

The effect of activated M ϕ 1 on $\gamma\delta$ T cell-mediated killing of gastric cancer cells *in vitro*

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Abstract. A clear understanding of the interactions between classically activated macrophages (M ϕ 1) and $\gamma\delta$ T cells may improve current therapeutic approaches, including that of immunotherapy for treating certain types of cancer. The present study aimed to expand the current knowledge by showing the effect of culture supernatants of M ϕ 1 on the proliferation, cell surface marker expression and tumor suppression effects of $\gamma\delta$ T cells, and by exploring the potential mechanisms involved. *In vitro*, M ϕ 1 were cultured by GM-CSF and IFN- γ . The isopentenyl pyrophosphate method was used to amplify human peripheral blood $\gamma\delta$ T cells. The surface markers of macrophages and $\gamma\delta$ T cells were detected by flow cytometry. The proliferation of $\gamma\delta$ T cells induced by the culture supernatants of M ϕ 1 was investigated using the MTT assay. The lactate dehydrogenase method was used to detect the cytotoxicity of $\gamma\delta$ T cells on the SGC-7901 gastric cancer cell line. Ten days after cultivation, the percentage of $\gamma\delta$ T cells from the repertoire of naive cells, expanded from 4.21 to 91.27%. The percentage of cells expressing CD44 was 94%. The percentage of CD68 on cultured M ϕ 1 was increased from 17.7 to 73.2%. The culture supernatants of M ϕ 1 increased the proliferation of $\gamma\delta$ T cells compared with the control group (33.8% vs. 0, $P<0.01$). The culture supernatants of M ϕ 1 increased the cytotoxicity of $\gamma\delta$ T cells compared with the control group (70.18 vs. 47.25%, $P<0.01$). In conclusion, the supernatant of cultured M ϕ 1 promotes the proliferation of $\gamma\delta$ T cells and their cytotoxic effect on the SGC-7901 gastric cancer cell line.

Introduction

Gastric carcinoma is a common human malignancy with a high incidence, including in countries such as China (1). The morbidity and mortality associated with gastric carcinoma is the leading cause of all malignancies worldwide (2). A recent epidemiological study revealed that the 5-year survival rate

of gastric carcinoma was approximately 90% (3). Thus, it is important to study and improve its prevention and treatment alternatives.

Immunotherapy is a relatively new treatment method based on the manipulation of various immune cells for cancer treatment. This treatment can improve the ability of recognition and presentation of tumor antigens by the immune system, resulting in a reduction in the incidence of tumor recurrence and metastasis (4-6). However, to achieve this, immunotherapy should be able to direct the innate immunity and adaptive response that exerts an antitumor effect (7,8). Mononuclear cells can be differentiated into classically activated macrophages (CAMs/M ϕ 1) by granulocyte macrophage colony-stimulating factor (GM-CSF) and play a key role in the induction of the specific immune response and immunological regulation (9,10). The $\gamma\delta$ T cells are an important subset of the T-cell population of the innate immune system *in vivo*, and have an important role in tumor immune surveillance (11). Currently, studies concerning the interactions of M ϕ 1 and $\gamma\delta$ T cells are mostly limited to inflamed tissues (12,13). To the best of our knowledge, the influence of M ϕ 1 on the antitumor effect of $\gamma\delta$ T cells has yet to be reported.

We hypothesized that M ϕ 1 can signal $\gamma\delta$ T cells to achieve a comprehensive cellular immunotherapeutic effect that may improve the elimination of cancer cells. To test this hypothesis, we used the supernatant of *in vitro* cultured M ϕ 1 to signal $\gamma\delta$ T cells and documented the effects on their proliferation, cell surface marker expression and cytotoxicity against gastric cancer cells. In addition, we examined the possible mechanisms involved in the findings and the opportunity for more direct tests for an immunotherapeutic approach.

Materials and methods

Materials. The human SGC-7901 gastric cancer cell line was obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). rhGM-CSF was purchased from Promega Corp. (Madison, WI, USA), and rhIL-2 and rhIFN- γ were purchased from Xiamen Amoytop Biotech Co., Ltd. (Xiamen, China). RPMI-1640 medium, calf serum and trypsin were purchased from Gibco (Grand Island, NY, USA). Methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Isopentenyl pyrophosphate (IPP), PE-labeled mouse anti-human monoclonal antibody CD3, FITC-labeled mouse

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anti-human monoclonal antibodies CD68, CD44, and $\gamma\delta$ TCR were purchased from MultiSciences (Lianke) Biotech Co., Ltd. (Hangzhou, China). Interleukin (IL)-10, IL-12 were analyzed using the commercially available kit from Gibco. Lactate dehydrogenase (LDH) was assayed using the commercially available kit by Shino-Test Corp. (Tokyo, Japan).

Culture and identification of $\gamma\delta$ T cells. Approximately 10 ml of peripheral blood with heparin/EDTA as anticoagulant were drawn aseptically from healthy donors and added to lymphocyte separation medium. Centrifugation was performed at $1,500 \times g$ for 15 min and the peripheral blood mononuclear cells (PBMCs) were separated. The PBMCs were washed three times with normal saline (each wash consisted of centrifugation at $1,000 \times g$ for 10 min) and then added to RPMI-1640 medium supplemented with 10% calf serum, 5% human AB serum, IL-2 150 kU/l and IPP 2 μ g/l. The $\gamma\delta$ T cells were cultured according to the method described by Chen *et al* and Liu *et al* (14,15). The $\gamma\delta$ T cells that had been cultured for 10 days were then collected to detect the cell surface markers $\gamma\delta$ TcR and CD44, and to determine the growth and cytotoxicity of $\gamma\delta$ T cells.

Macrophage culture. Six healthy volunteers were chosen and 200 ml blood were drawn from each volunteer. PBMCs were separated with lymphocyte separation medium. RPMI-1640 complete medium was used to dilute PBMCs to 2×10^9 /l, and the diluted PBMCs were seeded in 6-well plates with 5 ml for each well. The cells were then cultured at 37°C for 2 h with 5% CO₂. The unattached cells were washed with phosphate-buffered saline (PBS) and the cells that adhered were washed with warm saline only once. The attached cells were then cultured with RPMI-1640 medium that contained 700 kU/l GM-CSF and 10% FBS at 37°C with 5% CO₂. Half of the medium was changed once every 2 days, interferon (IFN)- γ of 166 kU/l was added on the sixth day, incubated for 24 h and M ϕ 1 was retrieved.

Detection of the expression of macrophage surface marker CD68 using flow cytometry. M ϕ 1 cultured for 7, 10 and 13 days were digested with trypsin and the cell concentrations were adjusted to 5×10^9 /l with PBS. Centrifugation, washing and resuspension were performed with PBS. Approximately 100 μ l of the cells were then resuspended in centrifuge tubes. CD68 was labeled with a fluorescent marker (FITC-labeled) to a final concentration of 5 mg/l and incubated in the dark at 4°C for 20 min. The unattached label was washed off with PBS and the cell phenotype was detected using a flow cytometer (Amnis Corp., Seattle, USA).

Detection of the effect of M ϕ 1 culture supernatant on $\gamma\delta$ T cell proliferation using MTT. $\gamma\delta$ T cells cultured for 10 days were diluted to obtain a final concentration of 1×10^9 /l. Subsequently, 0.2 ml of the cell suspension were added to each of the wells in a 96-well plate. Each group had 5 wells in replicates. The plates were incubated at 37°C in the presence of 5% CO₂ for 24 h. This was followed by addition of the culture supernatants of M ϕ 1, which were cultured for 10 days in each well. No M ϕ 1 supernatant was added to the control group. The cells were then cultured for another 72 h under the same

conditions and 20 μ l of MTT was added into each well and incubated for 4 h. The supernatant was removed and 100 μ l of DMSO was added per well, and mixed for 10 min. When the precipitate was completely dissolved, the absorbance (A) of each well was detected at 570 nm wavelength using ELISA. The cell proliferation rate was calculated using the formula: cell proliferation = value for test group/value for control group - 1) \times 100%.

Detection of the effect of M ϕ 1 culture supernatant on $\gamma\delta$ T cell phenotype using a flow cytometer. $\gamma\delta$ T cells, cultured for 10 days with M ϕ 1 supernatant (test group) or without M ϕ 1 supernatant (control group) were collected. In the test group, the volume ratio of M ϕ 1 culture supernatant to RPMI-1640 medium was 1:8. The concentration of cells was adjusted to 2×10^9 /l with PBS and 100 μ l of cell suspension was taken in centrifuge tubes. FITC-labeled $\gamma\delta$ TCR and anti-human CD44-FITC antibodies were added to the centrifuge tubes at a final concentration of 5 mg/l, incubated in the dark at 4°C for 20 min, washed with PBS and detected using a flow cytometer.

Measurement of tumor cytotoxic effect of $\gamma\delta$ T cells following treatment with M ϕ 1 culture supernatant. Cell cytotoxicity was detected using the LDH-release method. SGC-7901 gastric cancer cells were cultured to the logarithmic phase using high-glucose Dulbecco's modified Eagle's medium (DMEM). The cells were collected and washed with Hank's balanced salt solution twice and then prepared at a concentration of 2×10^8 /l. The $\gamma\delta$ T cells that were induced for 72 h at different concentrations (either 1:4, 1:8, 1:32 or 1:64) of M ϕ 1 supernatant to RPMI-1640 were used as effector cells at a concentration of 2×10^9 /l. The SGC-7901 gastric cancer and effector $\gamma\delta$ T cells were mixed at a ratio of 10:1. After centrifugation at 500 rpm for 5 min, the cells were incubated at 37°C for 6 h in the presence of 5% CO₂. The cells were mixed gently and centrifuged at 1,500 rpm for 10 min. Each group of cells had replicates. The control group consisted of $\gamma\delta$ T cells that had no M ϕ 1 supernatant added to them. After the incubation period, the culture supernatants of each group of cells were collected and used to detect the activity of LDH (U/l). The cytotoxicity of $\gamma\delta$ T cells was calculated using the formula: cytotoxicity of $\gamma\delta$ T cells = (LDH units of test tubes - LDH units released from effector cells)/(LDH units of maximum release tubes - LDH units released from target cells) \times 100%.

Statistical analysis. SPSS 19.0 (Chicago, IL, USA) was used to analyze the results. The values were presented as mean \pm standard deviation (SD). The t-test was used to compare the two groups, while one-way analysis of variance was used for comparison between groups. $P < 0.05$ was considered statistically significant.

Results

Cultures, $\gamma\delta$ T cell phenotype and assessment of cell purity. PBMCs had adherent growth after culturing for 24 h, in the $\gamma\delta$ T cell induction system. The cell colony size markedly increased after 48 h of cultivation. A single adherent cell layer was apparent after 10 days of cultivation. The individual cells presented a fusiform shape and few suspended cells were found.

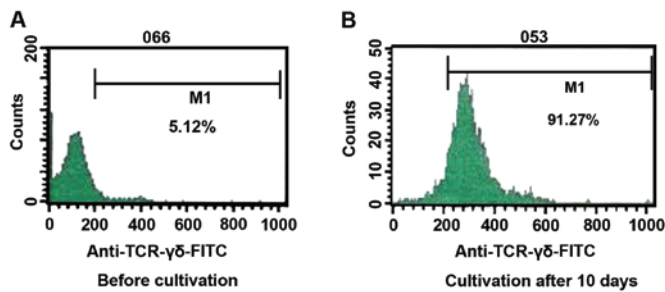


Figure 1. Percentage of $\gamma\delta$ T cells after culturing for 10 days.

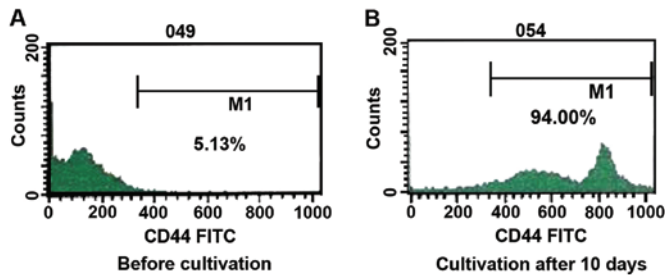


Figure 2. Expression of CD44 on $\gamma\delta$ T cell surface.

The cells collected before and after cultivation were labeled with mAb fluorescence, and then detected and analyzed by flow cytometry. Prior to cultivation, the ratio of $\gamma\delta$ T cells was 5.12% of the total, and the expression of CD44 was 5.13%. After cultivation for 10 days, the percentage of $\gamma\delta$ T cells was 91.27% and the percentage of expression of CD44 was 94.00% a reliable indicator of the purity of the $\gamma\delta$ T cells (Figs. 1 and 2).

Cultures, M ϕ 1 phenotype and assessment of cell purity. In the M ϕ 1 induction system, PBMCs were shown to have adherent growth after culturing for 24 h. Cell colonies of 3-6 cells became apparent after 48 h of cultivation. A bigger colony and few single adherent cells were observed after 7 days of culture and each individual cell appeared irregular in shape. Morphological characteristics of macrophages were apparent after 7 days. The cells appeared large in volume and oval or irregular in shape. The nuclei of the cells were oval or irregular in shape. Loose chromatin was present inside irregular

nuclear membranes with protrusions. The cytoplasm and pseudopodia were abundant and stained with gray blue. The percentage of expression on the cell surfaces of CD68 among the cells was 17.7% before cultivation, and 73.2% after cultivation (Figs. 3 and 4).

Effect of M ϕ 1 culture supernatant on the proliferation of $\gamma\delta$ T cells. The proliferation of $\gamma\delta$ T cells in the test groups that received different concentrations of M ϕ 1 supernatant was significantly higher than that in the control group which did not receive any supernatant ($P<0.01$). Within the supernatant:medium 1:1 to 1:8 ratio groups, the proliferation of $\gamma\delta$ T cells increased with decreasing concentrations of M ϕ 1 supernatant, reaching a peak (33.8% of cells) at the 1:8 ratio. The proliferation decreased by small degrees when the volume ratios used were $<1:16$ (Table I).

Effect of M ϕ 1 culture supernatant on the expression of the $\gamma\delta$ T cell surface marker $\gamma\delta$ TCR. The percentage of expression of the $\gamma\delta$ T cell surface marker $\gamma\delta$ TCR was 97.3% after treatment with M ϕ 1 culture supernatant, while that of the control group was only 91.27%. This difference was found to be statistically significant ($P<0.05$) (Fig. 5).

Effect of M ϕ 1 culture supernatant on the tumor cytotoxic effect of $\gamma\delta$ T cells. The tumor cytotoxic effect of $\gamma\delta$ T cells in the test groups treated with different concentrations of M ϕ 1 supernatant was always higher than the effect in the control groups ($P<0.01$). Within the groups treated with 1:4 to 1:16 supernatant:medium ratios the tumor-cytotoxic effect of $\gamma\delta$ T cells was higher in the group treated with a 1:16 solution, reaching a peak (70.18%) at the volume ratio of 1:16, while the proliferation ratio was decreased slightly when the volume ratio used was $<1:16$ (Table II).

Discussion

Macrophages have many functions including phagocytosis, antigen presentation, and secretion of cytokines. They occupy a central position within the cellular and molecular networks composed of immune and non-immune cells and various cytokines. Macrophages are also key players in the induction and regulation of specific immune responses (16).

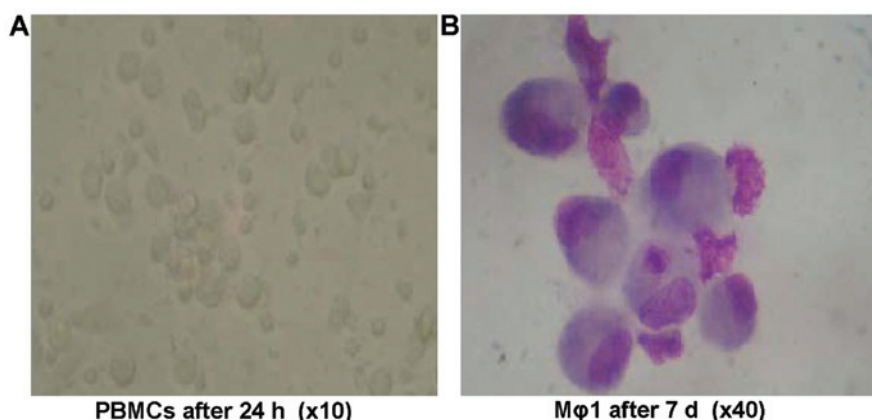


Figure 3. M ϕ 1 morphology before and after cultivation.

Table I. Effect of M ϕ 1 culture supernatant on the proliferation of $\gamma\delta$ T cells.

M:1640 (volume ratio)	$\gamma\delta$ T cell proliferation (A value) of the M ϕ 1 culture group	$\gamma\delta$ T cell proliferation percentage (%) of the M ϕ 1 culture group
Control	0.430 \pm 0.026	0.00
1:1	1.647 \pm 0.097 ^a	283.32 ^a
1:2	1.829 \pm 0.036 ^a	325.68 ^a
1:4	1.871 \pm 0.021 ^a	335.53 ^a
1:8	1.882 \pm 0.029 ^a	338.09 ^a
1:16	1.554 \pm 0.044 ^a	261.60 ^a
1:32	1.569 \pm 0.011 ^a	265.09 ^a
1:64	1.040 \pm 0.069 ^a	142.12 ^a
1:128	0.914 \pm 0.050 ^a	112.08 ^a

^aP<0.01 vs. control group. M, macrophage supernatant; A, absorbance; 1640, RPMI-1640 medium.

Table II. Effect of M ϕ 1 culture supernatant on the tumor cytotoxic effect of $\gamma\delta$ T cells.

M:1640	M ϕ 1 culture group	Control group
1:4	61.16 \pm 2.11 ^a	46.12 \pm 1.23
1:8	65.23 \pm 1.23 ^a	45.55 \pm 0.96
1:16	70.18 \pm 0.94 ^a	47.25 \pm 1.02
1:32	59.94 \pm 1.36 ^a	46.21 \pm 0.79

M, macrophage supernatant; 1640, RPMI-1640 medium. ^aComparison with control group and M ϕ 1 culture that had the same effector-target ratio, P<0.05.

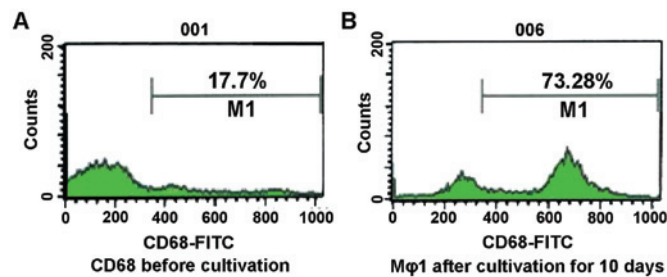
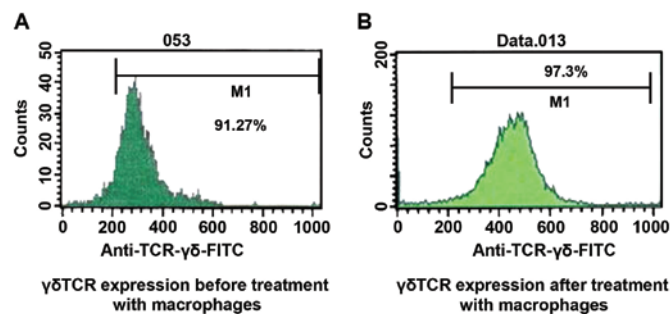


Figure 4. Expression of CD68 surface marker before and after cultivation.

Figure 5. $\gamma\delta$ TCR expression before and after treatment with macrophages.

Baron-Bodo *et al* cultured M ϕ 1 treating them with GM-CSF and IFN- γ and proved that IFN- γ activates M ϕ 1 and enhances their antitumor effects (17). GM-CSF induces macrophage proliferation and enhances their cancer cytotoxicity on cells *in vitro*. In addition, GM-CSF induces the macrophages to secrete inflammatory cytokines (18,19). CD68 is a transmembrane glycoprotein with a molecular weight of 110 kDa that is distributed on the cell surface of macrophages. Although there are only scarce CD68 glycoproteins on the surface of monocytes, their expression is increased significantly as monocytes differentiate into macrophages, allowing the marker to be used for the detection of human macrophages (20). In the present

study, it was found that the expression of CD68 on the surface of the cells had increased after cultivation. The percentage of the cells carrying the CD68 marker increased from 17.7% to 73.2%. This finding suggests that our experiment successfully transformed human PBMCs into macrophages.

$\gamma\delta$ T cells are an important subset of the innate immunity population of T cells *in vivo*, and account for 5-15% of T cells in peripheral blood. $\gamma\delta$ T recognize various antigens in an MHC unrestricted manner and are widely distributed in the epithelial tissues of the digestive and respiratory tracts. Nevertheless, there are some $\gamma\delta$ T cells circulating in peripheral blood (21). Previous studies have shown that $\gamma\delta$ T cells play an important role in tumor immune surveillance and immunotherapy (22-26).

$\gamma\delta$ TCR, expressed on the surface of $\gamma\delta$ T cells, is a receptor mainly associated with inflammation, tumor and other immune responses. Dalton *et al* showed that the interaction of macrophages and $\gamma\delta$ T cells is characteristic of the V δ 1 subtype with induction of the TCR (27,28). $\gamma\delta$ TCRs affect the antigen-presenting function, cytokine secretion profile, inflammatory reaction and tumor immunity action of macrophages. In addition, the cytotoxicity of $\gamma\delta$ T cells correlates with the presence of ligands on tumor cells, such as MICA/MICB and ULBP1-4. In the present study, treating $\gamma\delta$ T cells with M ϕ 1 culture supernatant resulted in an increased expression of the cell surface marker $\gamma\delta$ TCR, suggesting that M ϕ 1 can upregulate the expression of $\gamma\delta$ TCR.

The MTT experiment showed that when $\gamma\delta$ T cell cultures were treated with a M ϕ 1 culture supernatant mixture, the proliferation of the $\gamma\delta$ T cells was higher than the proliferation of the control cells grown without M ϕ 1 culture supernatant. The proliferation of $\gamma\delta$ T cells reached a peak, becoming 33.8% of the cells in the repertoire, at the supernatant:media volume ratio of 1:8. It was concluded that, the M ϕ 1 supernatant promotes the growth of $\gamma\delta$ T cells and provides a new approach for the improvement of immunotherapy methods. Furthermore, the present findings showed that M ϕ 1 culture supernatant adds strength to the cell-killing effect of $\gamma\delta$ T cells on human SGC-7901 gastric adenocarcinoma cells *in vitro*. We suggest that the effect may be due to the hyper-secretion by the M ϕ 1 of Th1-promoting cytokines, such as IL-12, which promote T-cell proliferation. IL-12 was first described as a maturation factor of cytotoxic T lymphocytes and an activator of the natural killer (NK) cells alone. However, the variety of functions of IL-12

increased to include, inducing peripheral blood lymphocytes to produce IFN- γ , enhancing the cytotoxicity of NK cells, promoting the T-cell proliferation and releasing the cytokines IFN- γ and TNF- α . Thus, IL-12 plays a role in the immune killing of tumor cells. It is possible that IL-12, which is present in the M ϕ 1 supernatant, activates the $\gamma\delta$ TCR of $\gamma\delta$ T cells, which in turn upregulates the expression of FasL, which induces the apoptosis of gastric cancer cells. The present study excluded the direct cell-cell contact effects by using only the supernatant of activated macrophages and $\gamma\delta$ T cells.

The findings of the present study have shown that activated macrophages can induce human $\gamma\delta$ T cells to kill SGC-7901 gastric cancer cells. Thus, the immunotherapeutic treatment of gastric carcinoma combining activated macrophage supernatant and $\gamma\delta$ T cells is a potentially successful anticancer strategy. Such treatment may kill tumor cells immediately, bypassing the restrictions imposed by MHC-restricted killing of cancer cells. Additionally, it may improve the functions of the antitumor immune network by ensuring the interaction between various immune cells and enhancing the effect of cytokines. Nevertheless, the exact mechanisms of action and the cell pathways involved remain to be investigated prior to considering the implementation of such a method in anti-cancer therapy.

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