RESEARCH ARTICLE

# Interleukin-7 Enhances the *in Vivo* Anti-tumor Activity of Tumor-reactive CD8+ T cells with Induction of IFN-gamma in a Murine Breast Cancer Model

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## Abstract

**Interleukin-7 (IL-7) is a potent anti-apoptotic cytokine that enhances immune effector cell functions and is essential for lymphocyte survival. While it known to induce differentiation and proliferation in some haematological malignancies, including certain types of leukaemias and lymphomas, little is known about its role in solid tumours, including breast cancer. In the current study, we investigated whether IL-7 could enhance the *in vivo* antitumor activity of tumor-reactive CD8+ T cells with induction of IFN-γ in a murine breast cancer model. Human IL-7 cDNA was constructed into the eukaryotic expression plasmid pcDNA3.1, and then the recombinational pcDNA3.1-IL-7 was intratumorally injected in the TM40D BALB/C mouse graft model. Serum and intracellular IFN-γ levels were measured by ELISA and flow cytometry, respectively. CD8+ T cell-mediated cytotoxicity was analyzed using the MTT method. Our results showed that IL-7 administration significantly inhibited tumor growth from day 15 after direct intratumoral injection of pcDNA3.1-IL-7. The anti-tumor effect correlated with a marked increase in the level of IFN-γ and breast cancer cells-specific CTL cytotoxicity. *In vitro* cytotoxicity assays showed that IL-7-treatment could augment cytolytic activity of CD8+ T cells from tumor bearing mice, while anti-IFN-γ blocked the function of CD8+ T cells, suggesting that IFN-γ mediated the cytolytic activity of CD8+ T cells. Furthermore, *in vivo* neutralization of CD8+ T lymphocytes by CD8 antibodies reversed the antitumor benefit of IL-7. Thus, we demonstrated that IL-7 exerts anti-tumor activity mainly through activating CD8+ T cells and stimulating them to secrete IFN-γ in a murine breast tumor model. Based on these results, our study points to a potential novel way to treat breast cancer and may have important implications for clinical immunotherapy.**

**Keywords:** Interleukin-7 - breast cancer - CD8+ T cells - IFN-γ - anti-tumor activity

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## Introduction

Cytokines are molecular messengers that allow the immune effector cells to communicate with one another to generate a coordinated, robust, but self-limited response to a target antigen (Lee et al., 2011). Over the past two decades, the growing interest in harnessing the immune system to eradicate cancer has been accompanied by heightened efforts to characterize cytokines and exploit their vast signaling networks to develop cancer treatments (Pellegrini et al., 2010). Cytokines directly stimulate immune effector cells and stromal cells at the tumor site and enhance tumor cell recognition by cytotoxic effector cells, and are crucial for the survival and proliferation of a broad range of T lymphocytes, resulting in the maintenance of a polyclonal naive T cell pool with the ability to respond to tumors (Klebanoff et al., 2004; Lee et al., 2011). Numerous animal tumor model studies have demonstrated that cytokines have broad anti-tumor activity and this has been translated into a number of cytokine-based approaches for cancer therapy (Krawczenko et al., 2005). Recent years have seen a number of cytokines, including GM-CSF, IL-2, IL-12, IL-15, IL-18 and IL-21, from bench to clinical trials, have been used for patients with advanced cancer (Krawczenko et al., 2005; Li et al., 2007).

IL-7 is a single-chain protein of 19 kDa produced predominantly by bone marrow and lymphoid stromal cells, which belongs to a type I short-chain cytokine of the hematopoietin family, a group that also includes IL-2, IL-3, IL-4, IL-5, GM-CSF, IL-9, IL-13,IL-15,and M-CSF (Li et al., 2007; Liu et al., 2007). It was first recognized as a protein with growth promoting effects on B-cell progenitors in vitro and later for its critical role in

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lymphopoiesis. Recently, it has been termed an "immuno-regenerative cytokine" recognizing its modulatory role in peripheral T-cell homeostasis (Fry et al., 2002; Krawczenko et al., 2005). The biological effects of IL-7 on target cells are mediated by binding to the high-affinity IL-7 receptor complex that is composed of the ligand binding IL-7 receptor a-chain and the common shared γ-chain (Vudattu et al., 2007). Naive T cells express high levels of the IL-7 receptor CD127 and respond rapidly to IL-7 stimulation, making this molecule an attractive agent for restoration of T cell activities in tumor-bearing hosts. For the recent years, IL-7 has been widely used as an immunotherapy for inflammation and tumor as for its multi-biological property (Appasamy, 1999; Krawczenko et al., 2005; Pellegrini et al., 2010; Lundstrom et al., 2012), functions as a pre-pro-B cell growth-stimulating factor, inducing proliferation of CD4/CD8 subtype in thymus, stimulating the differentiation of PBMC into LAK cells. There are several reports from both murine and human studies describing the anti-tumor activity of IL-7. In lung cancer-bearing mice (Andersson et al., 2009), IL-7 treatment led to marked reduction in tumor burden with extensive lymphocytic infiltration of the tumors and enhanced survival. The antitumor responses were accompanied by the enhanced elaboration of IFN-γ and the IFN-γ inducible CXCR3 targeting antiangiogenic chemokines CXCL9 and CXCL10. In melanoma-bearing mice (Wu et al., 1993; Krawczenko et al., 2005), after treatment with IL-7 in combination with local hyperthermia, the maximal anti-tumor effect was reached. The anti-tumor effect was also observed in patients with metastatic melanoma. The application of exogenous IL-7 augmented LAK cell proliferation and activity. Interleukin-7 cDNA transfered in non-small-cell lung cancer decreases tumor proliferation (Sharma et al., 1996), modifies cell surface molecule expression, and enhances antitumor reactivity. However, little is known about the antitumor effects of IL-7 against breast cancer. An understanding of the cellular mechanism of IL-7-mediated anti-breast cancer responses will enable the design of strategies for its optimum utility in tumor control.

In this study, we investigated the relative mechanism involved in how IL-7 performed antitumor activity in a murine breast tumor model. Our results demonstrated that the tumor growth was significantly inhibited following IL-7 administration to mice bearing breast cancer from day 15 after direct intratumoral injection of pcDNA3.1-IL-7. Accompanying the in vivo antitumor responses of IL-7 administration was marked by increasing the level of IFN-γ and enhancing CTLs cytotoxicity. IL-7 administration could augment cytolytic activity of $CD8^+$ T cells from mice bearing breast tumor, however, antibody to IFN-γ neutralized the cytolytic activity of $CD8^+$ T cells. Furthermore, in vivo neutralization of $CD8^+$ T lymphocytes by CD8 antibody reversed the anti-tumor benefit of IL-7. The neutralization to IFN-γ and depletion of $CD8^+$ T cells demonstrated that the in vivo mechanism underlying the anti-tumor effects of this regimen was partly dependent on the production of IFN-γ by the $CD8^+$ T cells and not their direct cytolytic capability.

## Materials and Methods

*Materials*

Antibodies including PE-anti-mouse IFN-γ, PE-Cy5-anti-mouse CD3, FITC-anti-mouse CD8, FITC-anti-mouse CD4, FITC-anti-mouse F4/80, FITC-anti-mouse pan-NK (CD49b) were purchased from BioLegend. Mouse IFN-γ ELISA kits was from eBioscience, CD8 antibody was purchased from BD Bioscience. Human IL-7 mAb (Clone 7417) and IFN-γ antibody were from R&D Systems. pcDNA3.1 (-)/Myc-His A Vector from Amersham Biosciences. DH-5α competent cells (E.Coli) were made by our own laboratory. Mouse breast cancer cell line TM40D was from China Center for Type Culture Collection (CCTCC). Wild-type BALB/C mice (4-6 weeks old, female) were from Hubei Province Centre for Disease Control and Prevention. The animal experimental protocols were performed in compliance with all guidelines and were approved by the Institutional Animal Care and Use Committee of Wuhan University.

*IL-7 Eukaryotic Vector Construction, Transient transfection, and Expression*

The human IL-7 expressing vector construction was according to previous articles. IL-7 cDNA (GenBank: accession, BC047698) was amplified and subcloned in-frame between the HindIII and EcoRI restriction sites of pcDNA3.1 (-) Myc-His A (designated as pcDNA3.1). The construction was confirmed by restriction enzyme digestions and DNA sequencing analysis. The primer sequences were (forward) 5'-GCAAGCTTATGTTCCATGTTTCTTTT-3' and (reverse) 5'-CCGGATCCTTATATACTGCCCTTCAA-3', where the underlined oligonucleotides represent EcoRI and HindIII sites, respectively, to facilitate cloning. cDNA encoding human IL-7 was obtained by reverse transcription and PCR amplification of RNA derived from the human peripheral blood. Murine TM40D cells were grown in 75 $mm^2$ flasks and transfected with 5 μg of pcDNA3.1, pcDNA3.1-IL-7 (designated as pcDNA3.1-IL-7), detailed steps according to Lipofectamine TM2000 (Invitrogen) manual. After 48 h of incubation at 37°C in a $CO_2$ incubator, cell supernatants were collected and analyzed by SDS-PAGE and Western blot.

*Model Construction of Mice Bearing Breast Tumor and Evaluation of Local Tumor Growth*

TM40D cells in the exponential growth phase were harvested and washed in PBS, before in vivo injection, their viability exceeded 95%, as determined by trypan blue staining. To induce localized tumors, syngeneic BALB/c mice were subcutaneously (s.c.) challenged into the forth Mammary Gland with $1\times10^6$ TM40D breast cancer cells (Murphy et al., 1993). The indicated doses (100 $\mu$g/100 $\mu$l) of pcDNA3.1, pcDNA 3.1-IL7 and/or PBS were then injected s.c. into the tumor injection site at day 5, and then stimulated by gene pulse transfection apparatus. Tumor size was determined every other day in 30-day interval with caliper, and the tumor volume in $mm^3$ was calculated by the formula: Volume = $(\text{width})^2 \times \text{length}/2$.

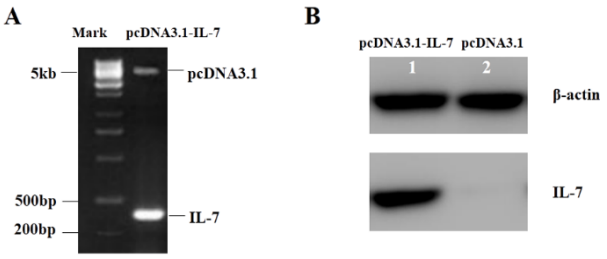


**Figure 1. pcDNA3.1-IL-7 Recombinant Plasmid Construction and Expression of Secreted IL-7 Proteins in TM40D Cells.** (A) Recombinant plasmid pcDNA3.1-IL-7 was digested by Hind III and EcoR I, then run DNA gel electrophoresis. (B) Cell culture supernatants from TM40D cells transfected with pcDNA3.1-IL-7 (lane 1) or empty vector pcDNA3.1 (lane 2) for 48 h were analysed by Western blot using human IL-7 mAb

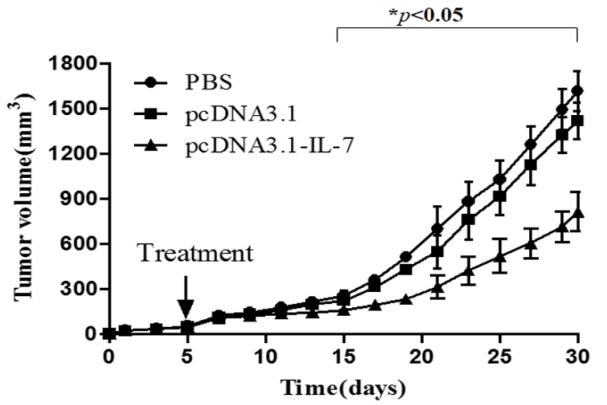


**Figure 2. IL-7 Significantly Inhibited Tumor Growth.** On the day 5 after inoculation with $1\times10^6$ TM40D cells, BALB/C mice were treated with PBS, empty vector pcDNA3.1 and pcDNA3.1-IL-7 (100μ g/100 μl) via directly injected into tumor tissue. Mean tumor size in each mouse group was quantified every other day. Bars, SD. The mean tumor size in pcDNA3.1-IL-7 mouse group was significantly smaller than that in PBS group or pcDNA3.1 group ($P < 0.05$) from day 15. *$p$ value was determined by comparing tumor size in pcDNA3.1-IL-7 group with PBS or pcDNA3.1 group

*IFN-γ-Specific ELISA*

For in vivo analysis of level of IFN-γ, the serum of each mouse group was harvested at day 30 post injected tumor cells, and then collected for analyzing cytokine levels by using an Ag-capture ELISA specific kits (eBioscience) according to the protocol recommended by the manufacturer.

*Flow Cytometry*

Splenocytes of each mouse group were harvested at day 30 post injected tumor cells, to detect the intracellular IFN-γ production, cells were incubated for 5h in the presence of PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and monensin (1000X; Biolegend). Cells were then stained with PE-Cy5-labeled anti-CD3, FITC-labeled anti-CD4, FITC-labeled anti-CD8, FITC-labeled anti-pan-NK (CD49b), or FITC-labeled F4/80 at recommended usage, and following fixed with 4% paraform- -aldehyde for 30 min. After permeabilized and stained with PE-labeled anti-IFN-γ, the intracellular IFN-γ production was analyzed on a BD C6 flow cytometer.

*In vitro Cytotoxicity Assay*

Target cells (TM40D) were prepared at $4\times10^5$/ml, $CD8^+$ T cells were isolated from splenocytes of treated mice, set 3 longitudinal groups to obtain effector-target ratios from 25:1 to 100:1, then 100$\mu$l of each cell solution was placed in triplicates into 96-well plate and incubated 48h. TM40D cells and $CD8^+$ T cells each alone was simultaneously seeded as two control groups. As for pcDNA3.1-IL-7 + anti-IFN-γ group, upon cells co-cultured, IFN-γ secretion was blocked by mouse IFN-gamma mAb (5 μg/ml, Clone H22, R&D Systems), and $CD8^+$ cells kill activity was detected by MTT method. Counted cell killed percentage (%) by using the formula: lysis percentage (%) = [1-(experimental OD – $CD8^+$ T cells OD)/ TM40D cells OD] × 100.

*In vivo $CD8^+$ T cells Depletion Assay*

Set three groups of mouse model (6 mice/group), PBS, pcDNA3.1-IL-7 + CD8 Ab Isotype and pcDNA3.1-IL-7 + Anti-CD8 (CD8 Antibody), respectively. And for pcDNA3.1-IL-7 + anti-CD8 group or pcDNA3.1-IL-7 + CD8 Ab Isotype group, mice were intravenously injected with anti-CD8 mAb (clone 2.43; BD Bioscience, 250 μg/mouse) or CD8 Ab Isotype (Rat IgG2a, ϰ) 24h before plasmid treatment. Tumor size (volume) was determined every other day in 30-day interval.

*Statistical Analysis*

The data were analyzed using Prism 5.0 software (GraphPad). Experiments were repeated three times. The data presenting the differences between the groups were analyzed by one-way or two way ANOVA with Bonferroni post-test to compare tumor volume in the treatment groups. $P < 0.05$ indicated that the value of the test sample was significantly different from that of the relevant controls.

## Results

*Construction of recombinant pcDNA-3.1-IL-7 plasmid and IL-7 expression in murine TM40D cells*

To construct the recombinant plasmids, the DNA fragment of the entire IL-7 gene sequence from splenocytes was amplified and cloned into the eukaryotic expression vector pcDNA3.1. Restriction enzyme digestion analysis (Figure 1A) showed that the relative molecular mass (Mr) of each inserted DNA fragment was identical to the value predicted. Recombinant plasmid was then transient transfected into TM40D cells, harvested cells after 48h. IL-7 expression was determined by western-blotting. Western blot analysis showed that IL-7 was expressed by TM40D cells (Figure 1B).

*IL-7 significantly inhibited mouse breast tumor growth*

To determine whether IL-7 has anti-tumor activity, the mice were treated with 100$\mu$g/100$\mu$l of pcDNA3.1(control vehicle), pcDNA3.1-IL-7 or PBS by using direct intratumoral injection at day 5 after TM40D cells injection; tumor size was then determined every day in 30-day interval. As shown in (Figure 2), tumor growth of

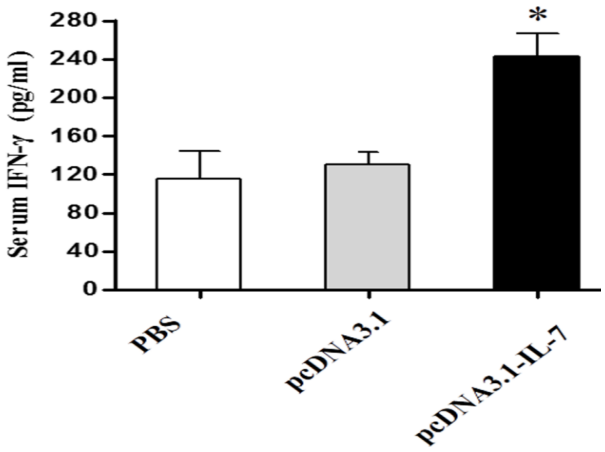


**Figure 3. Increased Serum IFN-γ Level in pcDNA3.1-IL-7-treated Mice.** On the day 5 after inoculation with $1\times10^6$ TM40D cells, mice were treated with PBS, empty vector pcDNA3.1 and pcDNA3.1-IL-7 (100 μg/100 μl) via directly injected into tumor tissue. (n=6 mice/group). The sera were harvested on day 30 and then analyzed IFN-γ level by ELISA. *$p$<0.05, pcDNA3.1-IL-7 group v.s. PBS or pcDNA3.1 group. Data shown are mean±SD from six independent experiments

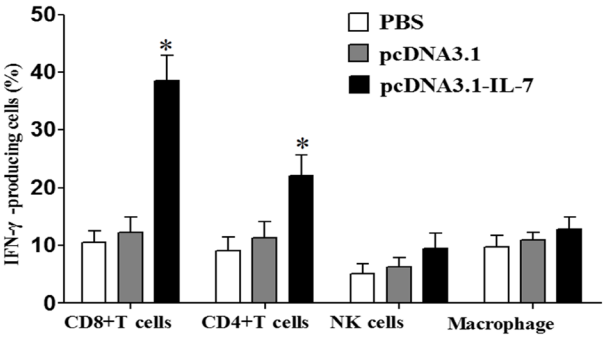


**Figure 4. IL-7 Induces Intracellular IFN-γ Production *in vivo* from CD4⁺ and CD8⁺ T Cells, but Not from NK Cells and Macrophage.** Spleens were harvested from BALB/c mice 30 days post TM40D cells injection. The intracellular IFN-γ production in splenic CD8⁺ T cells, CD4⁺ T cells, NK cells and macrophage was measured by flow cytometry. Data shown are mean±SD from six independent experiments. *$p$<0.05, pcDNA3.1-IL-7 v.s. PBS or pcDNA3.1 group

pcDNA3.1-IL-7 treated group was significantly inhibited since day 15 when compared with control groups. This result demonstrates that IL-7 was effective against TM40D -induced breast tumor.

*Increased serum IFN-γ level in IL-7-treated tumor bearing mice*

IFN-γ is a cytokine whose biological activity is conventionally associated with cytostatic/cytotoxic and anti-tumor mechanisms during cell-mediated adaptive immune response, and plays an important role in the host response to restrict tumor growth, so we examined the effect of exogenous IL-7 administration on IFN-γ secretion in tumor-bearing mice. The levels of serum IFN-γ was measured 30 days after mice were injected with breast tumor cells TM40D via s.c. ELISA analysis showed that serum IFN-γ levels were significantly increased in the pcDNA3.1-IL-7-treated group (242.3±10.1 pg/ml) (*$p$<0.05, Figure 3) compared to the pcDNA3.1 empty vector group (133.6±9.4 pg/ml) or PBS-treated

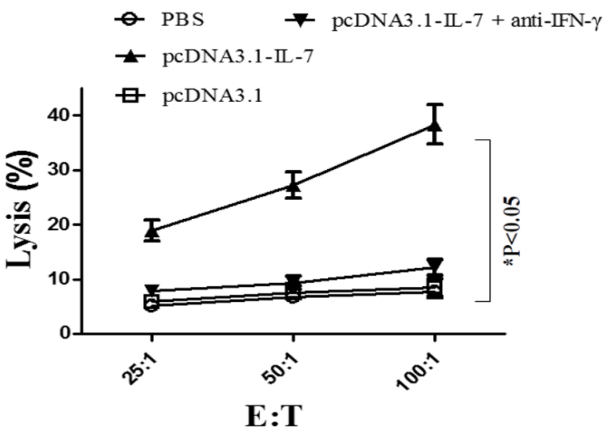


**Figure 5. As Shown, CD8⁺ T Cells, Namely Effector Cells, Were Isolated from Splenocytes of Tumor Bearing Mice on Day 30.** TM40D cells were used for target cells. Cells were co-cultured for 48h, then detecte CD8⁺ T cells cytolytic activity by MTT method. As for pcDNA3.1-IL-7+anti-IFN-γ group, IFN-γ antibody added in the first time cells co-cultured.*pcDNA3.1-IL-7 v.s. pcDNA3.1, pcDNA3.1-IL-7 + anti-IFN-γ or PBS groups, $p$<0.05

**Table 1. IL-7 Augments IFN-γ-mediated CD8⁺ T Cells Cytolytic Activity (Cytotoxic assay analyzed by MTT method)**

| Groups | Cell lysis (%) | | |
|---|---|---|---|
| | E:T=25:1 | E:T=50:1 | E:T=100:1 |
| PBS | 5.2±0.6 | 6.8±0.9 | 7.7±1.1 |
| pcDNA3.1 | 5.9±0.9 | 7.6±1.2 | 8.5±1.6 |
| pcDNA3.1-IL-7 | 18.6±1.9* | 27.3±2.4* | 38.3±3.6* |
| pcDNA3.1-IL-7+anti-IFN-γ | 7.8±0.9 | 9.3±1.2 | 12.2±1.5 |

*pcDNA3.1-IL-7 v.s. pcDNA3.1, pcDNA3.1-IL-7 + anti-IFN-γ and PBS groups, $p$<0.05. E, effector cells (CD8⁺ T cells were isolated from splenocytes of tumor bearing mice on day 30); T, target cells (TM40D cells). IL-7 expressed in vivo significantly increased CD8+ T cells lytic activity, but IFN-γ Ab neutralized this function

group (115.2±11.8 pg/ml). Our finding reveals that IL-7-treatment greatly enhances anti-tumor cytokine IFN-γ production in vivo and IFN-γ may serve as a role that facilitated IL-7 constraining tumor growth.

*IL-7 induces the intracellular IFN-γ production of CD4⁺ and CD8⁺ T cells*

To further assess the role of IFN-γ in the IL-7-mediated antitumor activity, we then employed flow cytometry analysis using dual fluorescence-labeled Ab to examine the types of immune cells (CD4⁺ cells, CD8⁺ cells, NK cells and Macrophage) producing IFN-γ in spleen of IL-7-treated mice. Intracellular cytokine staining analysis showed that IL-7 treatment significantly induced the production of intracellular IFN-γ in CD8⁺ T cells (Figure 4), as well as in CD4⁺ T cells. But has no comparable difference found in NK cells and Mϕ, this finding hinted that IL-7 may mainly prompt adaptive immune response to accomplish its anti-tumor activity.

*IL-7 augments CD8⁺ T cells cytolytic activity*

The above results suggest that IL-7 inhibits local tumor growth, and stimulates IFN-γ secretion in serum

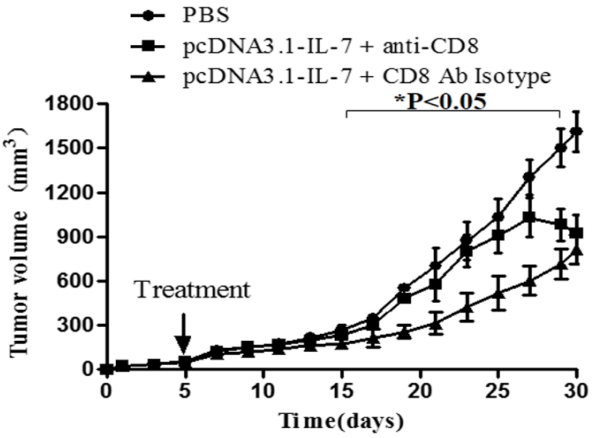


**Figure 6. CD8 antibody neutralized IL-7 antitumor effect.** On the day 5 after inoculation with $1\times10^6$ TM40D cells, BALB/C mice were treated with PBS, pcDNA3.1-IL-7 + anti-CD8 or pcDNA3.1-IL-7 + CD8 Ab Isotype via directly injected into tumor tissue, respectively. CD8 Ab was intravenously injected via tail vein 24h before plasmid treatment. Mean tumor size in each mouse group was quantified every other day. Bars, SD. The mean tumor size in pcDNA3.1-IL-7 + CD8 Ab Isotype mouse group was significantly smaller than that in PBS group or pcDNA3.1-IL-7 + anti-CD8 group ($*p < 0.05$) from day 15 to 28

or produced by CD8+ T cells, so we wondered whether IL-7 could enhance CD8+ T cells cytolytic activity. Ex vivo cytolytic assays showed that CD8+ T cells from IL-7-treated group had higher levels of cytolytic activity compared with control group at the same E/T ratio. Cytolytic activity of CD8+ T cells was more visible along with E/T ratio increased, especially when E/T was 100:1. However, anti-IFN-γ could block cytolytic activity performed by CD8+ T cells when previously incubated with it (Table 1 and Figure 5). So we could drew a conclusion that IL-7 inhibits breast tumor growth by inducing CD8+ T cells to secrete IFN-γ, and then IFN-γ performed its anti-tumor activity directly or indirectly by mediated CD8+ T cells cytolytic activity.

*In vivo injected CD8 Ab reversed IL-7 anti-tumor activity*

In order to figure out whether CD8+ T cell is a major component which IL-7 employed to anti-tumor. 24h before pcDNA3.1-IL-7 injection, mice were injected with CD8 mAb (250μg/mice) or Iso-type IgG control, and then measured tumor size according to previous schedule. We observed that anti-CD8-treatment reversed the inhibitory role of IL-7 in tumor growth, a significant difference of tumor size was noticed when compared anti-CD8-treatment with Iso-type from day 15 to day 28 (Figure 6). So we also confirmed that CD8+ T cells play important role in antitumor and is consistent with previous reports (Tanaka et al., 1999).

## Discussion

The potential for IL-7 to expand the naive T cell pool and memory cell pool by driving homeostatic expansion, could be applied therapeutically (Rosenberg et al.,2006). Using this rationale, studies have been performed to evaluate whether IL-7 can increase the number of responding T cells and improve anti-tumor immune responses (Krawczenko et al., 2005; Yang et al., 2005). Three main immunotherapeutic approaches have been used to evaluate IL-7 anti-tumor immunity, the vaccine strategies, adoptive transfer of tumor specific T cell therapy strategies and the gene transfer therapy strategies. Severial studies have been used to use IL-7 as an adjuvant together with vaccines to evaluate anti-tumor immunity in a lymphopenic environment (Krawczenko et al., 2005; Pellegrini et al., 2009). IL-7 and IL-15 are known to be increased under lymphopenic conditions, and studies have shown both using vaccine strategies and adoptive T cell therapy strategies (Rubinstein et al., 2008).

Several investigators have used gene transfer techniques to increase local production of IL-7 in tumor cells or within the tumor microenvironment (Krawczenko et al., 2005). IL-7 gene transfer enhances the immune response to tumor cells and can support tumor regression. Finke et al (Finke et al., 1998) applied adenovirus-enhanced CD3 receptor-mediated IL-7 gene transfer in SCID mouse bearing human lymphoma. An episomally replicating plasmid containing human IL-7 cDNA under the control of a cytomegalovirus promoter was used for transfecting cytokine-induced killer (CIK) cells (Pellegrini et al., 2011). A report (Sharma et al., 2003) described the intratumoral administration of IL-7 gene-modified DCs. Another group (Verhoeyen et al., 2003) described the application of lentiviral vectors for IL-7 gene transfer into resting primary T lymphocytes.

As considered that tumor vaccine produced by ex vivo way is complicated and time consuming, even more, viral vector application have the possibility influenced by viral replication and immune reaction mediated by recipient (Anderson et al., 2007), we adopted simple and effective gene-treatment by direct intratumoral injection of pcDNA3.1-IL-7 in the TM40D BALB/C mice grafts model to evaluate the antitumor effect of IL-7. From day 15 after direct intratumoral injection of pcDNA3.1-IL-7, IL-7 significantly inhibited tumor growth compared with empty vector pcDNA3.1 or PBS treatment. This result is in agreement with the previous studies about IL-7 antitumor activities.

IFN-γ, which is mainly produced by T cells and NK cells, plays a major role in the antitumor activity (Zaidi et al., 2011). The expression of serum and intracellular IFN-γ were deteced in our study. Accompanying the anti-breast cancer responses of IL-7-treament were increases the level of IFN-γ. Compared with PBS or empty vector pcDNA3.1 group, IL-7 administration group significantly stimulated IFN-γ production. In order to figure out the types of immune cells producing IFN-γ following IL-7-treated mice, intracellular cytokine staining was used to analyze the percentage of IFN-γ-producing cells among CD4+ T cells, CD8+ T cells, NK cells and Macrophage (Mϕ) in spleen. We found that IL-7 mainly induced CD4+ T cells and CD8+ T cells to secrete IFN-γ, but had minimal effect on NK cells and Mϕ. In additoin to increased cytokine IFN-γ production, immuno-histochemistry analysis showed that tumor tissue was infiltrated by plenty of inflammatory cells, CD4+ T cells and CD8+ T cells. Many facets of T lymphocyte activities are suppressed,

thus allowing tumors to progress in immune-competent hosts (Jicha et al., 1991; Klebanoff et al., 2006; Andersson et al., 2009). Our results indicate that IL-7 treatment of breast cancer-bearing mice restores T cell activities and stimulates IFN-γ production. IL-7 treatment caused a significant reduction in tumor burden that correlated with increases in the $CD8^+$ T cell population and stimulating them to secrete high level IFN-γ.

Cytotoxic T lymphocytes (CTLs) can play a prominent role in the anti-tumor immune responses of both mice and humans (Lynch et al., 1994). Many studies have demonstrated that γ-chain receptor cytokines, including IL-2, IL-7 and IL-15, act at different stages of the immune response to promote the survival, proliferation and effector function of $CD8^+$ T cells (Strengell et al., 2003; Krawczenko et al., 2005; Andersson et al., 2009). IL-7 is critical for the homeostatic expansion of naive $CD4^+$ and $CD8^+$ T cells in immunodeficient hosts and can also mediate the survival of memoryT cells (Roato et al., 2006). IL-7 has been demonstrated to enhance both antitumour and anti-viral CTL as well as lymphokine-activated killer (LAK) activity (Unsinger et al., 2010). In this report, we studied the ability of IL-7 to support the activation of in vitro $CD8^+$ T cell CTL present in spleen of breast cancer-bearing mice. We separated splenic T cells from each group as effector cells, and used TM40D cells as target cells to evaluate $CD8^+$ T cells cytolytic activity in vitro. As showed in Table 1, compared with PBS or empty vector pcDNA3.1 group, $CD8^+$ T cells from IL-7 treated tumor- bearing mice had enhanced cytolytic activity, especially when E:T (effector cells:target cells) ratio was 100:1. To explore whether the CTL direct by $CD8^+$ T cells, the importance of IFN-γ was assessed in neutralization experiments. When anti-IFN-γ was added into the co-culture system, neutralization of IFN-γ partially reversed the antitumor benefit of IL-7. The neutralization of antibodies to IFN-γ demonstrated that the in vitro mechanism underlying the antitumor effects of this regimen was partly dependent on the production of IFN-γ by the $CD8^+$ T cells and not their direct cytolytic capability.

T cells played a crucial role in the generation of an efficient IL-7-mediated anti-tumor immune response which is supported by the evidence from neutralization experiments (Ferrari et al., 1995; Geiselhart et al., 2001; Lai et al., 2011). With the aim of confirming the rolling part of $CD8^+$ T cells more precisely, we depleted $CD8^+$ T cells in vivo, and found that IL-7-treatment was not working well in controlling tumor growth, and IFN-γ level in sera decreased significantly. Neutralization of $CD8^+$ T cells led to the complete reversal of the anti-tumor immune responses, indicating $CD8^+$ T cell effectors for the full anti-tumor benefit of IL-7. As shown in Figure 6, after 28 days, tumor growth was inhibited in $CD8^+$ T cells depleted mice bearing breast cancer, the possible reason maybe that cytokines have network effects, and IL-7 can function on another part of immune system like $CD4^+$ T cells to inhibit tumor growth in an advanced stage (Lo et al., 2003; Terabe et al., 2004), or only as a result of the degradation of CD8 antibody. Those possibilities need to be further confirmed.

Taken together, the enhancement in IL-7-dependent anti-breast cancer immune responses requires both activited $CD8^+$ T cells and high level IFN-γ to exert their cytolytic function. Though the fact that IL-7 significantly reduced tumor burden mainly dependent on $CD8^+$ T cells to secret IFN-γ to perform their cytolytic function, there may be still have another ways for IL-7 to realize its antitumor capacity. Nevertheless, the findings from the current study may stimulate the development of an immunotherapeutic approach using IL-7 for the stimulation of adaptive immunity against breast cancer.

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The authors declare that they have no competing interests.

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