Study of the biological features of *in vitro* cultured γδ T cells

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Abstract. The aim of the present study was to investigate the biological features of *in vitro* cultured γδ T cells. The γδ T cells were *in vitro* cultured and on different culture days cell proliferation, phenotype, killing activity and the secretion of cytokines were analyzed. Cell numbers were counted by an automated cell counter, phenotype of the cells and cytokines were analyzed by flow cytometry, and killing activities of the cells against gastric cancer SGC-7901 cells were tested using the cell counting kit-8. From days 7 to 14, *in vitro* cultured γδ T cells enter the exponential phase. On day 14, maximum proliferation fold was observed, and on day 10, the maximum specific growth rate μmax was achieved. Flow phenotype cluster of differentiation 3^+-T-cell receptor $\gamma\delta^+$ of the γδ T cells in the first 7-17 days achieved a higher proportion and showed no significant differences between 10 days. Secretion of the cytokines interferon-γ and tumor necrosis factor-α gradually increased in the first 7-14 days. The maximum was achieved on day 14, and subsequently began to decrease. The cytolytic activity of the γδ T cells to kill the SGC-7901 cells in the first 7-14 days had an improved killing effect, a slight decline from the first 17 days; in the effector cell to target cell (E:T) ratio 20:1, 10:1 and 5:1 conditions, γδ T cells kill SGC-7901 cells more effectively than 1:1 and 1:2. In conclusion, γδ T cells cultured in the first 7-14 days are suitable for clinical transfusion, and the optimal transfusion time is day 10. An E:T ratio >5:1 is preferred.

Introduction

Treatment with γδ T cells is one of the commonly used cellular adoptive immunotherapies. The γδ T cells were recognized as one special type of cell that is between innate and adaptive immune cells (1). The cells mainly locate at the mucosae of the respiratory tracts, intestine and urogenital tract and subcutaneous tissues. The majority of the γδ T cells do not express cluster of differentiation 4 (CD4) or CD8. They can specifically recognize antigens in a major histocompatibility complex-nonrestricted manner, playing an important role in the defense of the body against infection, autoimmune disease and antitumor processes (2). γδ T cells are only 0.15-5% of the peripheral blood T lymph cells, and therefore, the γδ T cells that are used in clinical cellular adoptive immunotherapies have to be large-scale cultured *in vitro*. However, the biological features of *in vitro* cultured γδ T cells are rarely reported. Currently, numerous studies of the features of clinically used cytokine-induced killer (CIK) cells have been reported. Rettinger *et al* (3) found that the maximum specific growth rate of CIK was achieved on day 10 and the cell number began to decrease on day 30. The proliferation rate and activity of the cells were closely associated with the cell number and cell density, and the proliferation of CIK cells is time restricted. The study of Yang *et al* (4) showed that extended culture time can increase the cytotoxic activity of CIK cells; ~28-day culture time is the optimum time for CIK cell culture, increasing the effective cell number and significantly increasing the killing effect against tumor cells. However, the growth features of the cells reported in these studies are not consistent. Additionally, there are significant differences between types of immune cells. Therefore, the present study examined the biological features of *in vitro* cultured γδ T cells, comparing the growth rates, phenotype, secreted cytokines and killing activity of the γδ T cells cultured for different times. The optimal status of the *in vitro* cultured γδ T cells was investigated, which provides information for clinical treatment to determine the optimal culture time and the number of reinfused cells.

Materials and methods

Materials. Fetal bovine serum (FBS), trypsin, RPMI-1640 medium and OpTmizer medium were purchased from Gibco (Grand Island, NY, USA). Gastric cancer SGC-901 cells were purchased from SIBCB (CAS, Shanghai, China). Zoledronate was purchased from Novartis Pharma Schweiz (AG, Rotkreuz, Switzerland) and interleukin (IL)-2 was purchased from Beijing SL Pharmaceutical Co., Ltd. (Beijing, China). Lymphocyte Separation Medium was purchased from PAA Laboratories (Linz, Austria), phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA),

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and brefeldin A (BFA) was purchased from eBioscience Inc. (San Diego, CA, USA). PE-conjugated anti-T-cell receptor (TCR)-γδ antibody (347907), APC-conjugated anti-human CD3 (555342), FITC-conjugated interferon (IFN)-γ (554551), APC-conjugated tumor necrosis factor (TNF)-α (554514) and the Cytofix/Cytoperm™ Fixation/Permeabilization kit were purchased from BD Biosciences (Ann Arbor, MI, USA). Cell counting kit-8 (CCK-8) was purchased from Beyotime Intitute of Biotechnology (Shanghai, China). The automated cell counter Countess® was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). The Thermo MK3 microplate reader was purchased from Thermo Scientific (Rockford, IL, USA) and the BD Accuri C6 cytometer was purchased from BD Biosciences.

Culture of human γδ T cells. Peripheral blood (100 ml) was taken from 4 healthy volunteers who provided written informed consent. Ethical approval was provided from the Ethics Committee from the Department of Oncology, Tianjin Union Medical Center (Tianjin, China). Mononuclear cells were separated and washed with normal saline 3 times. Subsequently, the cells were suspended with OpTmizer medium supplied with 1.33 μl zoledronic acid and 1,000 U/ml IL-2. The culture of cells was maintained in a humidified incubator at 37˚C and 5% CO_2. Cell numbers were counted every 3-4 days by an automated cell counter and adjusted to $1x10^6$ cells/ml. The multiplication factor and growth rate were calculated.

Detection of the phenotypes and cytokines secreted by the cultured γδ T cells. The cells were harvested on days 7, 10, 14 and 17. The cells were washed with PBS and adjusted to $1x10^6$ cells/ml and incubated with the PE-conjugated anti-TCR-γδ antibody and APC-conjugated anti-human CD3 antibody. Phenotypes were analyzed by flow cytometry. The γδ T cells were supplied with 20 ng/ml PMA, 0.5 μg/ml ionomycin and 3 μg/ml BFA, and were subsequently cultured in a humidified incubator at 37˚C and 5% CO_2 for 6 h. Cells were harvested and incubated with fluorescein-conjugated antibody at 4˚C in the dark for 20 min, and were washed with PBS twice. The cells were resuspended by permeabilization buffer and incubated at 4˚C for 1 h. The cells were washed with wash buffer twice and incubated with fluorescein-conjugated antibody at 4˚C in the dark for 20 min. Cells were subsequently resuspended with 1% paraformaldehyde dissolved in PBS following washing with wash buffer twice. Cytokines secreted by γδ T cells were analyzed by flow cytometry.

Detection of killing activity of γδ T cells against SGC-7901 cells by the CCK-8 kit. SGC-7901 cells in the exponential phase were harvested and washed with RPMI-1640 medium supplied with 5% FBS and adjusted to $5x10^4$ cells/ml, serving as the target cells. γδ T cells were harvested on days 7, 10, 14 and 17. The cells were washed with RPMI-1640 supplied with 5% FBS and were diluted to $1x10^6$, $5x10^5$, $2.5x10^5$, $5x10^4$ and $2.5x10^4$ cells/ml, serving as the effector cells. Subsequently, the effector cells and the target cells were mixed with ratios (E:T) of 20:1, 10:1, 5:1, 1:1 or 1:2 in triplicate. The cell mixtures were cultured in a humidified incubator at 37˚C and 5% CO_2 for 24 h. The killing activities were detected by the CCK-8 kit and Thermo MK3 microplate reader at 450 nm.

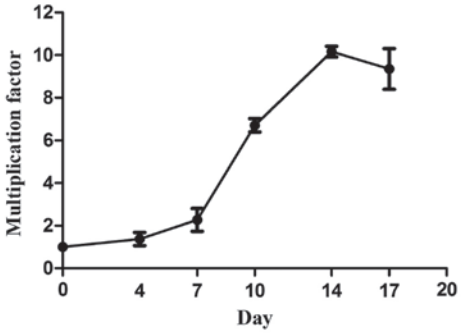

Figure 1. Proliferation rates of γδ T cells.

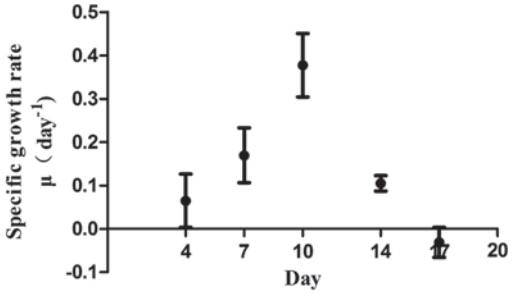

Figure 2. Specific growth rates of γδ T cells.

Statistical analysis. Data were analyzed by SPSS 19.0 (IBM, Corp., Armonk, NY, USA) using a pair-matching t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

In vitro growth of γδ T cells at different time-points. The γδ T cells cultured in OpTmizer medium were counted on days 0, 4, 7, 10, 14 and 17 by an automated cell counter. On days 7-14, the cells entered the exponential phase. On day 14, the maximum multiplication factor (10.146±0.252) was observed. The proliferation rate decreased following day 14. Specific growth rate μ reached a maximum (0.377±0.073) on day 10 and subsequently decreased. On day 17, a negative specific growth rate was observed (Figs. 1 and 2).

Phenotypes of γδ T cells on different days. The γδ T cells were harvested on days 7, 10, 14 and 17. Phenotypes of these cells were analyzed by the C6 cytometer. High percentages (61.2±0.052 to 67.4±0.055%) of γδ T cells were acquired at these points in time, without a significant difference (Figs. 3 and 4).

Cytokines secreted by γδ T cells at different points in time. The γδ T cells were harvested on days 7, 10, 14 and 17. The secretion of IFN-γ and TNF-α by the cells was analyzed by the C6 cytometer. The amount of IFN-γ and TNF-α increased gradually from day 7 to 14 and reached the peak

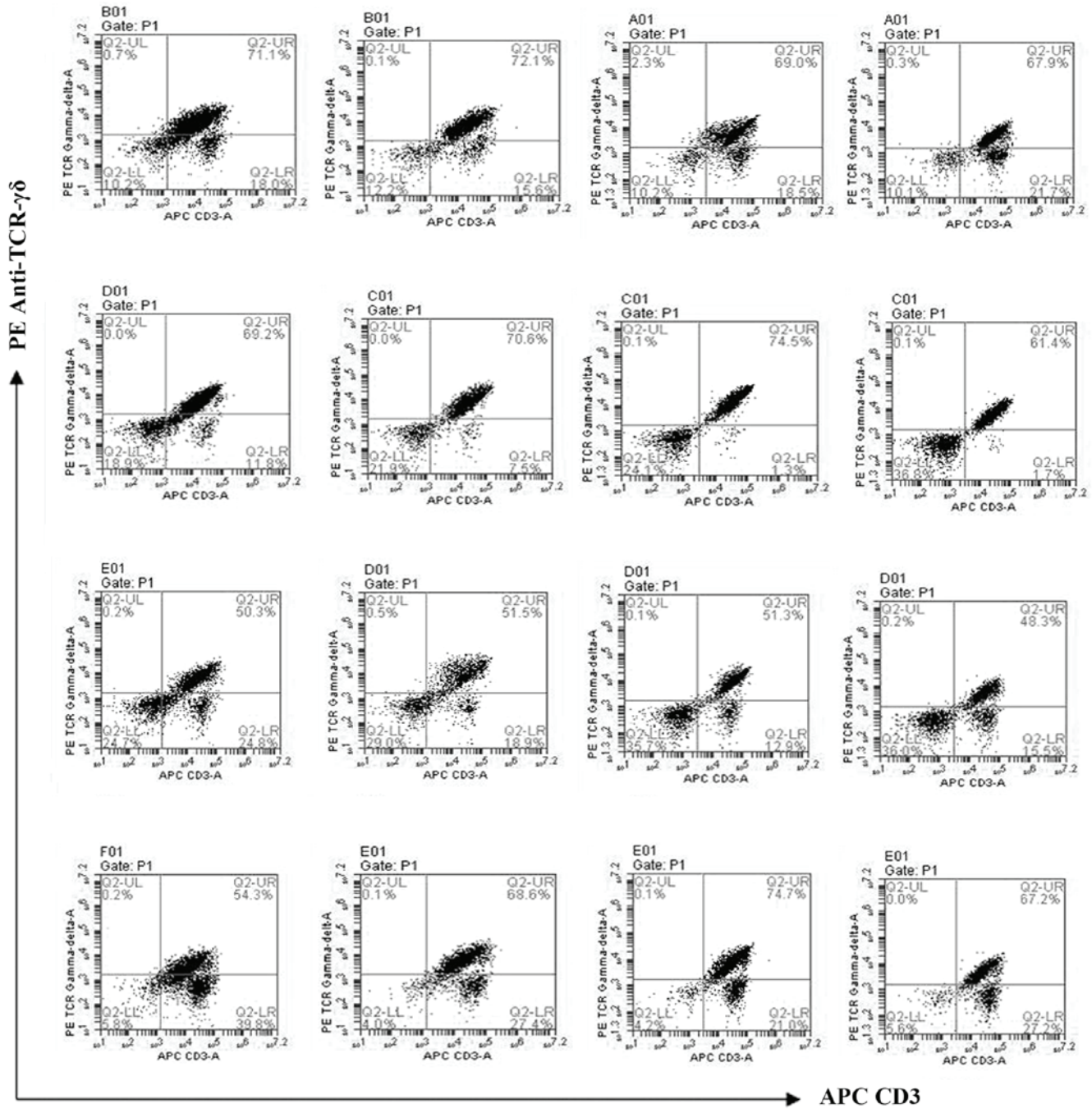

Figure 3. Phenotype of γδ T cells.

on day 14. Following this, IFN-γ and TNF-α decreased gradually. Compared with day 7, the percentages of IFN-γ and TNF-α secreted by the γδ T cells were significantly higher ($P<0.05$) (Figs. 5 and 6).

Killing activities of γδ T cells at different points in time. The γδ T cells were harvested on days 7, 10, 14 and 17. Subsequently, they were mixed with SGC-7901 with the ratios of 20:1, 10:1, 5:1, 1:1, or 1:2, and the killing activities of the γδ T cells were analyzed. The results showed that the killing activities of γδ T cells harvested on days 7, 10 and 14 were efficient; however, there was no significant difference ($P>0.05$). On day 17, the killing activities of the γδ T cells against SGC-7901 with the ratios of 10:1 and 5:1 decreased. On days 7, 10 and 14, the killing activities of γδ T cells with the ratios of 20:1, 10:1, 5:1 and 1:1 were higher than that of the cells with the ratios of 1:1 and 1:2. On day 17, the killing activities of γδ T cells with the ratios of 20:1 and 10:1 were higher than that of the cells with the ratios of 5:1, 1:1 and 1:2 (Fig. 7; Tables I and II).

Discussion

Since the discovery of the γ receptor and δ receptor of the T cells in the 1980s, the roles of γδ T cells in immunoregulation and immunosurveillance have been identified (5,6). Viey *et al* (7)

Table I. Killing activity of γδ T cells cultured with different E:T ratios to SGC-7901 cells on different days.

E:T ratio	n	Day 7	Day 10	Day 14	Day 17
20:1	4	91.7±4.9[a,b]	94.6±1.1[a,b]	97.0±1.5[a-c]	90.7±1.6[a-c]
10:1	4	93.8±1.9[a,b]	96.9±1.6[a,b]	90.8±3.4[a,b]	80.9±2.5[a-f]
5:1	4	92.7±2.9[a,b]	91.9±3.6[a,b]	83.1±4.2[a,b]	73.1±2.4[d,e]
1:1	4	73.8±2.7	70.1±2.0	68.4±2.0	67.9±1.7
1:2	4	66.3±2.3	63.3±1.6	65.5±2.2	60.9±2.7

P<0.05 [a]vs.1:1; [b]vs.1:2; [c]vs. 5:1; [d]vs. day 7; [e]vs. day 10; [f]vs. day 14. Data are mean % ± standard deviation. E:T, effector cell to target cell.

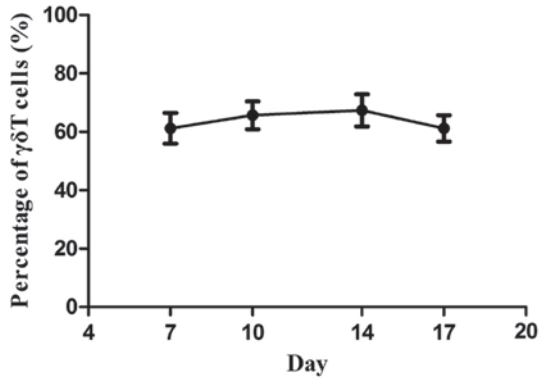

Figure 4. Percentage of γδ T cells.

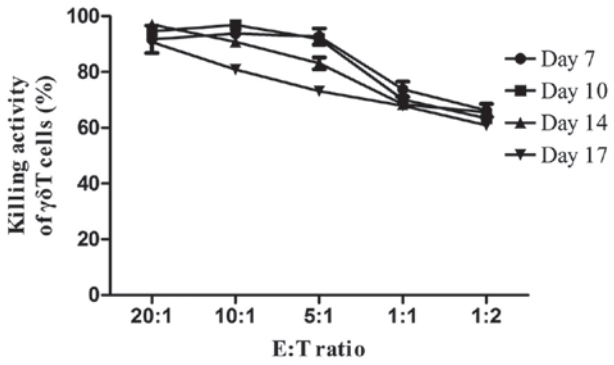

Figure 7. Killing activity of γδ T cells cultured in different days to SGC-7901 cells with different effector cell to target cell (E:T) ratios.

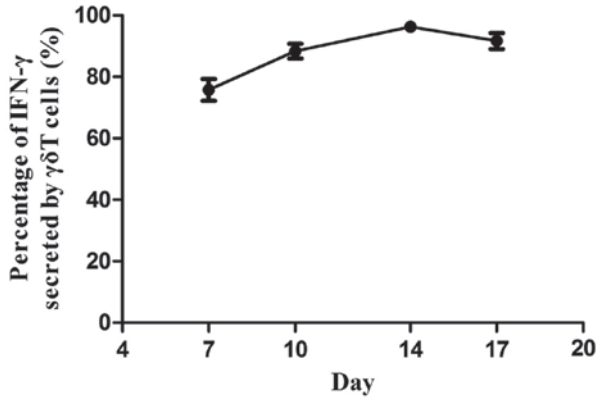

Figure 5. Percentage of interferon (IFN)-γ secreted by γδ T cells.

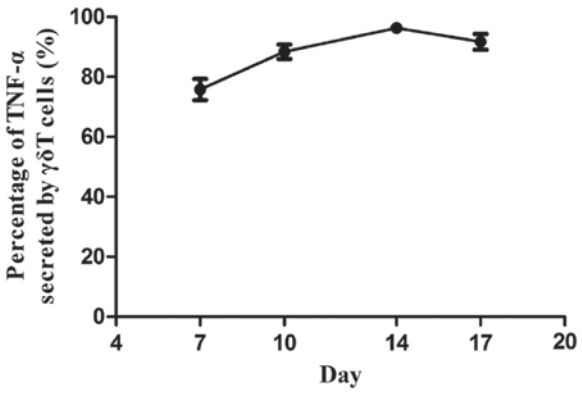

Figure 6. Percentage of tumor necrosis factor (TNF)-α secreted by γδ T cells.

found that the cultured γδ T cells were able to kill tumor cells. Currently, research of γδ T cells in the immunotherapy against tumors has increased (8-10). However, the biological features of *in vitro* cultured γδ T cells are rarely reported. In addition, understanding the state, phenotypes, secreted cytokines and killing activities will provide information for further study and clinical treatment.

Specific growth rate (μ) is one important index that indicates the dynamics of cell growth (11). In the present study, cell number reached the peak on day 14. However, the maximum specific growth rate (μmax) was achieved on day 10. This indicated that although the total number of the cells increased, the growth rate decreased after day 10. On day 17, the specific growth rate (μ) became negative and the total number of γδ T cells began to decrease.

The main effector cells of γδ T cells are the cells with the phenotype of $CD3^+TCR\gamma\delta^+$. These cells can express multiple cytokines following antigen stimulation, particularly Th1 cytokines (12), such as IFN-γ and TNF-α. In the present study, cytokines were analyzed by the C6 cytometer on different days. High percentages of γδ T cells were acquired on days 7, 10, 14 and 17 without a significant difference. Cytokines were also analyzed by the C6 cytometer, and the quantities of the cytokines secreted by the γδ T cells gradually increased from days 7 to 14 and reached the peak on day 14. Following this the quantities of the cytokines decreased.

To study the killing activities of the cells *in vitro*, γδ T cells that were cultured for different days were mixed with SGC-7901 cells in different ratios. Compared with the

cells cultured for 17 days, the γδ T cells that were in the exponential phase (days 7 to 14) showed higher killing activities. A more efficient effect occurred when the ratio was >5:1, which indicated that reinfused γδ T cells should be increased if possible.

In conclusion, it is suitable to reinfuse the γδ T cells in their exponential phase (days 7 to 14). The optimum time for reinfusion is ~day 10 and the ratio should be >5:1.

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