF-$\alpha$ and (**c**) IL-2 secretion by CD155Bi-armed ATCs in coculture with different prostate cancer cells. **d** Lactate dehydrogenase activity assay. All data are presented as the mean ± SD. Representative experiments of at least three experiments are shown. *$P < 0.05$, **$P < 0.01$
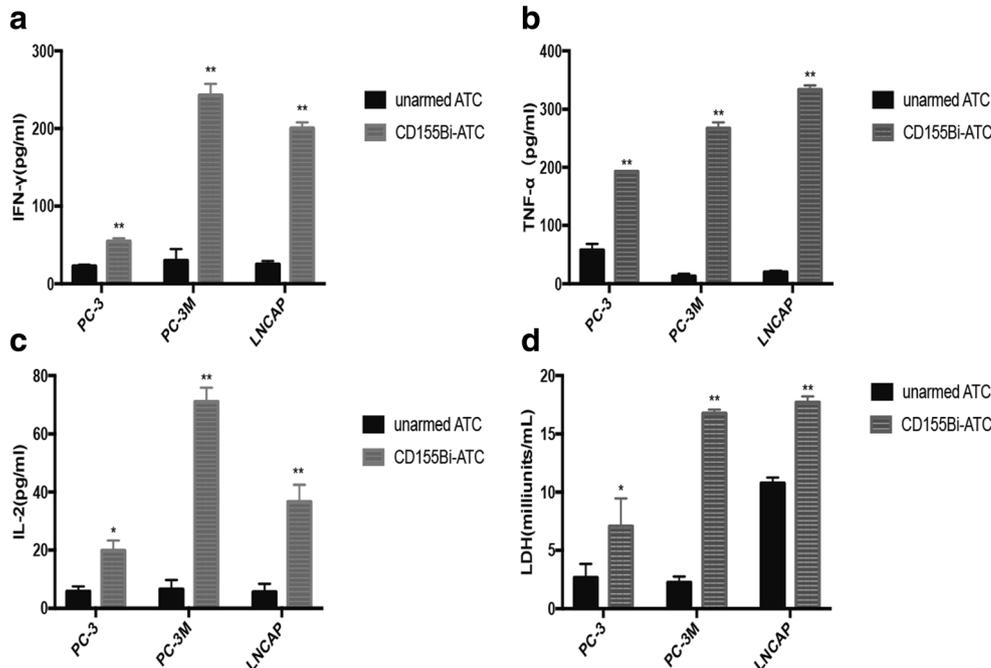
**Fig. 5** Cytotoxic effects of CD155Bi-armed ATCs from a prostate cancer patient on different prostate cancer cell lines. **a** IFN-γ, **b** TNF-α and (**c**) IL-2 secretion by CD155Bi-armed ATCs in coculture with different prostate cancer cells. **d** Lactate dehydrogenase activity assay. All data are presented as the mean ± SD. Representative experiments of at least three experiments are shown. *P < 0.05, **P < 0.01
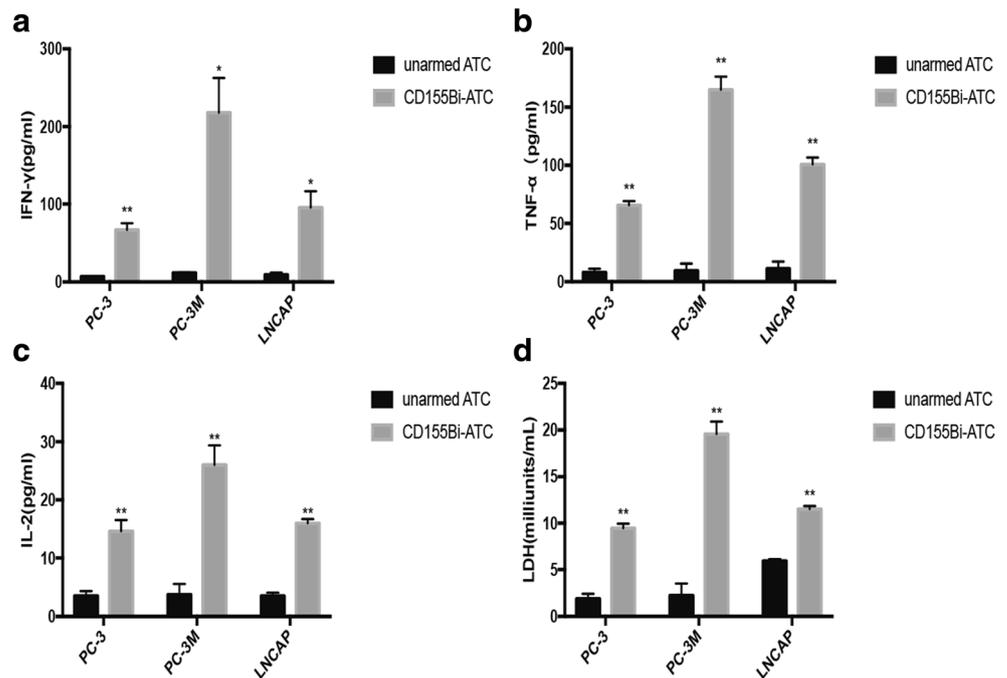


Previous studies have demonstrated that CD155 is involved in cell adhesion and migration, tumor cell invasion and metastasis, and embryonic development. Recently, it was discovered that CD155 is the ligand of the NK cell killing receptor CD226 [23], which has become a hot topic in NK cell tumor killing research. Expression of CD155 in tumor tissues and normal tissues differs, and high expression of CD155 can promote tumor metastasis [24], providing a basis for treatment strategies for CD155-positive tumors. For example, CD155 molecules are highly expressed by many breast cancer cell lines and primary tumor cells, and genetically engineered PV-loaded toxins have specific oncolytic effects and kill breast tumor cells [25]. Because CD155 is widely expressed, this treatment can be extended to a variety of tumor types [26].

An anti-CD3 × anti-PSA bispecific antibody was demonstrated to be effective against prostate carcinoma cells both in vitro and in vivo [27], indicating that bispecific antibodies may be a tool for prostate cancer immunotherapy. In this study, we show that the specific cytotoxicity of CD155-Bi-armed ATCs against CD155-positive prostate cancer cells was higher than that of control unarmed ATCs. After incubating CD155Bi-armed ATCs with prostate cancer cells, high concentrations of IFN-γ, TNF-α, and IL-2 secreted by these ATCs were detected. It is well known that these three cytokines are released from activated T cells and can directly kill tumors. In our in vitro cytotoxicity assay, ATCs armed with CD155-BiAb effectively killed cells without the addition of IL-2 to the culture. A significant increase in LDH secretion was also observed in the CD155Bi-armed ATC group compared to the unarmed ATC group when co-cultured with prostate cancer cells. Cells release LDH into the bloodstream after tissue damage or red blood cell hemolysis [28], and because LDH is a fairly stable enzyme, it has been widely employed to evaluate the presence of damage and toxicity to tissues and cells [29]. Additionally, the results of flow cytometry demonstrated that the level of CD69 expression among CD155Bi-armed ATCs was higher than that among control unarmed ATCs. CD69 is a marker of early T cell activation and a costimulatory molecule that increases T cell responses following TCR ligand interactions [30, 31]. Taken together, our data prove that the anti-CD3 × anti-CD155 bispecific antibody exerts cytotoxic effects on different prostate cancer cell lines in vitro; however, in vivo experiments are still needed to further verify the killing effect. In addition, CD155 expression in normal prostate and prostate cancer tissues also needs further validation.

In conclusion, combined with cytotoxicity and cytokine secretion assays in vitro, the ability of CD155Bi-armed ATCs to prevent the growth of prostate cancer cell lines suggests that these ATCs can be an effective strategy for the treatment of prostate cancer, with significant anti-tumor effects.

## Compliance with ethical standards

**Conflicts of interest** Huijun Zhao, Juan Ma, Ting Lei, Wanru Ma, Man Zhang declare that they have no conflicts of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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