ORIGINAL RESEARCH

Effect of ex vivo-expanded $\gamma\delta$ -T cells combined with galectin-1 antibody on the growth of human cervical cancer xenografts in SCID mice

Hua Li^{1*}

Yubin Wang^{2*}

FuXiang Zhou¹

**Hua Li and Yubin Wang contributed equally to this work.*

¹ Department of Medicine Oncology and Radiation Oncology, Zhongnan Hospital, Wuhan University, Wuhan, 430071, P.R.China.

² Wuhan University of Science and Technology, Wuhan, 430065, P.R.China.

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Abstract

Objective: To investigate the antitumor activity of *ex vivo*-expanded $\gamma\delta$ -T cells derived from tumor-infiltrating lymphocytes ($\gamma\delta$ TILs) from cervical cancer patients when combined with galectin-1 antibody and studied both *in vitro* and *in vivo*.

Methods: The presence of $\gamma\delta$ TILs in cervical cancer specimens was detected by immunohistochemistry and $\gamma\delta$ TILs were expanded using the solid-phase antibody method. The expression of galectin-1 by the human cervical cancer cell line, SiHa, was measured by Western blot and ELISA. *In vitro* cytotoxic activities of expanded $\gamma\delta$ TILs, with or without galectin-1 inhibitor, were determined using the LDH-release test. *In vivo* antitumor activity of $\gamma\delta$ TILs, combined with galectin-1 antibody, was evaluated using the SCID mouse model.

Results: $\gamma\delta$ TILs existed in the cervical cancer and the percentage of TCR $\gamma\delta^+$ cells in $\gamma\delta$ TILs after *ex vivo* expansion was $91.2 \pm 1.2\%$ detected by flow cytometry. SiHa cell expressed and secreted galectin-1 as measured by Western blot and ELISA. Expanded $\gamma\delta$ TILs from human cervical cancer demonstrated marked cytotoxicity to SiHa or Hela cells. In comparison with non-treated group, the cytotoxicity of $\gamma\delta$ TILs towards SiHa or Hela cell was significantly increased when effector and target cells were incubated with either lactose or galectin-1 antibody at E/T ratio of 1:1

($p < 0.05$). $\gamma\delta$ TILs, in combination with galectin-1 antibody treatment, significantly suppressed the growth of xenografts in SCID mice, in comparison with all other groups ($p < 0.05$). $\gamma\delta$ TILs alone also showed the ability to inhibit tumour growth *in vivo*, but were more efficient when combined with specific antibody ($p < 0.05$).

Conclusion: Taken together, our results suggest that $\gamma\delta$ -T cells, combined with galectin-1 antibody treatment, could be a more effective adoptive immunotherapy for patients with cervical cancer than traditional adoptive immunotherapy methods.

List of Abbreviations

FCM	Flow cytometry
ELISA	enzyme linked immunosorbent assay
TILs	tumor-infiltrating lymphocytes

Unlike $\alpha\beta$ -T cells, which require the recognition of specific processed peptide antigens presented by major histocompatibility complex (MHC) class-I or class-II molecules (adaptive immunity), $\gamma\delta$ -T cells, appear to recognize and respond to a variety of stress-induced self-antigens commonly displayed by cells

that have undergone malignant transformation.¹⁻⁶ Thus, while incapable of recognizing tumor-specific antigens *per se*, $\gamma\delta$ -T cells can nonetheless recognize malignantly-transformed cells - particularly malignant cells of epithelial origin - through less specific mechanisms that require no prior antigen exposure or priming (innate immunity). Consequently, $\gamma\delta$ -T cells can recognize and lyse malignantly-transformed cells almost immediately upon encounter — consistent with their role as a component of the innate immune system.

Tumor-infiltrating lymphocytes (TILs) are broadly applied in clinical research of adoptive immunotherapy because cytotoxicity mediated by TIL is much higher than that mediated by lymphokine-activated killer cells. Preferential expansion and antitumor responses of subsets of $\gamma\delta$ TILs have been found in many kinds of cancers, including colorectal cancer,^{7,8} lung adenocarcinoma^{9, 10} and pancreatic cancer,¹¹ Whether the $\gamma\delta$ TILs are found in human cervical cancer or can be expanded *ex vivo* has not been determined.

Galectin-1, a member of the beta-galactoside-binding family, is involved in several pathologic processes, including cancer progression, metastasis and immunobiology.¹²⁻¹⁵ It was well established that galectin-1 secreted by tumor cells induces T cells apoptosis, thus playing a role in the immune escape mechanism during tumor progression through induction of apoptosis of tumor-infiltrating T cells.¹⁶ Rabinovich reported that galectin-1 contributes to the immune privilege of tumours by modulating survival or polarization of effector T cells, and suggested a potential molecular target for manipulation of T cell apoptosis with potential implications in immunotherapy.¹ Meanwhile, Kohrenhagen *et al.* found that the expression of galectin-1 increased with the histopathologic grade of cervical cancer tissues during the progression of cervical neoplasia.¹⁸ Thus, galectin-1 is an attractive target in the development of therapeutics for the treatment of cervical cancer.

In this study, we expanded the $\gamma\delta$ TILs found in human cervical tumors and observed the effect of *ex*

vivo-expanded $\gamma\delta$ TILs, combined with galectin-1 antibody treatment, on the growth of human cervical cancer xenografts in SCID mice.

Materials and Methods

Samples

CB.17 SCID female mice (Animal Biosafety Level 3 Laboratory of Wuhan University) were used at 4-5 weeks of age and were kept under specific pathogen-free conditions. SCID mice were housed in microisolator cages and all of the food, water and bedding were autoclaved before use.

The human cervical cancer cell lines SiHa and Hela were provided by Prof. Song SZ of Wuhan University of Science and Technology.

Tumour samples were obtained from the Department of Surgery, Oncology Hospital of Hubei Province. Samples were taken from 10 patients diagnosed pathologically with cervical cancer (age range 42-65). None of the patients had received chemotherapy, radiotherapy or Chinese traditional medical therapy prior to surgery.

Immunohistochemistry

For histological analysis, human tumor tissues were embedded in OCT and snap frozen. For OCT snap frozen specimens, 4 μ m thick sections of embedded tissues were cut, air-dried and acetone-fixed. Endogenous peroxidases were inhibited by incubation in 3% H₂O₂. After non-specific binding sites were blocked (5% normal goat serum in PBS for 15 min), the tissue sections were immunostained with anti-TCR $\gamma\delta$ monoclonal antibody (Immunotech, Paris, France) using the PAP method (DAKO Corp., Carpinteria, CA) and counterstained with Mayer's hematoxylin, as reported previously.¹⁹ Tissue sections, subjected to the same treatment but incubated with isotype control mouse IgG1 of anti-TCR $\gamma\delta$ monoclonal antibody, were used as negative controls.

Evaluation of staining

The brown-yellow staining of the cytoplasmic membrane was considered positive for $\gamma\delta$ TIL. For each sample, the distribution and intensity of cell staining were assessed by observation of the entire tumor areas, at least 10 high-power field (HPF). The percentage of cells expressing TCR $\gamma\delta$ was estimated by dividing the number of positively stained cells by the total number of tumor cells per HPF. The degree of immunoreactivity was quantified by using the total immunostaining score calculated as the sum of the positivity percentage of stained cells and the staining intensity. The percentage was scored on a scale from 0 to 3 as follows: '0' (<5%, negative), '1' (5-25%, sporadic), '2' (26-50%, focal), '3' (>50%, diffuse). The staining intensity was scored as '0' (not stained), '1' (weakly stained), '2' (moderately stained), and '3' (strongly stained). Samples with weighted scores of 0-2 were defined as negative; and score of 3 as positive. No folding or edging-effect fields were chosen during calculation of 100 cells per five fields. The assessment was performed independently by two experienced pathologists blinded to the sample data.

Hematoxylin-eosin stain

For routine histological analysis, human tumor tissues were fixed in 10% neutral pH formalin overnight at 4°C, embedded in paraffin, sectioned at 4 μ m thickness and stained with hematoxylin-eosin.

Expansion of $\gamma\delta$ TILs

Twenty-four-well tissue culture plates (Costar, Cambridge, USA) were immobilized by anti-TCR $\gamma\delta$ monoclonal antibody (Immunotech) as reported previously.²⁰

Tumor tissues were firstly rinsed with RPMI-1640 medium supplemented with 2 mM L-glutamine, 20 mM HEPES, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamycin (all from GIBCO-BRL). Necroses and surrounding normal tissues were

discarded from tumor tissues. Tumor tissues were minced thoroughly, washed three times with RPMI-1640 medium, and resuspended in RPMI-1640 – a medium supplemented with 10% (v/v) human serum derived from healthy cord blood, 2 mM L-glutamine, 20 mM HEPES, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamycin, and human rIL-2 (Pharmingen, San Diego, USA; 400 U/ml. The cord blood samples were from the Department of Obstetrics and Gynecology, Zhongnan Hospital. The infants had not undergone testing previously, but their mothers had been tested before to disclose any infection. The cord serum samples were pooled, sterilized by filtrating through a 0.22 μ m film and stored at -20°C. The tumor tissues were then transferred to 24-well plates immobilized by an anti-TCR $\gamma\delta$ monoclonal antibody. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and supplemented every other day with the same medium. The single-cell suspension of TILs could be established after 3–4 weeks of culture and was maintained in culture in the presence of complete medium.

Phenotypic Analysis of TILs

Phenotypic studies were performed by FCM analysis. After cultured for 30 days, a total of 5×10^5 TILs were washed in PBS containing 1% BSA and 0.1% sodium azide, and were incubated at 4°C for 30 min with phycoerythrin (PE)-conjugated anti-TCR $\gamma\delta$ (IgG1, clone IMMU 510, Immunotech) or PE-conjugated isotype controls (clone 679.1Mc7, Immunotech). Cells were washed twice with PBS and analysed with a flow cytometer (Beckman, Brea, CA, USA).

Western blots

Two million cells or 0.1 mg tissue were lysed with 500 μ L SDS-PAGE 1 $\times$ loading buffer. After boiling for 5 min, samples were separated by 12.5% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Skim milk powder solution (10%, w/v) was used in blocking. Monoclonal anti-Gal-1 antibody

(Clone, 25C1, Vector Laboratories, Peterborough, UK) was used at a dilution of 1:200 at room temperature in TBST buffer (50 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) with 3% fetal bovine serum (Hyclone). The membrane was incubated with primary antibody for 1 h at room temperature, washed three times, and then incubated with 1:3000 fold diluted HRP-conjugated goat anti-mouse IgG. Signals were visualized using enhanced chemiluminescence (ECL). The galectin-1 mAb was replaced by mouse IgG1 (ZhongYi Biological Technology, Wuhan, China) for a negative control. Galectin-1 highly expressed cervical cancer tissue was set as a positive control.

ELISA assay

2×10^6 SiHa cells were seeded in six-well-plate with 2 mL culture medium. The culture medium of SiHa cells was collected after 6 h by centrifugation, and Gal-1 content determined with an ELISA kit (ZhongYi Biological Technology, Wuhan, China). The culture medium RPMI1640 was used as a negative control.

In vitro cytotoxicity assay

Cytotoxicity measurements were made using the Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, USA). All measurements were done in triplicate in a total volume of 0.1 mL phenol red free media per well in U-bottom 96-well microtitre plates. Serial dilutions of $\gamma\delta$ TILs were added to 10^4 target SiHa cells or Hela cells (EXP). Cells were incubated for 6 h. After cell lyses, LDH-concentration of the supernatant was measured in 50- μ L aliquots. Spontaneous LDH release of effector cells (SFE) was measured without addition of target cells. Spontaneous LDH release (SFT) and maximum LDH-release (MFT) of target cells were determined by induction of cell lyses with 9% Triton-X-100. The specific cell lyses was calculated by the formula: Percentage of specific cell lyses = $(\text{EXP} - \text{SFE} - \text{SFT}) / (\text{MFT} - \text{SFT})$. For galectin-1

inhibition detection, 10^4 $\gamma\delta$ TILs and 10^4 SiHa cells or Hela cells were incubated with galectin-1 mAb (1:100) or lactose (1 mM) for 6h. Specific cell lyses was detected as described above.

Transplantation and growth of human tumors in SCID mice

Forty mice were equally divided into five groups. The SiHa cells were resuspended in 0.2 mL RPMI 1640 containing 10% FBS (Hyclone). Mice were injected subcutaneously into the right flank with 2×10^6 cells/mouse of the SiHa cellular suspensions. $\gamma\delta$ TILs were injected s.c. simultaneously with SiHa cells, in a methodological approach similar to the classical Winn assay,²¹ at doses of 2×10^6 cells/mouse. Galectin-1 mAb (10 μ L) or isotype control mouse IgG1 were diluted in 50 μ L RPMI-1640 medium and injected intratumorally every three days, ten times in all. Tumor growth was monitored by measuring maximal and minimal diameters by caliper, and tumor weight was estimated with the formula: tumor weight (mg) = $\text{length (mm)} \times \text{width}^2 \text{ (mm)} / 2$, as described previously.²²

Statistical analysis

The significance of differences among groups was determined by the Student's *t* test.

Results

$\gamma\delta$ -T cells infiltrated the cervical tumour

$\gamma\delta$ TILs have been found in many kinds of cancers, but this is the first report of their presence in human cervical cancer. First, anti-TCR $\gamma\delta$ antibody was used to confirm the existence of $\gamma\delta$ -T cells in the cervical cancer tissues from cervical cancer patients by immunohistochemistry. Interestingly, we observed that there were numerous $\gamma\delta$ -T cells infiltrating the cervical cancer tissues and many fewer in the paraneoplastic tissues (Fig. 1A-D): this enabled us to expand the $\gamma\delta$ TILs of the cervical cancer *in vitro*. Consistent re-

FIGURE 1. $\gamma\delta$ TILs were detected in human cervical cancer using an anti-TCR $\gamma\delta$ antibody. Ten cases were analysed . (a) $\gamma\delta$ -T cells had infiltrated in the human cervical cancer tissues, as the arrows indicated, but were hardly present in the paraneoplastic tissue (b)(40 $\times$), (c) the isotype antibody of anti-TCR $\gamma\delta$ antibody was used as a negative control and (d) HE stain of the human cervical cancer tissue sample (10 $\times$).

sults were acquired using semiquantitative method to assess the distribution and intensity of cell staining as described above. The results showed positive immunostaining in all the slides of cervical cancer tissues (weighted score >3) (data not shown).

Characterization of $\gamma\delta$ -T Lymphocytes after expansion by immobilized anti-TCR $\gamma\delta$ antibody

After 30 days of *ex vivo* expansion, the phenotypic analysis of TILs was detected by FCM. The percentage of TCR $\gamma\delta$ positive TILs was $91.2\pm 1.2\%$, consistent with a prior report^[23] (Fig. 2A). These results indicated a sufficient expansion of $\gamma\delta$ TILs for the further studies.

FIGURE 2. (a) The phenotypic analysis of TILs from 10 cases after 30 days of ex vivo expansion was detected using FCM. The percentage of TCR $\gamma\delta$ -positive TILs was $91.2 \pm 1.2\%$. The experiments were repeated three times and representative data are shown. The expression of galectin-1 in SiHa cells was detected by Western blot (b) and ELISA (c). Galectin-1 highly expressed cervical cancer tissue was set as a positive control and mouse IgG1 as isotype control in the Western blot.

SiHa cell expressed and secreted galectin-1

Increased expression of galectin-1 in the cervical tumour and Hela cells has been reported^[18,24], but whether the cervical tumor cell line SiHa expressed the galectin-1 was still unknown. ELISA and Western blot indicated that the SiHa cells not only expressed the galectin-1 protein but also secreted it into the supernatant (Fig. 2B, 2C.).

Inhibition of galectin-1 enhanced the cytotoxicities of $\gamma\delta$ TILs to SiHa cell and Hela cell

As shown in figure 3A and C, $\gamma\delta$ TILs from cervical cancer tissue demonstrated marked cytotoxicity to allogeneic cell lines SiHa and Hela at very low ratios of effector to target cells (5:1, 1:1 and 1:5) (Fig. 3A, 3C). Galectin-1, secreted by SiHa or Hela cells or expressed on SiHa or Hela cells, may impact the function of $\gamma\delta$ TILs; therefore, lactose, an inhibitor of

galectin-1, and galectin-1 antibody were added to the assay. The results showed that in comparison with the non-treated group, the cytotoxicities of $\gamma\delta$ TILs to SiHa or Hela cells were significantly increased when effector and target cells were incubated with the inhibitor of galectin-1 (either lactose or galectin-1 antibody) at E/T ratio 1:1 ($p < 0.05$), whereas no difference was seen between the control group (medium only) and isotype IgG of anti-galectin-1 (Fig. 3B, 3D).

$\gamma\delta$ TILs combined with galectin-1 antibody inhibited the growth of human cervical tumor xenografts in SCID mice

To further assess the therapeutic potential of administering of $\gamma\delta$ TILs combined with galectin-1 antibody for treatment of cervical cancer, a human xenograft model of cervical cancer was used. Mice were inoculated s.c. with 2×10^6 cells/mouse of the SiHa cellular

FIGURE 3. Inhibition of galectin-1 enhanced the cytotoxicities of $\gamma\delta$ TILs to SiHa and Hela cells.(a,c) $\gamma\delta$ TILs were set as effector cells(E), SiHa or Hela target cells (T) were incubated in different E/T ratios. Cell lysis was determined by measuring LDH activity from lysed cells. The $\gamma\delta$ TILs from each case (10 cases in total) were used in the experiment. The data shown were calculated as mean $\pm$ s.e.m. and the experiments were repeated three times. (b) 10^4 $\gamma\delta$ TILs and 10^4 SiHa or Hela cells were incubated with galectin-1 mAb(1:100) or lactose(1mM) for 6h. Specific cell lysis was detected as described above. $\gamma\delta$ TILs and SiHa or Hela cells were incubated with RPMI-1640 medium for 6h served as negative control. The cytotoxicities of $\gamma\delta$ TILs to SiHa or Hela cell were significantly increased when effector and target cells incubated together with the inhibitor of galectin-1 (either lactose or galectin-1 antibody), compared with negative control or isotype IgG control ($p < 0.05$).

suspension, together with 2×10^6 cells/mouse of the $\gamma\delta$ TILs. In the antibody treated group, various antibodies were mixed in the cellular suspensions for the initial injection. For the remaining antibody treatments, antibodies were intratumourally injected every three days, ten times in total. SiHa cells xenografted in SCID mice without treatment were set as control. As the re-

sults shown in figure 4, $\gamma\delta$ TILs combined with galectin-1 antibody treatment could significantly suppress the growth of cervical cancer xenograft in SCID mice, in comparison with all other groups. $\gamma\delta$ TILs alone also inhibited tumour growth *in vivo*, but less efficiently than $\gamma\delta$ TILs combined with specific antibodies. Interestingly, galectin-1 antibody applied

* vs SiHa+ $\gamma\delta$ TIL+Gal-1 mAb group, $p < 0.01$

** vs SiHa+ $\gamma\delta$ TIL+Gal-1 mAb group, $p < 0.05$

FIGURE 4. $\gamma\delta$ TILs, combined with galectin-1 antibody, inhibited the growth of human cervical tumor xenografts in SCID mice. CB.17 SCID female mice were injected with 2×10^6 cells/mouse of the SiHa cellular suspensions and $\gamma\delta$ TILs were injected s.c. simultaneously at doses of 2×10^6 cells/mouse. In the antibody treated groups, galectin-1 mAb or isotype control mouse IgG1 were injected. Tumor growth was monitored by the tumor weight. $\gamma\delta$ TILs, combined with galectin-1 antibody treatment, can significantly suppress the growth of SiHa cells in SCID mice in comparison with all other groups ($p < 0.05$).

alone also appreciably inhibited tumour growth *in vivo* 30 days after implantation of SiHa cells.

Discussion

Cervical cancer is the second most common cancer among women worldwide, with approximately 500,000 diagnosed cases and 250,000 deaths every year.²⁵ Cervical cancer is the leading cause of death from cancer in many developing countries.²⁶ This high incidence, coupled with the lack of effective therapies to treat cervical tumors, creates a pressing need for more efficient therapeutic methods.

Previous research has shown that $\gamma\delta$ T cells could be a new candidate for adoptive immunotherapy in the future treatment of patients with cancer. With advantages including non-MHC restriction and direct facili-

tated recognition, $\gamma\delta$ T cells might play an important role in adoptive immunotherapy with immune cells and overcome some disadvantages of $\alpha\beta$ T cells in the treatment of tumours.²³ Martinet *et al.* indicated that the successful harnessing of $\gamma\delta$ cells for cancer therapies requires us to consider the pitfalls already identified along the development of other T cell-based cancer immunotherapies.²⁷ Demotte *et al.* recently demonstrated that it was due to the secretion of galectin-3 by tumor cells that TILs, within the tumor, were anergic or functionally blocked.²⁸ The expression of galectin-3 is increased in many kinds of cancers but is decreased in cervical cancer;²⁹ however, the expression of galectin-1, which functions similarly to galectin-3, is increased in cervical cancer patients.^[18,30] We hypothesize that the efficiency of $\gamma\delta$ T cells-based cancer adoptive immunotherapy could be enhanced when galectin-1 secreted by cervical tumor cell was blocked at the same time.

As reported, freshly isolated $\gamma\delta$ TILs do not demonstrate any cytotoxicity to tumor cell lines, but they will immediately acquire the cytotoxic ability after culture *in vitro*.²⁰ Therefore, different from the $\gamma\delta$ TILs in the tumor location, *ex vivo* expanded $\gamma\delta$ TILs are not anergic or functionally blocked by the immune suppressive factors coming from the tumor. It is well known that $\gamma\delta$ TILs cells are capable of killing a variety of malignant cells innately; without the requirement of the recognition of tumor-specific antigens and that expanded $\gamma\delta$ TILs have capacity of killing autologous and allogeneic tumor cells.^{20,23} Consistent with these findings, the results shown in the Fig. 3 and Fig. 4 confirm that the *ex vivo*-expanded $\gamma\delta$ TILs have ability to kill SiHa cells. Moreover, this ability can be enhanced when immune suppressive factors secreted from the tumor cells are blocked, as seen with galectin-1 in cervical cancer.

In this study, we first identified the infiltrating $\gamma\delta$ lymphocytes in human cervical tumour tissue by immunohistochemistry and then expanded $\gamma\delta$ TILs *in vitro*. Our data indicate that, as previously shown in other tumors, $\gamma\delta$ TIL exists in cervical cancer tissue,

and that the $\gamma\delta$ TILs can be effectively expanded *ex vivo*. After determining the expression of galectin-1 in SiHa cells by ELISA and Western blot, we investigated the cytotoxicities of $\gamma\delta$ TILs to SiHa and Hela cells combined with inhibitors of galectin-1 *in vitro*. The results indicated that the cytotoxicities of $\gamma\delta$ TILs to SiHa and Hela cells were significantly increased when added with galectin-1 inhibitors, either specific antibodies or lactose. With the *in vivo* experiments, the SiHa cell could hardly grow in the SCID mice with the treatment of $\gamma\delta$ TILs combined with Gal-1 mAb. Taken together, the data above indicated that neutralization the galectin-1 in the tumor microenvironment could enhanced the anti-tumor ability of $\gamma\delta$ TILs.

As reported, inhibition of *gal-1* gene expression in a rat glioma cell line arrests tumour growth, suggesting that endogenous galectin-1 has growth-promoting activity.³¹ This may explain why Gal-1 antibody applied alone can appreciably moderate the growth of xenogeneic cervical cancer *in vivo*. It is of note that, in the cytotoxicity assay, the combination of $\gamma\delta$ TILs and lactose provided stronger inhibition of tumor cell growth than seen for $\gamma\delta$ TILs and galectin-1 mAb. This phenomenon probably stems from inhibition of other ligands of lactose, such as galectin-3, as galectin-1 is not the only ligand of lactose. Nevertheless, it seems that neutralization of galectin-1 mainly accounts for the enhanced cytotoxicity as there was no significant difference between the cytotoxicity of $\gamma\delta$ TILs with lactose and that with galectin-1 mAb.

These data underscore the potentially relevant role of $\gamma\delta$ TILs in the immunotherapy of human cervical cancer. Furthermore, our work provides concrete experimental evidence suggesting that $\gamma\delta$ T cells-based cancer immunotherapy combined with Gal-1 mAb treatment might be better than conventional $\gamma\delta$ T cells-based cancer immunotherapy. This might be more promising if we confirm a similar level of cytotoxicity of $\gamma\delta$ TILs to autologous tumor cells. In addition to Gal-1 mAb, other compounds might be tested includ-

ing, other galectin-1 ligands or agents that prevent the polymerization of galectin-1.

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Correspondence to:

FuXiang Zhou, Ph.D., M.D.,
 Department of Medicine Oncology and Radiation Oncology,
 Zhongnan Hospital, Wuhan University,
 Donghu Road, 169#, Wuchang,
 Wuhan, 430071,
 P.R.China.
 Email: fxzhou2010@hotmail.com
 Tel:+86 27 67813155