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*Original Article*

# IFNγ and TNFα synergistically promote galectin 9 secretion by human osteosarcoma cells MG-63 to prevent T cell killing

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**Abstract:** Objectives: Osteosarcoma is the most common bone tumor usually distributed in adolescence and the elderly. IFNγ and TNFα play double-sided roles in tumor immunity. The fundamental mechanism of IFNγ and TNFα in osteosarcoma remains elusive. We speculated that TNFα and IFNγ serve a role in regulating immune checkpoint molecule, Galectin 9, expression of MG-63 osteosarcoma cells. Methods: The human osteosarcoma cell line, MG-63, was stimulated with recombinant human IFNγ and TNFα. Cytokine stimulated MG-63 cells were cocultured with human peripheral T cells. Real-time PCR, flow cytometry and ELISA were used to detect related molecule expression. Results: IFNγ and TNFα up-regulate Galectin 9 expression of MG-63 cells synergistically. IFNγ and TNFα stimulated MG-63 cells induce CD4 and CD8 T cell apoptosis and inhibit cytokine production through the Tim-3/Galectin 9 pathway. A High level of serum Galectin 9 and highly expressed Tim-3 of peripheral T cells were detected in osteosarcoma patients. Conclusion: We found that Galectin-9 is induced by IFNγ and TNFα stimuli in osteosarcoma cells. Furthermore, Tim-3/Galectin-9 pathway contributes to the inducible immunomodulatory functions of osteosarcoma cells, which may provide a new clue to novel strategies for the osteosarcoma therapy.

**Keywords:** Galectin 9, T-cell immunoglobulin- and mucin domain-3, tumor necrosis factor alpha, interferon gamma, osteosarcoma

## Introduction

Osteosarcoma is the most common bone tumor, usually distributed in adolescence and the elderly [1]. Surgical resection and systemic chemotherapy are the current therapeutic approaches of osteosarcoma [2]. The genetic and cellular alterations of tumor cells usually induce tumor immune escape and metastasis. In recent years, identification of T cell immune checkpoints, including Programmed cell death protein 1 (PD-1), Lymphocyte-activation gene 3 (Lag-3) and T-cell immunoglobulin- and mucin domain-3 (Tim-3), has prompted the development of new approaches to cancer therapy [3-5]. However, the understanding of immune checkpoint in osteosarcoma is still limited. Therefore, it is crucial to discover the immunologic mechanism to develop novel immunotherapies for osteosarcoma.

Tim-3 is a type I trans-membrane protein and generally considered as an immune checkpoint belonging to the Tim family [6]. Tim-3 is expressed on Th1 cells, CD8 T cells, monocytes, dendritic cells, and some tumor cells, such as gastric cancer, melanoma, and NSCLC cells [7]. Tim-3 plays a critical role in suppression of Th1 responses. Furthermore, tumor-infiltrating lymphocytes (TILs) express a number of Tim-3 providing negative regulation to anti-tumor immune responses. Therefore Tim-3 is considered a potential candidate for tumor immunotherapy.

Galectin 9 is identified as Tim-3 ligand which induces the apoptosis of T helper 1 cells and peripheral tolerance [8]. Galectin 9 molecules can be synthesized intracellularly in antigen presenting cells, endothelial cells and mesenchymal stromal cells in response to inflammation. TNFα and IFNγ play essential roles in tumor surveillance. TNFα-induced Galectin 9

expression and prompts T cell apoptosis in astrocytes [9]. It was recently shown that Galectin 9 was upregulated intracellularly by mesenchymal stromal cells after exposure to TNFα and IFNγ [10]. Expression and secretion of Galectin 9 leads to apoptotic cell death of Tim-3+ lymphocytes in the tumor immune microenvironment [10, 11]. Therefore, it is critical to clarify the role of proinflammatory cytokine in Tim-3 and Galectin 9 interaction and anti-tumor immunity.

In this study, we investigated whether TNFα and IFNγ serve a role in regulating Galectin 9 expression of MG-63 osteosarcoma cells. We found that IFNγ and TNFα perform tumor immune-associated suppressive function by promoting Galectin 9 production of MG-63 cells in coculture with T cells. A high level of serum Galectin 9 and highly expressed Tim-3 of peripheral T cells was observed from patients with osteosarcoma. These findings suggest a critical role of proinflammatory cytokines in the immune regulation of osteosarcoma tumor immune microenvironment.

## Methods

### *MG-63 cell culture*

The study was approved by the institutional review board and ethics committee of Hebei Medical University. The human osteosarcoma cell line, MG-63 was purchased from National Infrastructure of Cell Line Resource of China. Cells were maintained in Minimum Essential Medium (Gibco, USA) containing penicillin, streptomycin and 10% fetal bovine serum. MG-63 cells were stimulated with 0.1, 1 or 10 ng/ml recombinant human IFNγ or TNFα (Peprotech) for 6 days.

### *Human sample collection*

This study recruited 17 patients with osteosarcoma and 14 healthy controls. The blood plasma from healthy controls or osteosarcoma patients was collected from fresh blood samples after centrifugation. The human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Thermo Fisher) density gradient centrifugation. PBMCs were maintained in RPMI-1640 medium (Gibco, USA) supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37°C with 5% $CO_2$.

### *Real-time PCR*

The TRIzol reagent (Invitrogen) was used to extract total RNAs from MG-63 cell lysates in accordance with the manufacturer's manual. RNAs were transcribed into cDNA by PrimeScript® RT reagent Kit (TAKARA). SYBR Green Master Mix (Applied Biosystems) was used for detection of cDNA. The primer sequences used in this study are Galectin 9 (forward: 5'-CTTTCATCACCACCATTCTG-3', reverse: 5'-ATGTGGAACCTCTGAGCACTG-3'), IFNγ (forward: 5'-CATCAGCAACAACATAAGCGTCA-3', reverse: 5'-CTCCTTTTCCGCTTCCTGA-3'), Gzmb (forward: 5'-AATGTGAAGC-CAGGAGATGTGTGC-3', reverse: 5'-CCGAAAGGAAGCACGTTT-GGTCT-T-3' and β-actin (forward: 5'-ACCAACTGGGAC-GATATGGAGAAGA-3', reverse: 5'-TACGACCAGA-GGCATACAGGGACAA-3') as previously described [12]. Real-time PCR was proceeded using 7500 Real-Time PCR System (Applied Biosystems).

### *Cell proliferation assay*

MG-63 cell proliferation was evaluated by MTT assays. Cells were plated at $3 \times 10^3$ cells/well in 96-well plates. MG-63 cells were washed with PBS and incubated in 0.5 mg/ml MTT solution for 4 h at 37°C. Subsequently, MTT solution was discarded, and 150 μl DMSO was added. After being mixed for 10 min at room temperature, cells were quantified spectrophotometrically at 490 nm in an ELx800™ plate reader (BioTek).

### *ELISA*

Galectin 9 concentrations of cell supernatant and blood plasma were quantified by ELISA assay. The human Galectin 9 ELISA kit (R&D) was used according to manufacturer's protocol. Plasma samples require a 4-fold dilution. VersaMax Microplate Reader (Molecular Devices) was applied to determine the 450 nm optical density of each well within 30 minutes.

### *Flow cytometry and cell sorting*

Single cell suspensions were stained with antibodies (BioLegend or BD Biosciences) for 30 min at 4°C in dark. The following anti-human antibodies were applied in this study: CD3 (HIT3a), CD4 (GK1.5), CD8 (SK1), Galectin 9

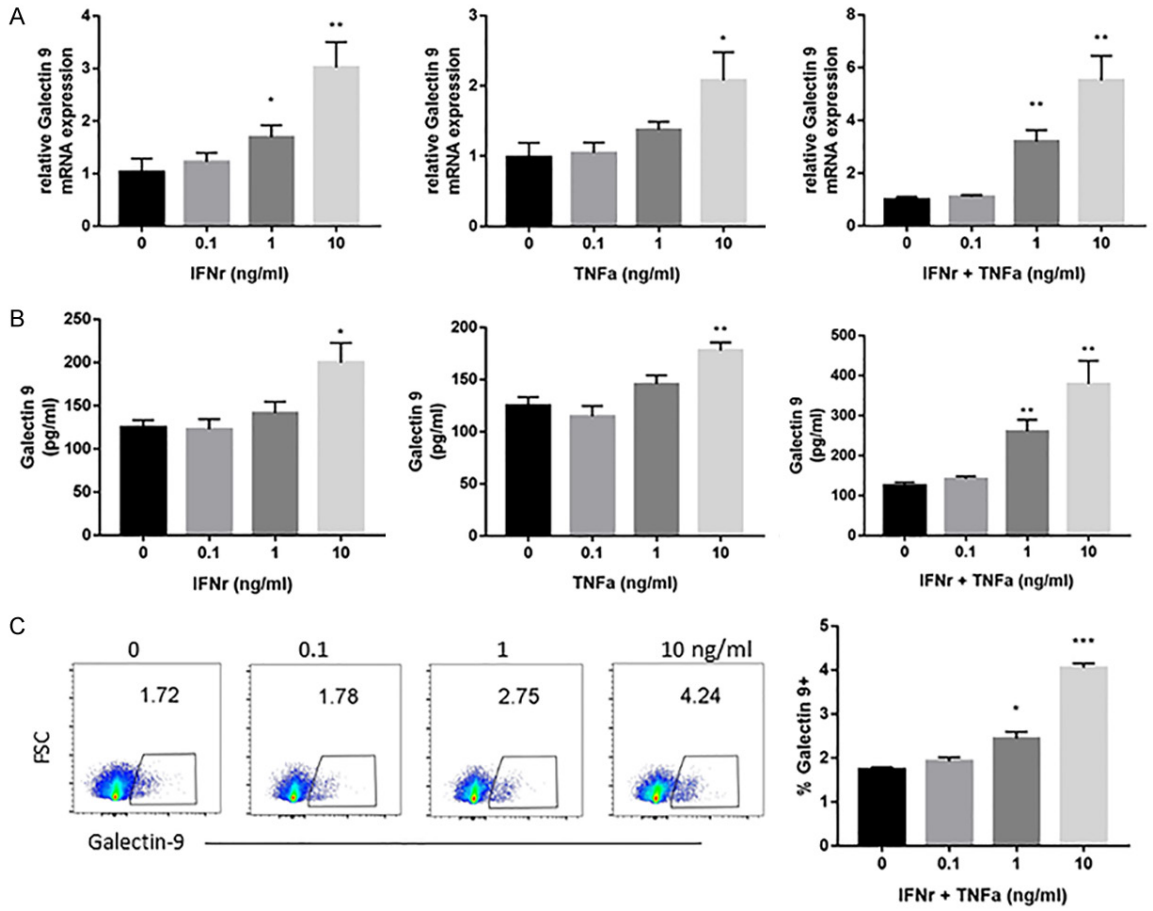


**Figure 1.** IFNγ and TNFα induce Galectin 9 production of osteosarcoma cells. Osteosarcoma cell line, MG-63 was cultured with different concentrations of IFNγ and TNFα for 6 days. A. Galectin 9 expression was analyzed by real-time PCR. B. Galectin 9 concentration of supernatant was analyzed by ELISA assay. C. Galectin 9 expression on MG-63 cells was analyzed by flow cytometry. The results are expressed as the proportion of Galectin 9+ MG-63 cells. n = 5. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

(clone: 9M1-3). The gating strategies of Tim-3 were set using isotype antibodies recommended by the manufacturer. Human PBMCs were sorted for $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^+$ T cells on an Aria II flow cytometer (BD Biosciences) to a purity of 97-99%. The Annexin V-PE 7-AAD apoptosis kit (BD) was used according to the manufacturer's protocol to measure MG-63 cells and T cell apoptosis.

*Statistics*

One-way ANOVA with Bonferroni or Tukey's post-test for multiple comparisons; 2-tailed, unpaired *t* test used for unmatched pairwise sample comparison (SPSS 23). Significant differences are shown as *: $P < 0.05$, **: $P < 0.01$, ***$P < 0.001$.

## Results

*IFNγ and TNFα synergistically up-regulate Galectin 9 in osteosarcoma cell MG-63*

To study the function of proinflammatory cytokine to osteosarcoma cells, we investigated Galectin 9 expression on osteosarcoma cell line, MG-63. As shown in **Figure 1A**, Real-time PCR revealed elevated Galectin 9 expression of MG-63 cells after IFNγ and TNFα stimulation. The Galectin 9 expression correlated with increasing concentrations of IFNγ and TNFα. The level of Galectin 9 secretion was examined by ELISA (**Figure 1B**). IFNγ and TNFα stimulation increased Galectin 9 concentration in MG-63 cell supernatant. Flow cytometry results also shown highly expressed surface Galectin 9 of

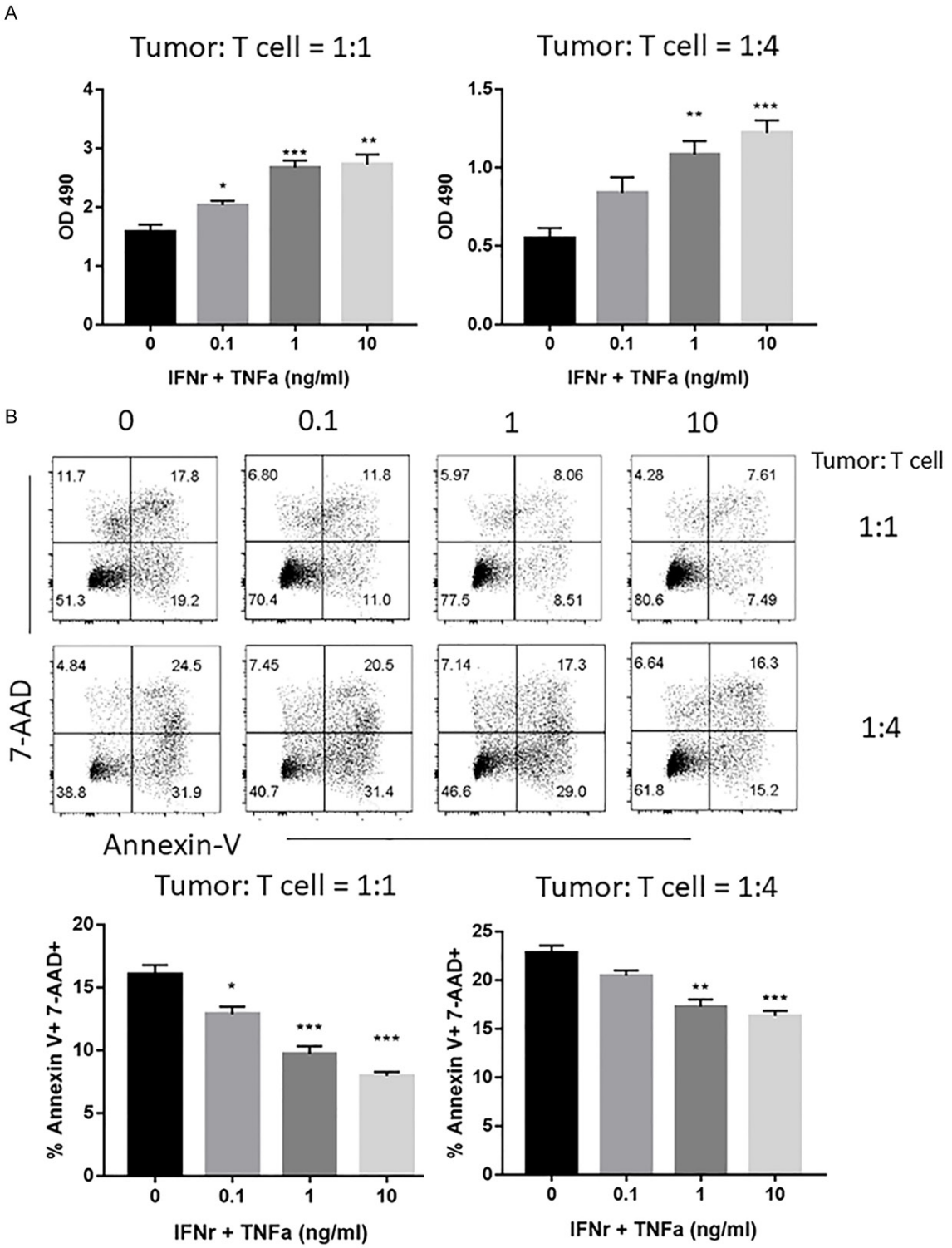


**Figure 2.** The role of IFNγ and TNFα in cell proliferation and apoptosis of MG-63 cells in the coculture with T cells. IFNγ and TNFα stimulated MG-63 cells were cocultured with T cells for 3 days. A. Proliferation of MG-63 cells was analyzed by MTT assay. B. Apoptosis of MG-63 cells was analyzed by flow cytometry. Annexin V+ 7-AAD+ populations refer to late apoptotic cells. *: P < 0.05, **: P < 0.01, ***: *P* < 0.001.

MG-63 cells with IFNγ and TNFα stimulation (**Figure 1C**). Moreover, we found that combined IFNγ and TNFα stimulation showed the highest Galectin 9 expression of MG-63 cells, which

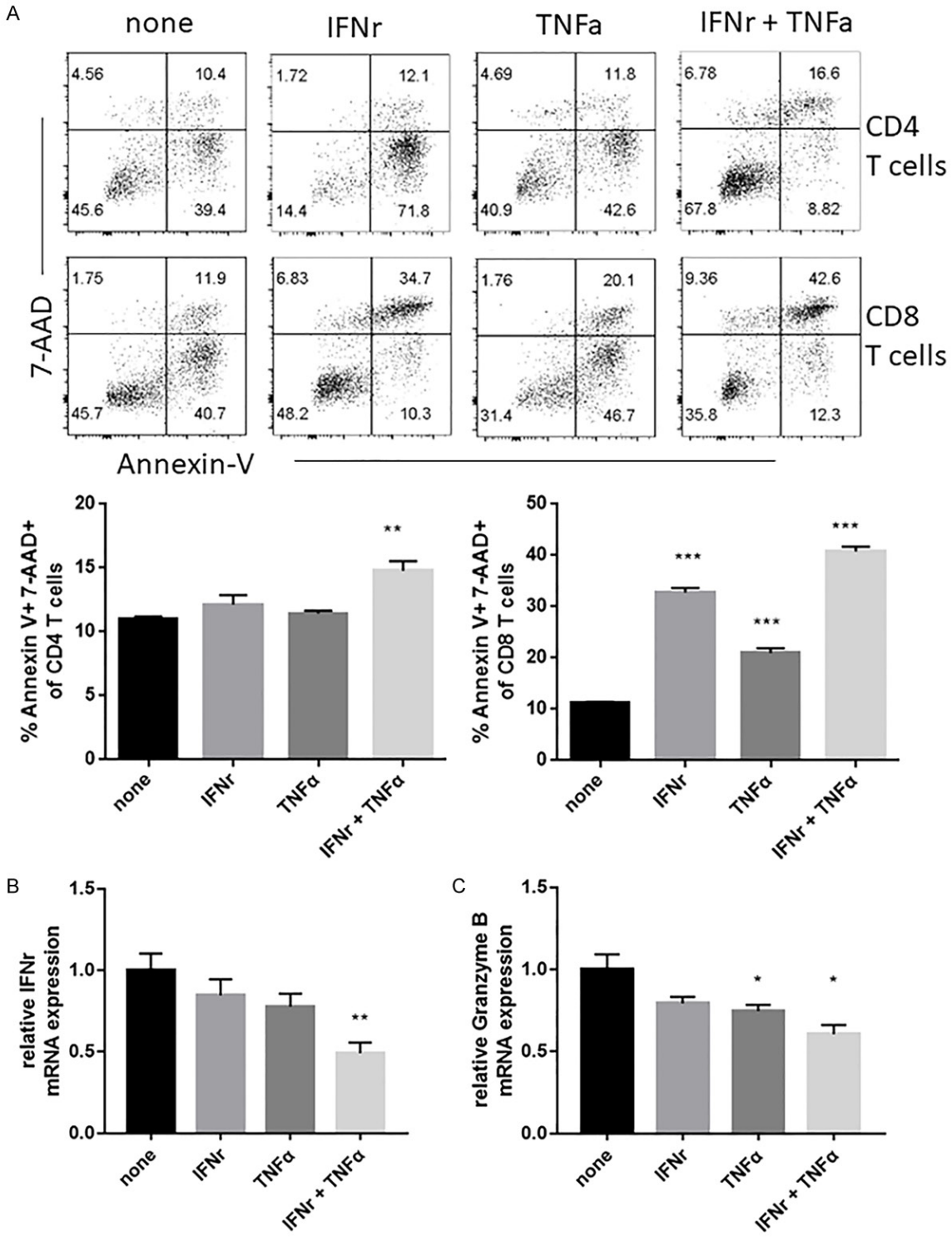


**Figure 3.** The role of IFNγ and TNFα in CD4 and CD8 T cell apoptosis in coculture of MG-63 cells and T cells. IFNγ or TNFα stimulated MG-63 cells were cocultured with CD4 or CD8 T cells for 3 days. A. Apoptosis of $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry. Annexin V+ 7-AAD+ populations refer to late apoptotic cells. B. IFNγ mRNA expression of CD4 or CD8 T cells were detected by real-time PCR. C. Granzyme B mRNA expression of CD4 or CD8 T cells were detected by real-time PCR. Asterisk, significant compared to none controls. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

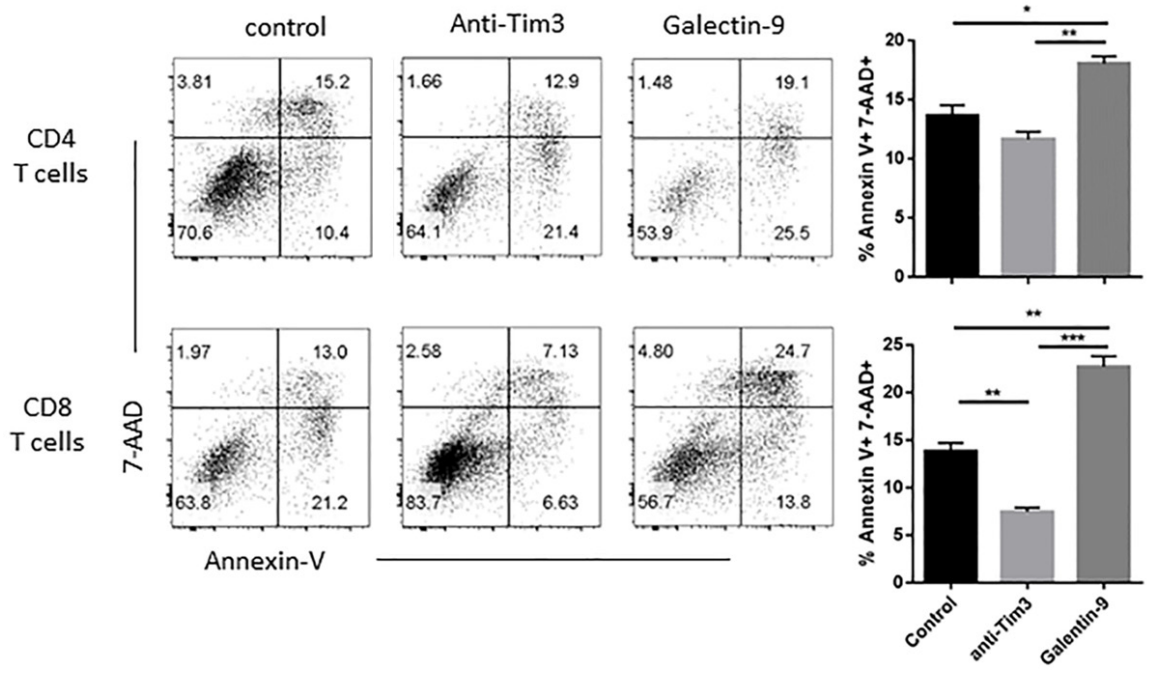


**Figure 4.** Galectin 9/Tim-3 pathway influenced CD4 and CD8 T cell apoptosis in the coculture of MG-63 cells and T cells. 10 ug/ml anti-human Tim-3 was applied to block Galectin 9/Tim-3 pathway. 1 ug/ml Galectin 9 was applied to activate Galectin 9/Tim-3 pathway. Apoptosis of $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry. Annexin V+ 7-AAD+ populations refer to late apoptotic cells. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

suggested a synergistic effect of IFNγ and TNFα.

*IFNγ and TNFα stimulated MG-63 cells mitigated cell killing of T cells*

MG-63 cells were stimulated by 0-10 ng/ml IFNγ and TNFα for 6 days. Then the supernatant was discarded to eradicate the influence of supernatant cytokines to T cells. IFNγ and TNFα stimulated MG-63 cells were cocultured with peripheral $CD3^+$ T cells from healthy donor. As shown in **Figure 2A**, we examined MG-63 cell proliferation by MTT assay. IFNγ and TNFα stimulation could significantly increase the proliferation of MG-63 cells cocultured with T cells. Furthermore, cell apoptosis of MG-63 cells also significantly decreased with IFNγ and TNFα stimulation (**Figure 2B**).

*IFNγ and TNFα stimulated MG-63 cells induce CD4 and CD8 T cell apoptosis and inhibit their immune function in the tumor microenvironment*

To investigate the influence of IFNγ or TNFα stimulated MG-63 cells to T cell subsets, we cocultured 10 ng/ml IFNγ or TNFα stimulated MG-63 cells with sorted CD4 or CD8 T cells *in vitro*. As shown in **Figure 3A**, similarly with results shown above, combined stimulation with IFNγ and TNFα on MG-63 cells induced significant apoptosis of CD4 and CD8 T cells. However, IFNγ or TNFα stimulated MG-63 cells induced more late apoptosis in CD8 T cells then that in CD4 T cells. To investigate the influence of IFNγ or TNFα stimulated MG-63 cells on functions of CD4 and CD8 T cells, we detected the effector molecule expression of CD4 and CD8 T cells by real-time PCR (**Figure 3B**). The expression of major Th1 CD4 T cell cytokine, IFNγ, decreased significantly in CD4 T cells cocultured with IFNγ + TNFα stimulated MG-63 cells. Similarily, the killing molecule of CD8 T cells, Granzyme B, decreased significantly in CD8 T cells cocultured with TNFα and IFNγ + TNFα stimulated MG-63 cells.

*The role of Tim-3/Galectin-9 pathway in MG-63 and T cell interaction*

Tim-3/Galectin 9 pathway is not the only immune checkpoint in tumor immune microenvironment, thus we applied Tim-3 blocking antibody and Galectin 9 to investigate the role of Tim-3/Galectin 9 pathway in the interaction of

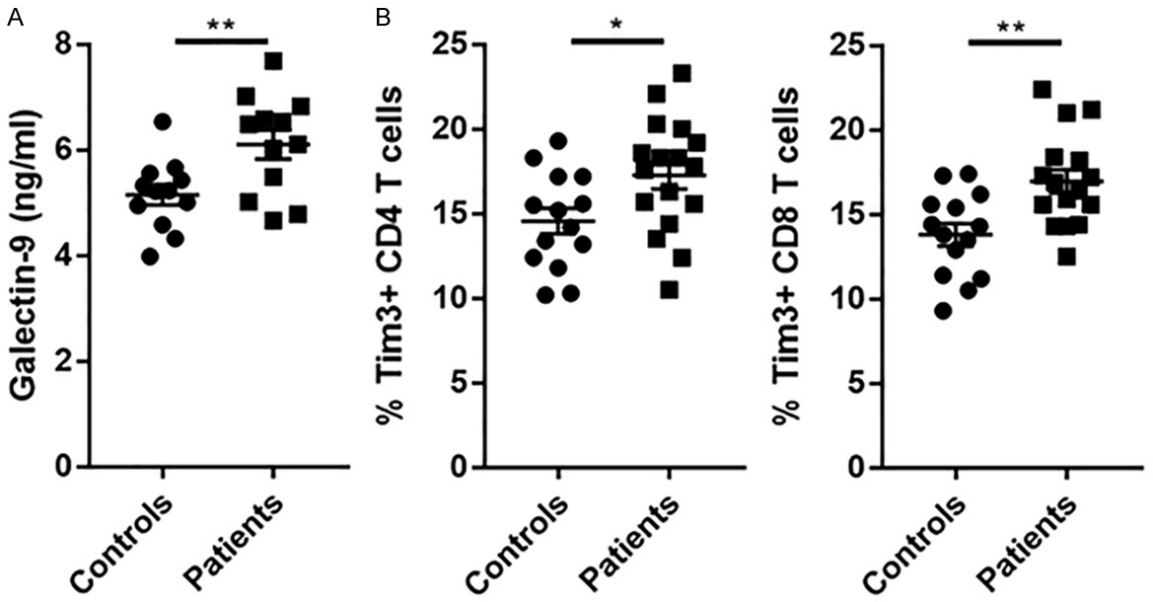


**Figure 5.** Serum concentrations of Galectin 9 and the proportion of Tim-3+ peripheral T cells presented in osteosarcoma patients. A. Serum concentration of Galectin 9 was detected by ELISA assay. B. Percentage of Tim-3+ cells on $CD4^+$ and $CD8^+$ T cells in patients and controls. Data were calculated from 17 patients and 14 healthy controls. *: $P < 0.05$, **: $P < 0.01$.

IFNγ + TNFα stimulated MG-63 cells and T cells. We blocked Tim-3 with 10 ug/ml anti-human Tim-3 monoclonal antibody. The Tim-3/Galectin pathway was activated by 1 ug/ml Galectin 9 protein. As shown in **Figure 4**, Tim-3 blocking inhibited CD8 T cell apoptosis significantly. Furthermore, the proportion of late apoptotic CD4 T cells was also reduced with Tim-3 blocking. On the other hand, Galectin-9 induced Tim-3/Galectin-9 activation promoted CD4 and CD8 T cell apoptosis significantly. These data indicated the crucial role of Tim-3/Galectin-9 pathway in T cell apoptosis of osteosarcoma tumor immune microenvironment.

*A high level of serum Galectin 9 and highly expressed Tim-3 of peripheral T cells from patients with osteosarcoma*

We examined peripheral Galectin 9 concentration of osteosarcoma patients and healthy controls. Compared to healthy controls, osteosarcoma patients expressed higher levels of Galectin 9 in peripheral blood (**Figure 5A**). As shown in **Figure 5B**, Tim-3 expression was significantly increased in peripheral $CD4^+$ and $CD8^+$ T cell in osteosarcoma patients than the healthy controls.

## Discussion

In this study, we investigated the function of IFNγ and TNFα in osteosarcoma cell line, MG-63. IFNγ and TNFα induced a high level of Galectin 9 in MG-63 cells respectively, and we found a synergistic effect of those two proinflammatory cytokines. IFNγ and TNFα stimulated MG-63 cells mitigation of cell killing function of T cells. Furthermore, IFNγ and TNFα stimulated MG-63 cell-induced CD4 and CD8 T cell apoptosis and inhibited the secreting of their effector cytokine, IFNγ and Granzyme B. Tim-3/Galectin 9 pathway played a central role in the immune regulation of IFNγ and TNFα stimulated MG-63 cells and T cells.

The immune system plays a crucial role in repression and elimination of cancer. However, the immune system is also restrained by several suppressive mechanisms. Immune activation and immune suppression consist the equilibrium of immune system and protect our body from infective diseases, tumors and autoimmune diseases. Immune checkpoints play an important role in homeostatic regulation of immune system. In the past several decades, remarkable advances in immunology have led to new immune checkpoint therapies, such as

CTLA4 and PD-1/PD-L1 [13]. Tim-3/Galectin 9 is one of the immune checkpoints, whose fundamental mechanism in tumor immune microenvironment remain elusive. We demonstrated that proinflammatory cytokine, IFNγ and TNFα, induced Galectin-9 secretion of osteosarcoma cells, which inhibited the immune function of T cells close to osteosarcoma cells. IFNγ is the uppermost immune-provoking cytokine produced by innate immune cells [14]. IFNγ combined with TNFα can arrest tumor cells in G0/G1 phase and drive cancer into senescence [15]. IFNγ can upregulate PD-L1, one of the immune checkpoints, in all types of cells [16]. This study shows that IFNγ and TNFα may inhibit anti-tumor immune response to osteosarcoma cells through Tim-3/Galectin 9 pathway. These results may lead to new strategies for the treatment of osteosarcoma.

The correlation of a high level of Tim-3 on $CD4^+$ T cells and $CD8^+$ T cells and pathological category of cancer and autoimmune disease is widely reported [17, 18]. Our data did also confirm this phenomenon in osteosarcoma patients. To figure out the molecular mechanism of the interaction of MG-63 cells and T cells, we used anti-Tim-3 antibody and Galectin 9 to block and activate Tim-3/Galectin 9 pathway. Compared to CD4 T cells, we found that CD8 T cells were influenced greater after coculture with IFNγ and TNFα stimulated MG-63 cells. Han et al. also demonstrated that patients with elevated Tim-3 expression in $CD8^+$ T cells revealed a higher tumor grade, while this relevance could not be observed in $CD4^+$ T cells [19]. This indicates the potential different roles of Tim-3/Galectin 9 pathway in $CD4^+$ and $CD8^+$ T cells.

In conclusion, we observed that IFNγ and TNFα induced a high level of Galectin 9 in MG-63 cells synergistically. The production of Galectin 9 induced CD4 and CD8 T cell apoptosis and inhibited the secretion of IFNγ and Granzyme B through the Tim-3/Galectin pathway. These results may provide a new clue to novel strategies for the treatment of osteosarcoma.

## Disclosure of conflict of interest

None.

## Abbreviations

IFNγ, Interferon gamma; TNFα, Tumor Necrosis Factor alpha; PD-1, Programmed cell death protein 1; Lag-3, Lymphocyte-activation gene 3; Tim-3, T-cell immunoglobulin- and mucin domain-3; TILs, tumor-infiltrating lymphocytes.

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