

# Irradiation increases the immunogenicity of lung cancer cells and irradiation-based tumor cell vaccine elicits tumor-specific T cell responses in vivo

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**Background:** During the past decades, great efforts have been built to develop lung cancer vaccines. Whole tumor cell lysate (TCL) are ideal sources of antigens for cancer vaccine design, which however have limited efficacy due to insufficient immunogenicity. Recently, radiotherapy has been closely related to immunotherapy. Numerous studies have demonstrated the regulatory effect of irradiation (IR) on tumor immune response.

**Purpose:** To explore the immunogenicity modulation effect of IR on lung cancer cells.

**Methods:** RNA-sequence and qPCR assay was used to evaluate the change of tumor antigens expression after repeated X rays radiation on A549 cells. Vaccine based on TCL of irradiated Lewis lung cancer cells (IR-LLC) was established; therapeutic effect of TCL (IR-LLC) was examined in xenografted tumor model of mice. Flow cytometry was conducted to evaluate the rate of immune cells in spleen; ELISA was used to detect the level of cytokines in plasma. Immunohistochemistry was performed to evaluate the infiltrations of T-cell in tumor tissues; TIMER analysis was used to explore the correlations between tumor antigen expressions and the abundances of immune infiltrates.

**Results:** IR upregulated the expression of tumor antigens in A549 cells. Compared to the control group and unirradiated tumor cell vaccine, TCL(IR-LLC) had a significantly stronger anti-tumor effect in mice bearing with LLC xenografts. TCL(IR-LLC) significantly increased matured DCs and total CD4<sup>+</sup> T cells but downregulated Tregs and PD-1<sup>+</sup> CD8<sup>+</sup> T cells in mice spleen; TCL(IR-LLC) vaccine upregulated the level of IFN- $\gamma$  and IL-4 while decreased IL-10 in serum; increased infiltrations of CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells were observed in the tumor tissues of mice immunized with TCL(IR-LLC). Tumor antigens including FN1, MFGE8, MMP2, MYL9 may contribute to the enhanced T-cell response.

**Conclusion:** This study confirmed the immunogenicity modulation effect of IR in NSCLC cells, indicating IR might be an effective strategy to enhance the anti-tumor immunity of cancer cell vaccine.

**Keywords:** lung cancer, whole tumor cell vaccine, irradiation, tumor antigen, T cell

## Introduction

Non-small-cell lung cancer (NSCLCs) accounts for approximately 80% of lung cancers, and is considered the leading cause of cancer-related mortality worldwide.<sup>1</sup> Surgical resection of NSCLC offers a favorable prognosis for patients with localized lung cancer.<sup>2</sup> However, approximately 75% of patients with NSCLC have reached stage III/IV at the time of diagnosis.<sup>3</sup> Although significant progress has been made in the treatments of advanced stage lung

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cancer, survival remains poor.<sup>4</sup> In recent years, along with the remarkable developments in the field of cancer immunology, new methods of immunotherapy have been studied to augment anti-tumor immune responses to target cancer cells with less damage to normal tissue.<sup>5</sup>

One of the major methods of immunotherapy is cancer vaccines. Commonly, whole tumor cell, DNA-, RNA-, and peptide-based vaccines are used to evoke antigen-specific immune response.<sup>6</sup> In lung cancer, single protein or peptide-based vaccines such as MUC-1 (stimuvax), MAGE-A3, and GM.CD40L have been studied in phase III clinical trials, the outcome of which was discouraging.<sup>7</sup> In this regard, whole tumor cell vaccine has attracted our attention since it contains a complete antigen-spectrum of tumor cells. However, due to the indigenous and poor immunogenic status of tumor cells, whole tumor cell vaccines have shown limited efficacy in early studies.<sup>8</sup> In this context, methods to enhance the immunogenicity of cancer cells have become critically important.

In recent years, radiotherapy has been closely related to immunotherapy. Stereotactic ablative radiotherapy (SABR), characterized by high dose and low fractionation regimen (more than 5 Gy dose per fraction), has achieved great success in the reduction of tumors outside the irradiated field or metastasis, showing an abscopal effect.<sup>9</sup> Previous studies showed that tumor cells could release large amounts of tumor-associated antigens (TAAs) following SABR, contributing to the increase of antigen presentation and robust host immune activation. Meanwhile, irradiation (IR) could also enhance tumor immunogenicity for its phenotypes modulation such as the increased expression of CEA and MUC-1, that ultimately enhances killing by cytotoxic T lymphocytes.<sup>9–11</sup> Thus, these radiation-induced changes caused by high-dose IR provide a solution to optimize traditional autologous tumor cell vaccines.<sup>12,13</sup> In past studies of cancer vaccines, IR was simply used as an inactivation method to prepare tumor cell vaccines, usually with a lethal radiation dose of up to 100 Gy, which caused tumor cells to be inactivated or unable to proliferate.<sup>14–16</sup> While, in this study, we aimed to explore the immunogenic modulation effect of IR on tumor cells and vaccines.

Therefore, the antigen expression of NSCLC cell line treated with IR was investigated in the present study. Further, the lysate of irradiated whole lung cancer cell was used as a vaccine and we observed a significantly higher anti-tumor response in the mouse model of Lewis lung cancer (LLC).

## Materials and methods

### Cell lines and cell culture

Human NSCLC A549 cell line and LLC cell line were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China). The A549 cells and LLC cells were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA), and DMEM (Thermo Fisher Scientific), respectively. Both were supplemented with 10% FBS (Thermo Fisher Scientific). The cells were incubated at 37°C under an atmosphere of 5% CO<sub>2</sub>.

### Cell IR

A549 cells and LLC cells were inoculated into culture flasks and irradiated with 8 Gy X-rays at exponential growth phase using a Trilogy linear accelerator (Varian Medical Systems, CA, USA) at a rate of 6 Gy/minute. The cells were then passaged 24 hours after IR, and the same procedure of 8 Gy IR was repeated five times. After the last IR, the irradiated A549 cells and LLC cells, termed "IR-A549" and "IR-

LLC" respectively, were collected for further experiments.

### RNA-sequence analysis

The mRNA-enriched total RNA was extracted from A549 cells and IR-A549 cells at log growth phase. Library preparations were sequenced on the BGISEQ-500 platform (BGI, Shenzhen, People's Republic of China), and 50 bp single-end reads were generated. Differential gene expression analysis was performed using DESeq and differentially expressed genes (DEGs) were selected based on fold change  $\geq 2$  and adjusted *P*-value  $< 0.001$ .<sup>11,17</sup>

### qRT-PCR

Total RNA was isolated with the Axyprep Multisource Total RNA miniprep Kit (Axygen, Union City, CA, USA) and reverse transcribed into cDNA with the PrimeScript RT Master Mix (TaKaRa, Dalian, People's Republic of China). Then the samples were subjected to RT-PCR using a SYBR Premix Ex Taq (Tli RNaseH Plus; TaKaRa) via the Fast 7300 RT-PCR system (Applied Biosystems, Thermo Fisher Scientific).

### Preparation of tumor cell lysates (TCL)

For preparation of TCL, both IR-LLC and LLC cells were digested in trypsin and suspended in PBS at a concentration of  $1.0 \times 10^7$  cells per mL, subjected to

five cycles of freezing and thawing (liquid nitrogen for 5 minutes, 37°C for 5 minutes) and then centrifuged at 2,500 rpm for 10 minutes. Afterwards, the supernatants were collected, termed “TCL(IR-LLC)” and “TCL(IR-LLC)” respectively. Protein concentration of TLC was quantified by the BCA assay (MAIBIO Shanghai, People’s Republic of China) and the final concentrations were adjusted and stabilized to 215 ng/ $\mu$ L.

## Xenograft mouse model and vaccination

The 6–8 week-old female C57BL/6J mice were purchased from the Shanghai Experimental Animal Center. All animal experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory animals with all experimental protocols approved by the Institution of Animal Care and Use Committee of Jinshan Hospital. The mice were subcutaneously injected with  $2 \times 10^6$  LLC cells in the left flank on day 0. One week later, mice bearing LLC tumors were divided into three groups ( $n=7$ ) and vaccinated with 0.1 mL PBS (as control group), TCL(LLC), and TCL(IR-LLC) respectively. The vaccination was administrated on day 7, 11, and 15 and tumor growth was measured with a caliper every 3 days. On day 20, all mice were euthanized, with tumors, blood, and spleens harvested for later experiments.

## Flow cytometry

Splenocytes were isolated from spleens of mice and collected through Ficoll-Hypaque purification. About  $5 \times 10^6$  cells were used for antibody staining using PE-conjugated anti-CD11 mAb and APC-conjugated anti-CD80 mAb or FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD279 mAb or FITC-conjugated anti-CD4 mAb and PE-conjugated anti-Foxp3 mAb APC-conjugated anti-CD25 mAb (eBioscience, San Diego, CA, USA). The cell counts for CD3+, CD4+, and CD8+ T lymphocytes were assessed with a flow cytometer (Beckman, Miami, FL, USA).

## Measurements for cytokines

Serum was isolated from the blood samples of the mice and serum cytokine levels were analyzed using ELISA. The level of IL-4 was measured using Mouse IL-4 ELISA Kit (Proteintech, USA); and the levels of IFN- $\gamma$  and IL-10 were measured using Mouse IFN- $\gamma$  ELISA Kit and Mouse IL-10 ELISA Kit (InTech, People’s Republic of China), respectively according to the manufacturers’ instructions.

## Immunohistochemistry and TUNEL assay

The tumor tissues of mice were immobilized with 4% paraformaldehyde and embedded in paraffin. The tissue sections were preincubated with BSA at room temperature, and later incubated overnight at 4°C with rabbit anti-mouse CD8 antibody or rabbit anti-mouse CD4 antibody (Bioss, People’s Republic of China). Afterwards, they were stained with DAB and finally examined under a microscope. For detection of tumor cell apoptosis, the sections were stained with TUNEL kit (KeyGen, Jiangsu, People’s Republic of China), and then incubated with DAPI (blue fluorescence) to stain nuclei. Finally, the sections were randomly observed under a microscope and examined by IPP 6.0 software.

## Analysis of tumor immune estimation resource (TIMER)

To obtain the immunity profile of genes, tumor-infiltrating lymphocytes (TILs) were profiled using a code provided by TIMER, a public resource at <http://cistrome.org/TIMER>. The correlations between expressions of the selected genes and the abundances of immune infiltrates were provided by gene module on TIMER.<sup>18</sup>

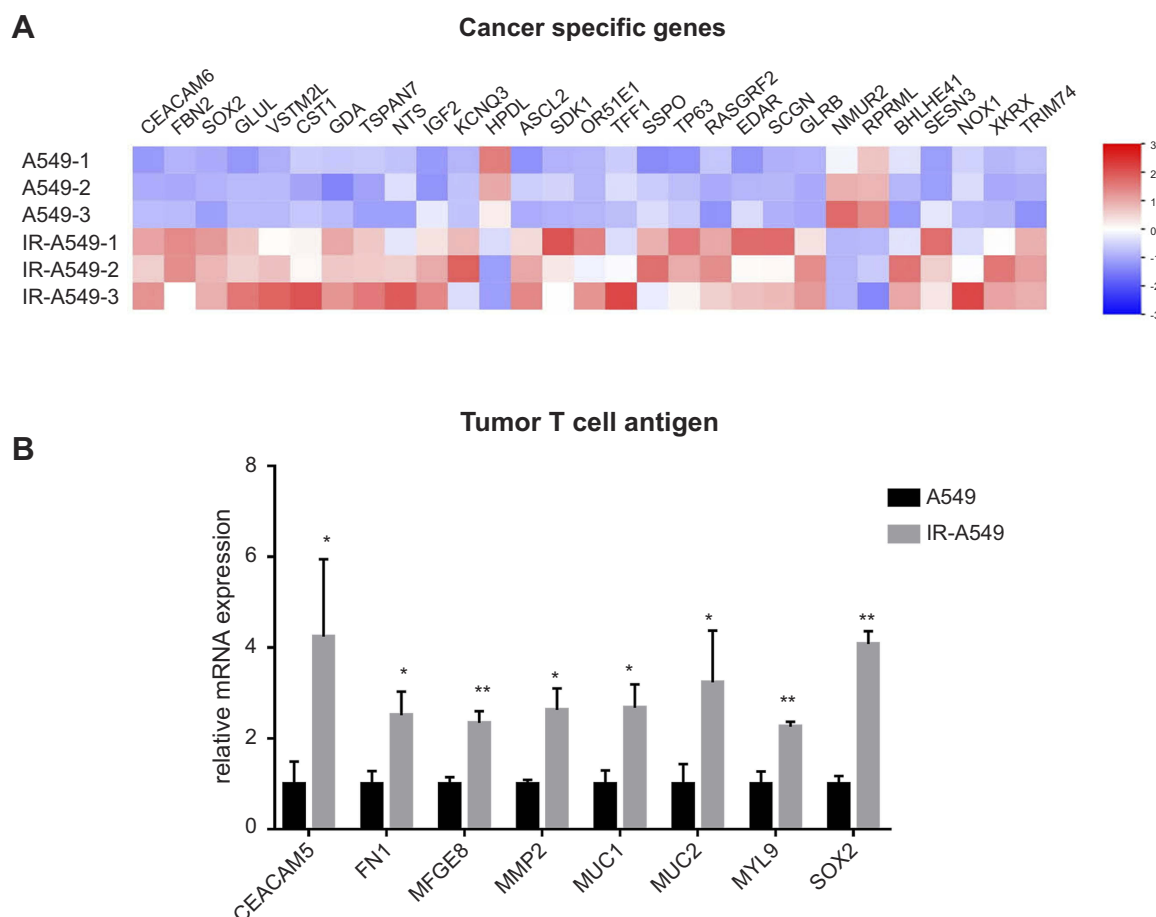
## Statistical analysis

All the experiments were performed at least three times. The results were presented as mean  $\pm$  SD. The statistical analysis of compared groups was assessed using 2-tailed unpaired Student’s *t*-tests or one-way ANOVA in case of more than three groups.

## Results

### Repeated IR upregulated the expression of tumor antigens in A549 cells

To evaluate the immunogenic modulation effect of IR, A549 cells were exposed to 8 Gy X-rays five times, termed “IR-A549”. The genomic profiles of A549 and IR-A549 cells were examined by RNA-sequence. A total of 308 DEGs (selected based on fold change  $\geq 2$  and adjusted *P*-value  $\leq 0.001$ ) was identified between A549 and IR-A549. Then, the expressions of cancer-specific genes (CSGs) and the potential tumor antigens, which may provide epitopes and targeted by the immune system were compared between these two groups.<sup>18,19</sup> CSGs have been identified in a previous study.<sup>18</sup> As shown in Figure 1A, the two groups displayed 29 differentially expressed CSGs ( $q < 0.001$ ), among which



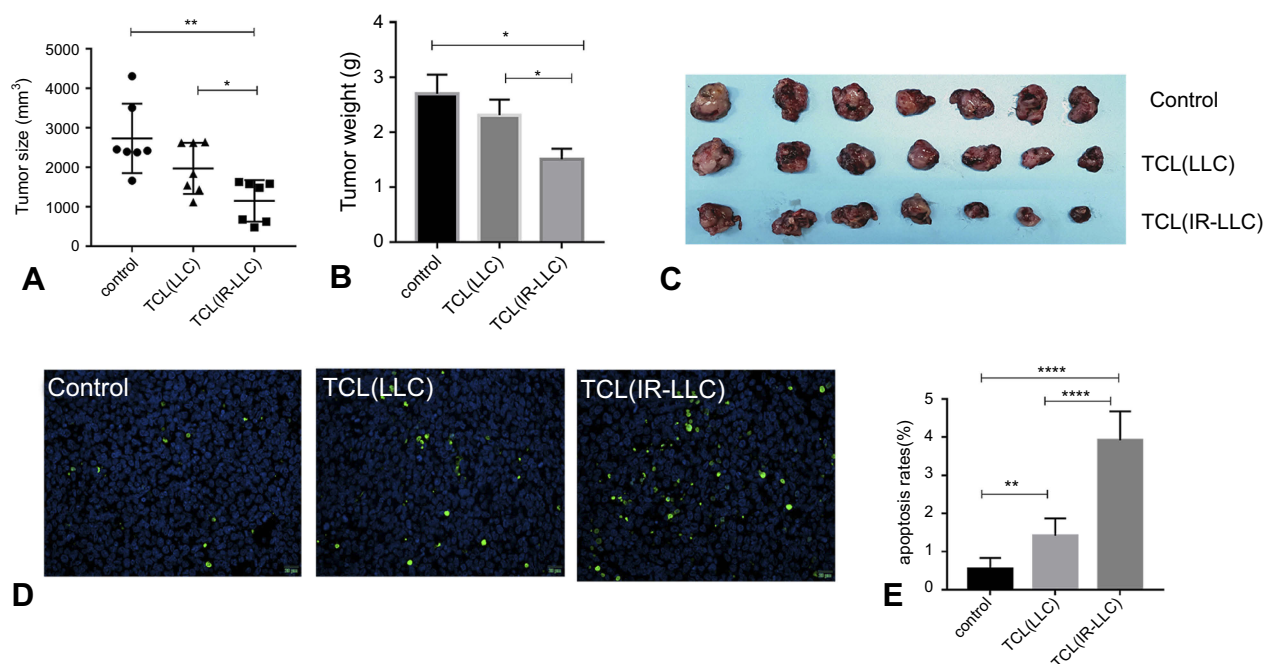
**Figure 1** Immunogenic modulation effect of irradiation (IR) in A549 cells. **(A)** Heat map of cancer-specific genes' mRNA expression was plotted using results of RNA sequence. Colored bars represent differential levels of mRNAs expressed in A549 and IR-A549 cells. **(B)** The mRNA levels of tumor T cell antigens were analyzed by qRT-PCR. The data shown are the representative of three experiments (\* $P<0.05$ ; \*\* $P<0.01$ ).

a large proportion was upregulated by IR. Next, the expression of the tumor antigens containing HLA ligands and T cell epitopes, which were provided in Tumor T cell antigen database, was analyzed.<sup>20</sup> A total of eight tumor antigens (FN1, MFGE8, CEACAM5, MUC1, SOX2, MMP2, MYL9, and MUC2) were found to be differentially expressed between A549 and IR-A549 cells. Consistently, the expression of these eight genes was all upregulated by IR and validated by qRT-PCR (Figure 1B).

### Therapeutic vaccination involving TCL (IR-LLC) showed enhanced anti-tumor effect in LLC mouse model

Next the effect of IR-based lung cancer vaccine was examined in vivo. For in vivo study, we used LLC cells instead of A549 cells as required for the mouse tumor model. Vaccine based on irradiated TCL(IR-LLC)

was established by repeated freezing and thawing. One week after the mouse LLC xenograft models were constructed, all mice were divided into three groups, including control group, TCL(LLC) group, and TCL(IR-LLC) group, injected with PBS, the TCL of unirradiated LLC, and TCL of irradiated LLC, respectively. The injection was administrated on day 7, 11, and 15 and mice were sacrificed on day 20. As shown in Figure 2A–C, significant differences in tumor volume and tumor weight were observed among the three groups, with TCL(IR-LLC) group ranking the lowest. Furthermore, the apoptosis of tumor cells was examined through TUNEL assay of the tumor tissues. Compared to control group, TCL(LLC) and TCL(IR-LLC) groups had increased apoptosis rates, and TCL(IR-LLC) group achieved the highest apoptosis rate (Figure 2D, E). These results indicated that TCL(IR-LLC) had the strongest anti-tumor effect, which may be explained by its role in increasing apoptosis of tumor cells.



**Figure 2** The vaccine inhibited tumor growth and increased tumor apoptosis in Lewis lung cancer (LLC) mouse model. **(A)** The tumor volumes of mice were examined at day 20, calculated with the formula: volume (mm<sup>3</sup>)=0.5× length(mm)×width<sup>2</sup> (mm). **(B)** The weight of isolated tumors at the end point of observation. Each dot represented each mouse. **(C)** The pictures of tumor tissues isolated from three groups. **(D)** Representative images of the TUNEL assay. The green fluorescence represented apoptotic cells, whereas the cell nuclei stained with DAPI was shown in blue. Scale bar, 20 μm. **(E)** The tumor cell apoptosis rate of the three groups. The data shown are the representative of three experiments (\**P*<0.05; \*\**P*<0.01; \*\*\*\**P*<0.0001).

**Abbreviations:** TCL, tumor cell lysates; IR, irradiation.

## TCL(IR-LLC) vaccine upregulated the immune response of dendritic cells and T-cells in the spleen

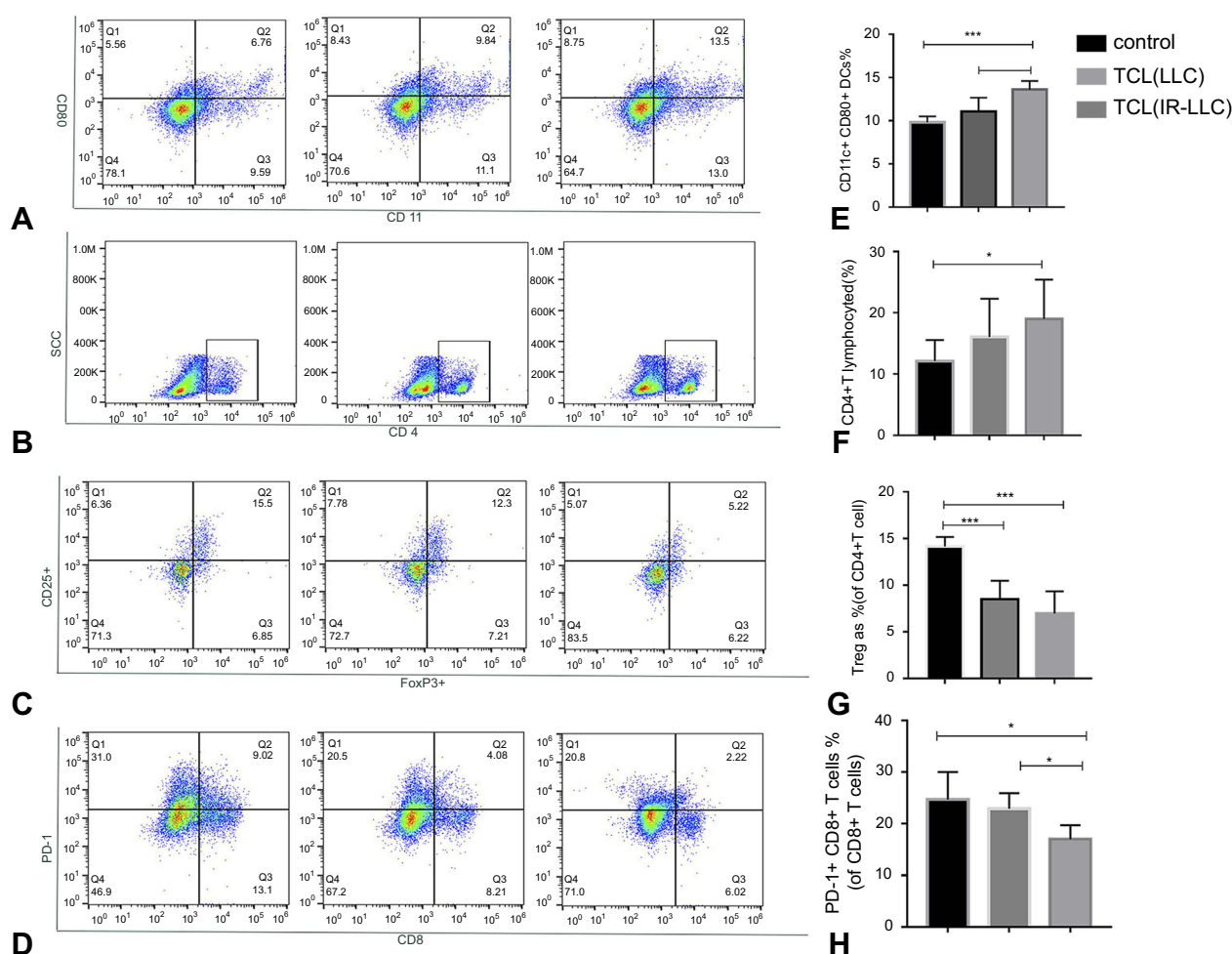
To investigate the possible mechanisms of anti-tumor effects induced by TCL(IR-LLC) vaccine, the changes in the number and fraction of immune cells were evaluated by flow cytometry in mononuclear cells isolated from the mouse spleens. More matured dendritic cells (Figure 3A and E) and significantly higher fraction of CD4<sup>+</sup> T cells in spleens (Figure 3B and F) were observed in mice immunized with TCL(IR-LLC) than the other two groups. Meanwhile, the immune suppression-related cells, including Treg cells and PD-1<sup>+</sup> T-cells, were downregulated by TCL(IR-LLC) injection. In Figure 3C and G, the percentages of CD25<sup>+</sup>Foxp3<sup>+</sup>Treg cells by gating on CD4<sup>+</sup> T-cells in the spleens were significantly reduced in the TCL (IR-LLC) vaccine group compared with the other groups. In addition, despite the insignificant difference in the amounts of CD8<sup>+</sup> T-cells among these three groups, the ratio of PD-1-expressing CD8<sup>+</sup> T-cells by

gating on CD8<sup>+</sup> T-cells was lowest in the group stimulated by TCL(IR-LLC) (Figure 3D and H). Collectively, these results suggested that TCL(IR-LLC) vaccine achieved better tumor-specific immune response of dendritic cells and T-cells in spleens.

## TCL(IR-LLC) vaccine changed the secretion of cytokines in serum

To further determine the immune status regulated by TCL (IR-LLC) injection, the secretion of several cytokines in serum of LLC mouse model was assessed by ELISA. IFN-γ is a key cytokine in anti-tumor immunity with its cytostatic and immune-provoking effects.<sup>21</sup> IL-4 is a key factor of Th2 cytokines involved in humoral immunity.<sup>22</sup> IL-10 is considered to be an important anti-immunity cytokine.<sup>23</sup> As shown in Figure 4A–C, among the three groups, the TCL(IR-LLC) group showed the highest concentration of IFN-γ and IL-4 (*P*<0.05), but the lowest concentration of IL-10 (*P*<0.05). These results revealed a stronger immune stimulating effect of TCL(IR-LLC) vaccine.





**Figure 3** The vaccine-activated immune response of dendritic cells and T-cells in the spleen. The numbers of immune cells in spleens were measured by flow cytometry. (A–D) Representative images of flow cytometry. (E–H) The statistical results of proportions of dendritic cells and T-cell subsets. (A and E) The fraction of CD11c+CD80+ dendritic cells in spleens. (B and F) The fraction of CD4+ T-cells in spleens. (C and G) The ratio of Treg cells in CD4+ T-cells. (D and H) The ratio of PD-1+ T-cells in CD8+ T-cells. The data shown are the representative of three experiments (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ).

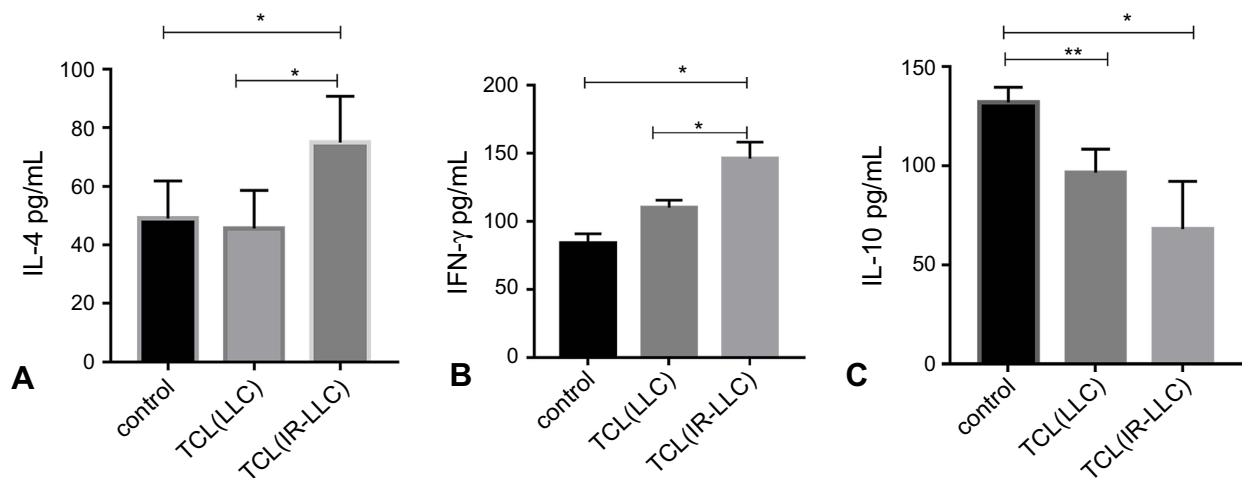
**Abbreviations:** TCL, tumor cell lysates; IR, irradiation; LLC, Lewis lung cancer.

## TCL(IR-LLC) vaccine increased the abundance of immune infiltration of T-cells in tumor tissue

To examine whether TCL(IR-LLC) vaccine could increase TILs, immunohistochemistry was performed to evaluate the infiltrations of CD4+ and CD8+ T-cells in tumor tissues from mice of each group. As demonstrated in Figure 5A–C, more tumor infiltrations of CD4+ T-cells and CD8+ T-cells were observed in mice immunized with TCL(IR-LLC) than the other two groups. In summary, these results indicated that TCL(IR-LLC) vaccine could activate more intensive tumor-specific T-cell responses.

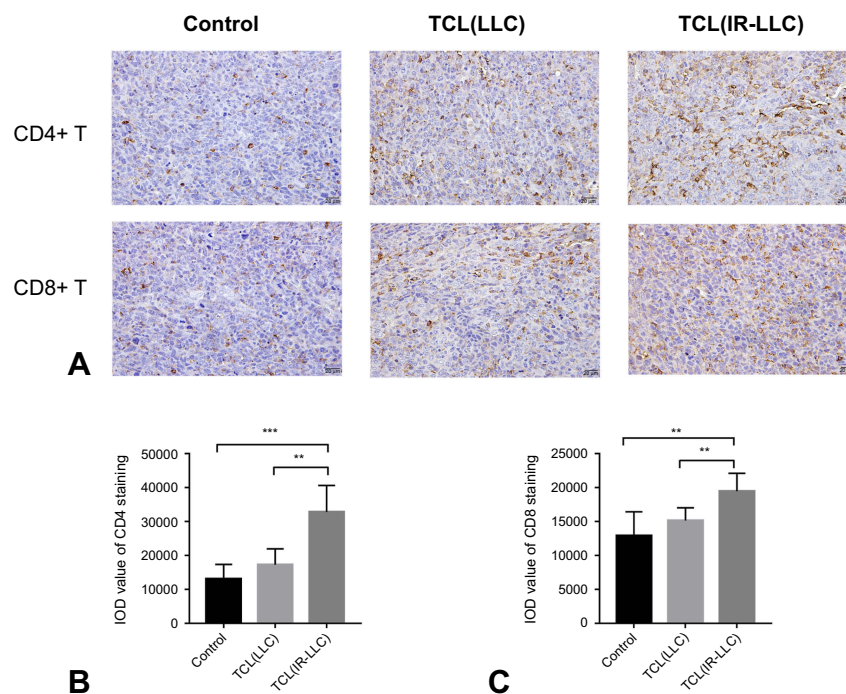
## Tumor antigens including FN1, MFGE8, MMP2, and MYL9 are positively associated with immune infiltrates

To further elucidate how IR modulates the immunogenicity of tumor cells and which antigens may contribute to induce effective immune response, we examined the tumor antigens overexpressed in irradiated A549 cells to explore their association with immune infiltrates. The correlations between the expressions of these genes and the abundances of immune infiltrates (CD4+ T-cells, CD8+ T cells, and dendritic cells) in lung adenocarcinoma were examined through TIMER analysis.<sup>18</sup> Figure 6 shows positive association of FN1, MFGE8, MMP2, and MYL9 with infiltration of CD4+



**Figure 4** The vaccine changed the secretion of cytokines in serum. The levels of cytokines in serum were examined through ELISA. (A–C) The levels of IL-4, IFN-γ, and IL-10 in serum. The data shown are the representative of three experiments (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

**Abbreviations:** TCL, tumor cell lysates; IR, irradiation; LLC, Lewis lung cancer.



**Figure 5** The vaccine increased the immune infiltration of T-cells in tumor tissue. (A) Representative images of immunohistochemistry staining for CD4+ and CD8+ T-cells of tumor tissues in three groups. (B) The statistical results of the IOD value of CD4 staining in three groups. (C) The statistical results of the IOD value of CD8 staining in three groups. The data shown are the representative of three experiments (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

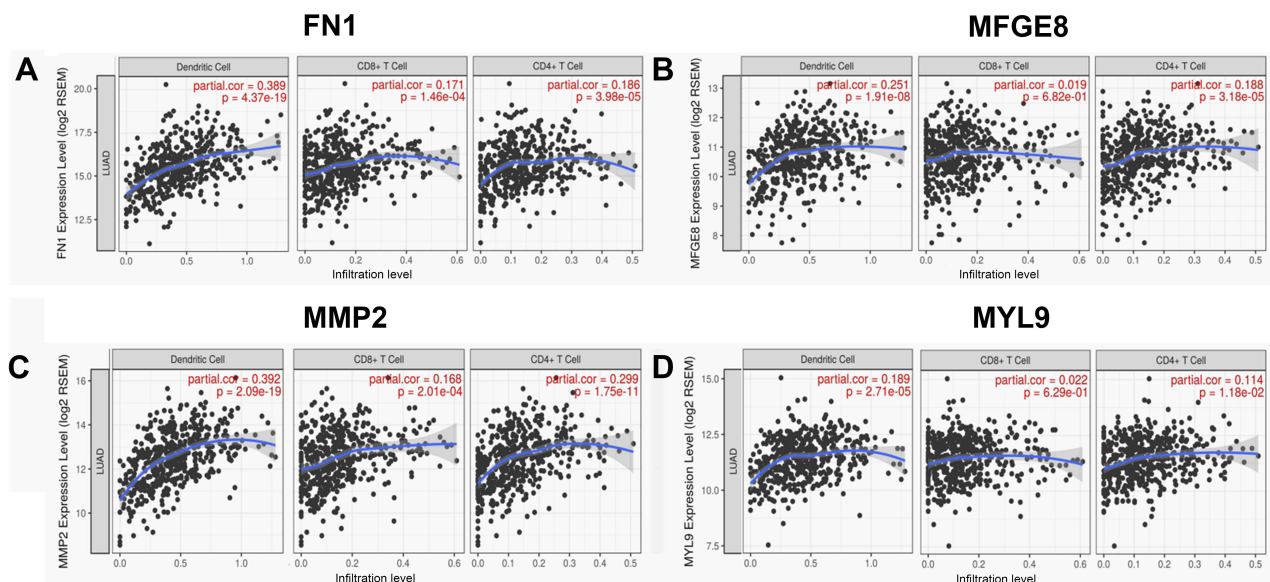
**Abbreviations:** IOD, integrated optical density; TCL, tumor cell lysates; IR, irradiation; LLC, Lewis lung cancer.

T-cells, CD8+ T cells, and dendritic cells. In contrast, the other genes including CEACAM5, MUC1, MUC2, and SOX2 showed no significant correlation or negative correlation with immune infiltration (Figure S1). Furthermore, the abundances of other immune infiltrates (B cells, neutrophils, macrophages) presented in Figure S1, indicated a trend roughly consistent with previously mentioned results.

These results suggested that IR increased the immunogenicity of tumor cells possibly through the upregulation of FN1, MFGE8, MMP2, and MYL9.

## Discussion

Immunotherapy plays a critical role in treatment of lung cancer. In this field, cancer vaccine, triggering the



**Figure 6** Four tumor antigens were positively associated with immune infiltrates. The correlations between gene expression (FN1, MFGE8, MMP2, MYL9) and immune infiltration level (CD4+ T cells, CD8+ T cell, and dendritic cells) in lung adenocarcinoma were examined by tumor immune estimation resource analysis. (A–D) The scatterplots showing the purity-corrected partial Spearman's correlation and statistical significance.

immune system to prevent tumor progression, has been researched for decades; however, the outcomes are always discouraging.<sup>6</sup> One of the causes is the difficulty in identifying the targeting tumor antigen peptide due to tumor heterogeneity.<sup>13,24,25</sup> Previous studies also showed the unsatisfactory effect of single protein or peptide-based vaccines.<sup>6,26,27</sup> In this regard, whole tumor cell vaccine seemed a better choice because it provides a wide spectrum of TAAs, the efficacy of which however, is also limited.<sup>8</sup> It may be attributed to the insufficient immunogenicity of tumor cells.<sup>7,26</sup> This study aimed to find a strategy for improving the immunogenicity of whole tumor cell vaccine. In recent years, the immune activation effect of IR has aroused much concern, especially the in situ vaccine effect of SABR in NSCLC.<sup>10</sup> IR-induced cell death was reported to result in the exposure of numerous TAAs from damaged tumor cells and cellular debris. In addition, IR was also proven, by recent studies, to alter the expressions of ICAM1, MUC1, and CEA in colon tumor cells lines, resulting in enhanced attack by cytotoxic T lymphocytes.<sup>28,29</sup> Consequently, we hypothesized that IR could be considered as a potential strategy to improve the immunogenicity of tumor cell vaccine. As demonstrated in previous studies, radiation-related immune response may be dose-dependent and only radiation beyond a certain threshold dose can initiate robust immune response. Hence, the dose of X-rays

used on tumor cells in this study was 8 Gy, the same as the “ablative” dose in SABR therapy of NSCLC.<sup>9,10</sup>

CSGs provide a high possibility of encoding peptides which can be targeted by circulating lymphocytes resulting in adaptive anti-tumoral immunity.<sup>19,20</sup> In the present study, most of the CSGs were upregulated after IR. Moreover, the expression of a series of tumor T cell antigens was proven to contain T cell epitopes and were consistently upregulated in irradiated A549 cells.<sup>19,20</sup> It indicated that IR might enhance the immunogenicity of lung cancer cells, presenting the potential of IR-modulated whole tumor cell vaccine for cancer vaccine development.

Unfortunately, the study of IR-treated human A549 vaccine could not be successfully conducted because constructing a xenograft A549 mouse model would require nude mice. However, the deficiency of T cell immunity in nude mice limits the study of vaccines. Thus, for in vivo experiments, we used LLC cells instead to construct a xenograft LLC mouse model and LLC vaccine. Therapeutic efficacy of TCL(IR-LLC) vaccine was validated. Compared to the traditional TCL(LLC) vaccine, IR-treated LLC vaccine TCL(IR-LLC) showed enhanced inhibitory effect of tumor growth by increasing tumor cell apoptosis. As known, tumor-specific T-cell response is crucial in anti-tumor immunity.<sup>30</sup> The activation of T-cell response is evoked by both innate immune cells and adaptive immune cells. Dendritic cells are known to be the chief antigen-presenting cells which load and cross-present tumor antigens onto MHC-I and later activate CD4+ T helper and CD8+



cytotoxic T-cells.<sup>31,32</sup> The results of the present study demonstrated that TCL(IR-LLC) vaccine contributed to a significant increase in matured dendritic cells in mouse spleens, which may be followed by a higher antigen presenting ability. Different subpopulations of T-cells have various functions of pro- and anti-tumor immunity.<sup>33</sup> CD4<sup>+</sup> T helper cells, especially Th1 cells, are essential for activation of CD8<sup>+</sup> cytotoxic T-cells and the priming of tumor-specific cytotoxic T lymphocyte responses, whereas Treg cells can suppress effector T-cell-mediated anti-tumor immunity.<sup>34</sup> In this study, the TCL(IR-LLC) group obtained an increase in the proportion of total CD4<sup>+</sup> T-cells but a decrease in the ratio of Treg cells in CD4<sup>+</sup> T-cells. On the other hand, PD-1 is a checkpoint molecule on T-cells that inhibit adaptive immune responses. This study showed that TCL(IR-LLC) vaccine led to an insignificant increase in the proliferation of CD8<sup>+</sup> T-cells and a significant decrease in the ratio of PD-1<sup>+</sup> cells in total CD8<sup>+</sup> cells. These results indicated that the anti-tumor immunity may be activated through the downregulation of these two types of immune suppressor cells. Th1 cytokine IFN- $\gamma$  and Th2 cytokine IL-4 play an important role in anti-tumor immunity through their cytostatic and T cell-related immune-provoking effects.<sup>21,22</sup> In contrast, IL-10 is an important anti-immunity cytokine, as well as a Treg-associated cytokine.<sup>23</sup> The results of cytokines' secretion in serum also showed the pro-immunity effect of the vaccine. Moreover, the immune infiltrates of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T-cells in tumor tissue displayed consistent results.

As the immunogenic modulation effect of IR on tumor cells had been determined through the study of A549 tumor antigen expression and the in vivo study of irradiated LLC vaccine, tumor antigens that correlated with the immune stimulation effect were further explored. Through TIMER analysis, FN1, MFGE8, MMP2, and MYL9 containing T-cell peptide were found to be positively correlated with immune infiltrates in lung cancer.<sup>18,20</sup> As these genes were also expressed in the tumor microenvironment, further study was required for validation, although partial Spearman's correlation was tumor purity-corrected. Further experiments are also needed to study the potential mechanism. Surprisingly, previous studies showed that FN1, MFGE8, MMP2, and MYL9 all promoted the malignant behavior of cancer cells and contributed to metastasis in different cancers.<sup>35–39</sup> Consequently, these four genes may be potential cancer immunotherapy targets for the prevention of progression, relapse, and metastasis. It will be more convincing if vaccines based on human irradiated autologous tumor cells are studied through human trials, and the immune activation effect of these genes is measured in vivo. This is included in our future research.

## Conclusion

In summary, this study demonstrated that IR was able to increase immunogenicity of lung cancer cells. Furthermore, irradiated lung cancer cell vaccine showed enhanced anti-tumor efficacy by eliciting intensive T-cell response. It is possible for FN1, MFGE8, MMP2, and MYL9 to contribute to the enhanced T-cell response. Further studies are required to confirm the relationship between immune response and these upregulated genes. It may provide a new insight into immunotherapy in the field of lung cancer.

## Acknowledgment

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## Disclosure

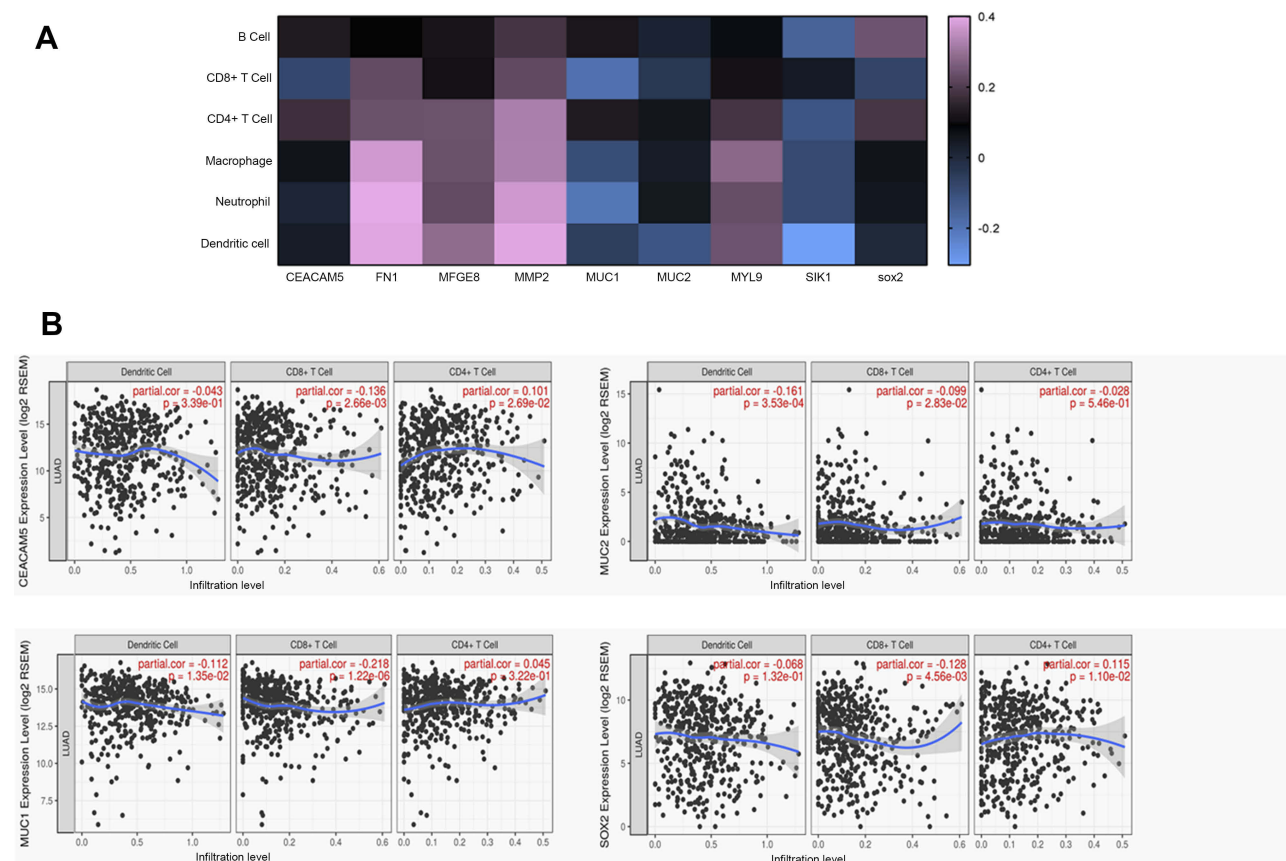
The authors report no conflicts of interest in this work.

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## Supplementary material



**Figure S1** The correlations between gene expression and immune infiltration level were examined by tumor immune estimation resource (TIMER) analysis. **(A)** Heat map plotted by the correlations between gene expression and immune infiltrate level in lung adenocarcinoma through TIMER analysis. Colored bars represent differential levels of Spearman's correlation. **(B)** The correlations between gene expression (CEACAM5, MUC1, MUC2, SOX2) and immune infiltration level (CD4+ T cells, CD8+ T cells, and dendritic cells) in lung adenocarcinoma.

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