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Original Article

Inhibitory effect of LY294002 on CD3mAb-activated T cells and Mtb-Ag-activated γδT cells via TCR signal transduction pathway

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Abstract: Objective: This study aimed to observe the effects of phosphatidylinositol 3 kinase (PI3K) specific inhibitor LY294002 on CD3 monoclonal antibody (CD3mAb) activated T cells and Mtb-Ag activated γδT cells, and to investigate the function of PI3K in TCR signal transduction pathway. Methods: Healthy human Peripheral blood mononuclear cell (PBMC) was isolated. CD3mAb, phorbol esters (PMA), ionomycin (IM), and Mtb-Ag were used to stimulate PBMC. Flow cytometry was used to detect the expression of CD69 in $CD3^+$ T cells and γδT cells at different time. PBMC were pretreated by LY294002, followed by double staining with CD3PE/CD69 FITC and γδPE/CD69 FITC. The CD69 was detected in $CD3^+$ T cells and in γδT cells. The total number of cells cultured for 10 days was counted. Results: After stimulation by CD3mAb for 24 h, the expression of CD69 in both $CD3^+$ T cells and γδT cells was both increased to about 56%. After stimulation by PMA and IM for 6 h, the expression of CD69 in both $CD3^+$ T cells and γδT cells was increased to about 99%. After stimulation by Mtb-Ag for 24 h, the expression of CD69 in $CD3^+$ T cells was increased to 16.0%, while 75.2% in γδT cells .The expression of CD69 in Mtb-Ag activated γδT cells and proliferation of γδT cells were obviously inhibited after pretreatment with LY294002. Conclusion: γδT cells are specifically activated by Mtb-Ag, and its activation depends on TCR pathway. The activation effects of CD3mAb on T cells and Mtb-Ag on γδT cells are both inhibited by LY294002.

Keywords: γδT cells, CD69 molecules, signal transduction, LY294002, CD3mAb, PMA, IM, Mtb-Ag

Introduction

There have been many studies on the immunological functions of cells, but it remains quite few for the mechanism by which cells develop the immunological functions, especially for the signal transduction pathways about cells activation by antigen stimulation. The existence of antigen presenting cells (APC) is essential for Mtb-Ag, but the absorption, processing and presenting of APC is not essential [1]. In the current study, we studied the inhibitory effects of a selective PI3K inhibitor (LY294002) on the CD3mAb-induced-T cells activation and Mtb-Ab-induced- T cells activation. We also investigated the function of PI3K in TCR signal pathways involved in the activation of CD3mAb-induced-T cells and Mtb-Ab-induced-T cells.

Materials and methods

Reagents and instruments

All the following reagents were obtained from commercial sources. lymphocytes separation medium (Chinese Academy of Medical Sciences Institute of Hematology), new born calf serum (Hangzhou Sijiqing Biological Company), cell culture medium RPMI 1640 (GIBCO, USA), CD3mAb (DACO, Denmark), PMA (Sigma, USA, SNP1585), IM (Sigma, USA, SNI0643), Mtb-Ag (kindly gifted by Dr. Henry Boom from School of

Medicine, Case Western Reserve University, USA), IL-2 (kindly gifted by professor Tian Zhigang, Shandong Academy of Medical Sciences), Anti-TCRγδPE (Becton Dickinson Company, USA, SN3437907), Anti-CD69 FITC (Ancell Company, USA, SN819010), anti-CD3 PE (Ancell Company, USA, SN9734008), anti-IL-2 FITC (Ancell Company, USA, SN615015), and LY294002 (Sigma, USA, SNL9908).

Instruments for this study included optical microscope (Olympus BH, Japan), inverted microscope (Wuguang, WJ12-50, XSB-14), CO_2 incubator (Harris hw0301T-VBA, USA), Flow cytometry (Coulter EPICS^R XL-MCL, Beckman-counter Company, USA), cell culture plate (Falcon Company, USA), refrigerated centrifuge (Heraeus 400R, Germany), and low temperature refrigerator (SANYO ULTRA LOW NDF-382, Japan).

PBMC isolation

Five milliliter of peripheral blood was acquired from five healthy volunteers. PBMCs were isolated by the use of lymphocyte separation medium and were cultured with RPMI1640, with the final concentration of $1\sim2\times10^6$/ml.

CD69 expression in $CD3^+$ T cells and γδT cells

Cells were added into the 24-well culture plate, followed by the addition of the stimulating reagents, including PMA (20 ng/ml) + IM (1 μg/ml), CD3mAb (5 μg/ml) and Mtb-Ag (5 μg/ml). Cells were collected after being cultured for 0, 6, 12, 24, 48 and 72 h. CD69 expressions were measured after being cultured at different time.

Effects of LY294002 on the CD69 expression in $CD3^+$ T cells and γδT cells

PBMC was added into 24-well culture plate. The different concentrations of specific signal inhibitor LY294002 (0, 0.4, 2 and 10 μM) were also added into 24-well plate. After being cultured for 30 min, CD3mAb (5 μg/ml) was added into $CD3^+$ T cells and Mtb-Ag (5 μg/ml) was added into γδT cells. Cells were collected after 24 h and stained by CD3PE/CD69 FITC and γδPE/CD69 FITC. CD69 expression in $CD3^+$ T cells and γδT cells was analyzed and cells treated with PMA (20 ng/ml) + IM (1 μg/ml) at the corresponding time points were also analyzed as the controls.

Effects of LY294002 on IL-2 expression in CD3mAb-activated-T cells

PBMCs were added into 24-well culture plates. The different concentrations of LY294002 (0.4, 2 and 10 μM) were also added into 24-well plate. After being cultured for 30 min, CD3mAb (5 μg/ml) and PMA (20 ng/ml)+ IM (1 μg/ml) were added into 24-well plate, respectively. Cell culture supernatant was collected and EIA Reagent Kits were used to analyze IL-2 production.

Effects of LY294002 on cell proliferation

LY294002 affected the proliferation of CD3mAb-activated-PBMC. PBMC was added into 24-well culture plate. The different concentrations of LY294002 (0, 0.4, 2 and 10 μM) were also added into 24-well plate. After being cultured for 30 min, CD3mAb (5 μg/ml) was added. IL-2 (50 u/ml) was added into the cell cultures every 3 d. Cells were collected after 10 d and counted. To verify whether LY294002 affected the proliferation of Mtb-Ag-activated-γδT cells, PBMCs were added into 24-well culture plate with the addition of the LY294002 with different concentrations (0, 0.4, 2 and 10 μM) respectively. Then Mtb-Ag (5 μg/ml) was added into the culture 30 min later. Every 3 d, IL-2 (50 u/ml) was added into the cell cultures. Cells were collected and counted after 10 d. The proportion of γδT cells was analyzed by γδPE single staining and counted for absolute number.

Flow cytometry analysis

Argon ion laser wavelength was 488 nm in flow cytometry and FSC/SSC was used for lymphocytes analysis. MinMDI Version 2.8 was used for data analysis.

Statistical analysis

Data are expressed as the mean ± SEM or raw numbers. The normality of the variables was assessed using the Shapiro-Wilk test. For data that were normally distributed, one-way analysis of variance (ANOVA) and the LSD post hoc multiple comparisons test were applied. The Kruskal-Wallis test and the Mann-Whitney U test were performed to compare data that were not normally distributed. The chi-square test was employed to compare expression rate of the cells. All data were processed by SPSS soft-

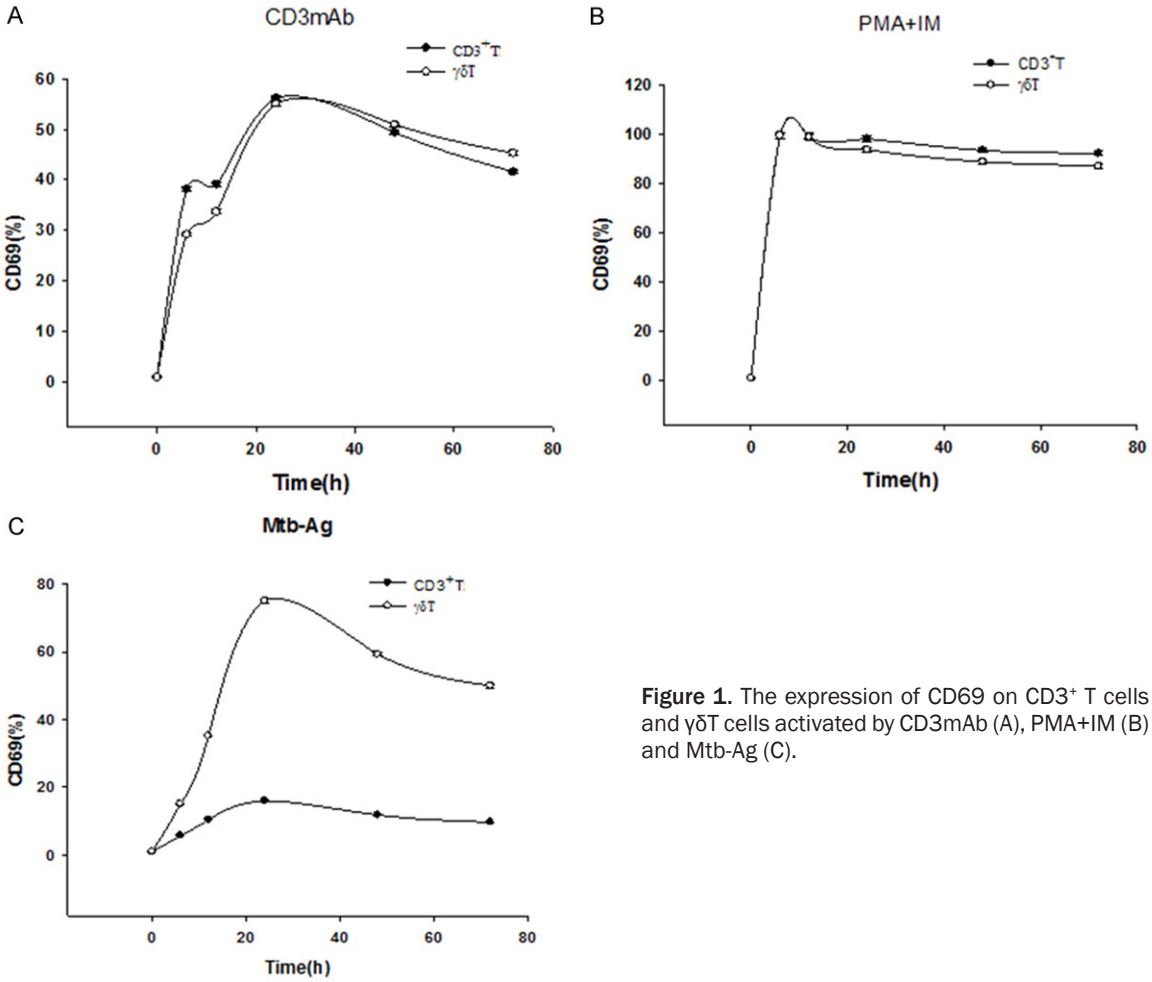

Figure 1. The expression of CD69 on $CD3^+$ T cells and γδT cells activated by CD3mAb (A), PMA+IM (B) and Mtb-Ag (C).

ware package for Windows version 13.0 (SPSS, Inc, Chicago, USA), All statistical tests were two-sided, and statistical significance was defined as $P<0.05$.

Results

CD69 expression in $CD3^+$ T cells and in γδT cells

At 0, 6, 12, 24, 48 and 72 h, CD69 expressions in $CD3^+$ T cells were analyzed, yielding 1%, 32.8%, 39.1%, 56.2%, 49.4% and 41.6% in CD3mAb stimulating group, 1%, 99.5%, 99.1%, 98%, 93.3% and 92.1% in PMA+ IM group, and 1%, 5.7%, 10.4%, 16%, 11.8% and 9.7% in Mtb-Ag group. CD69 expressions in γδT cells were analyzed, yielding 0.91%, 29.2%, 33.7%, 55.1%, 51.0% and 45.3% in CD3mAb stimulating group, 0.91%, 99.3%, 98.6%, 93.6%, 88.7% and 87.0% in PMA+ IM group, and 0.91%, 15.1%, 35.2%, 75.2%, 59.4% and 50.0% in Mtb-Ag group (**Figure 1**). $CD3^+$ T cells and γδT cells were activated rapidly after being stimulated by PMA+ IM and CD69 expression reached the peak within 6 h. The activation of $CD3^+$ T cells and γδT cells were slower in CD3mAb and Mtb-Ag stimulating than in PMA+ IM stimulating. And CD69 expression reached the peak at 24 h.

Inhibitory effects of LY294002 on T cells activation by CD3mAb

The effects of LY294002 on the CD69 expression in CD3mAb-activated-T cells were also observed. In flow cytometry analysis, CD69 expression in CD3mAb stimulating group was 52%, 46%, 33% and 19% by the use of LY294002 (0, 0.4, 2 and 10 μM). LY294002 showed significant inhibitory function, as showed in **Figure 2**. Whereas LY294002 showed no inhibitory function in PMA+ IM group and CD69 expression was more than 99%.

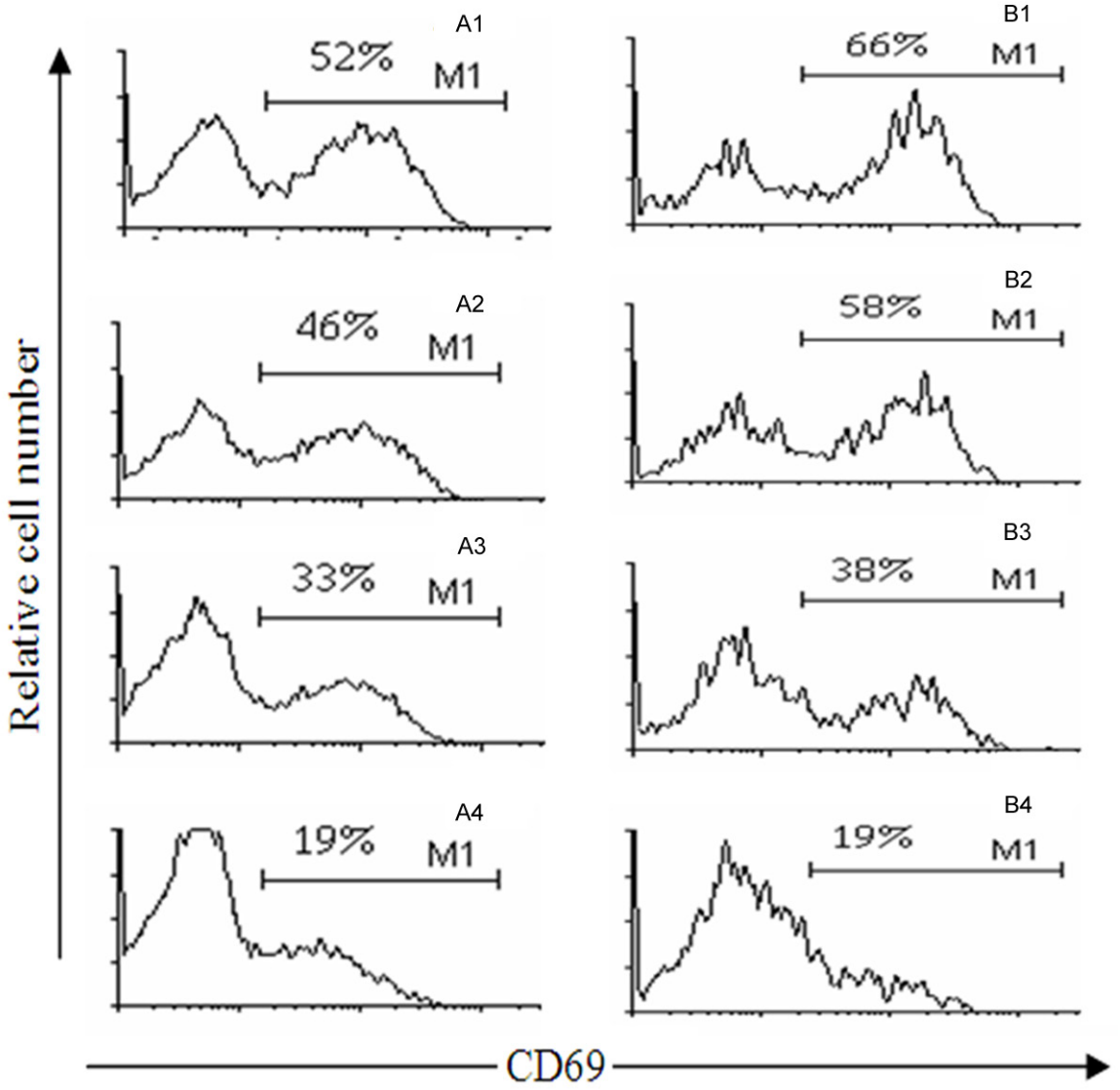

Figure 2. The inhibitory effects of LY294002 on CD69 expression of CD3mAb activated T lymphocytes and Mtb activated γδT cells.

We further investigated the inhibitory effects of LY294002 on γδT cells activation after Mtb-Ag stimulation and CD69 expression in Mtb-Ag-activated-γδT cells. With the presence of LY294002 (0, 0.4, 2 and 10 μM), CD69 expressions in γδT cells was 66%, 58%, 38% and 19% respectively, as shown in **Figure 2** (B1, B2, B3 and B4), while LY294002 expressed no inhibitory function in PMA+ IM group with the expression of CD69 was more than 99%.

Effects of LY294002 on IL-2 expression in CD3mAb-activated-T cells

IL-2 expression in CD3mAb stimulating group was 561 pg/ml, 477 pg/ml, 280 pg/ml and 36 pg/ml respectively after intervention by LY294002 (0, 0.4, 2 and 10 μM), whereas IL-2 expression in PMA+ IM group was 1,922 pg/ml, 1,928 pg/ml, 1,938 pg/ml and 1,915 pg/ml respectively. The results showed that IL-2 secretion following T cells activation through TCR pathway may be dependent on PI3K activation and not dependent on PMA+ IM stimulating activation (**Figure 3**).

Effects of LY294002 on T cells proliferation activated by CD3mAb

After the action of LY294002 (0, 0.4, 2 and 10 μM), the total number of cells reached 35.6×10^6, 31.1×10^6, 18.7×10^6 and 7.0×10^6

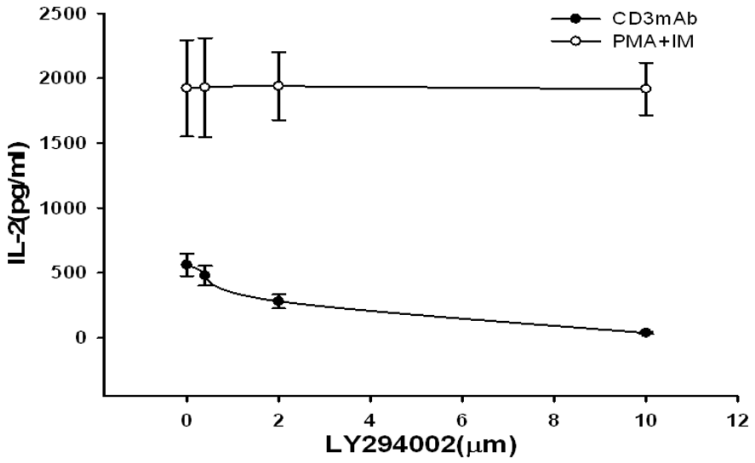

Figure 3. The inhibitory effect of LY294002 on T cells in IL-2 expression.

respectively (**Figure 4**). The effect of LY294002 on γδT cells proliferation after Mtb-Ag activation was also observed. With the presence of LY294002 (0 μM, 0.4 μM, 2 μM and 10 μM), the total cell number reached 9.8×10^6, 8.2×10^6, 4.0×10^6 and 1.9×10^6. γδT cells reached 7.03×10^6, 4.72×10^6, 1.26×10^6 and 0.17×10^6. The proportion of γδT cells to control was 71.8%, 57.6%, 31.7% and 9.1% (**Figure 4**).

Discussion

γδT cells were identified to be subsets of T lymphocytes in 1986. The proportion of γδT cells in adults peripheral blood is 0.5~5%. And γδT cells mainly exist in mucous membrane and subcutaneous tissues, for example, the proportion of γδT cells in human small intestine intraepithelial lymphocytes (IEL) is 10~18%; while the proportion of γδT cells in human large intestine IEL is 25~37%. The proportion of γδT cells in mice IEL is 50%. Mucous membrane and epithelial tissues have been proved to be the first defense to fight against pathogen invasion. Furthermore, carcinogenesis was often found in mucous membrane and epithelial tissues. Therefore, the high proportion of γδT cells existence in mucous membrane and epithelial tissues suggest that these cells are pivotal in anti-microorganism immunology, anti-parasite immunology and tumor immunology [2].

Our results showed γδT cells were activated by Mtb-Ag specifically. The proportion of γδT cells in newly isolated PBMC was 4.5~4.9%. After being stimulated by CD3mAb and cultured for 10 days. The proportion of γδT cells in PBMC turned to 1.37%. In contrast, after being activated by Mtb-Ag and cultured for 10 days, the proportion of γδT cells in PBMCs increased to 69.2~72.2% [1]. The results showed that Mtb-Ag could activate and promote the proliferation of the cells. PBMC was stimulated by Mtb-Ag and IL-2 was supplied for cell culture. In this way, a large amount of γδT cells were acquired rapidly and easily. After T cells activation, we also observed the gene expressions including instant gene expression (15~30 min after stimulation), early stage gene expression (30 min~48 h after stimulation) and later stage gene expression (2~14 d after stimulation). The activated markers on the surface of T cells included representative IL-2 receptor CD25, transferrin receptor CD71 and CD69. All these activating markerswere used as T cells activation markers in previous studies [1]. CD69 expression was found in the earliest stage and CD69 was named as activating induced molecules. CD69 was observed after 2 h of T cells activation, but was not detected in silent T cells. In contrast, CD3mAb activated cells through TCR-CD3 complex was slow and weak. Mtb-Ag also stimulated PBMC through TCR-CD3 complex. There was an obvious difference between the activation of total T cells and γδT cells. The expression ratio of CD69 in total T cells was 16%, while it was 75.2% in γδT cells. CD69 expression in both total T cells and γδT cells reached the peak at 24 h. Based on these results, Mtb-Ag acted as the selective stimulator for γδT cells, and the peak activation effect on CD69 were detected after 24 h.

LY294002 function of T cells and γδT cells was mediated by TCR signal transduction pathway. PI3K was proved to be an intracellular phosphatidyl inositol kinase by Sugimoto and Macara in1984 and constituted of one catalytic subunit (110 KD, p110) and one regulatory subunit (85 KD, p85). Amino terminal of p85 contains one SH3 structural domain and one proline rich domain which can combine with SH3 structural domain. Carboxyl terminal of p85 contains two SH2 structural domains and

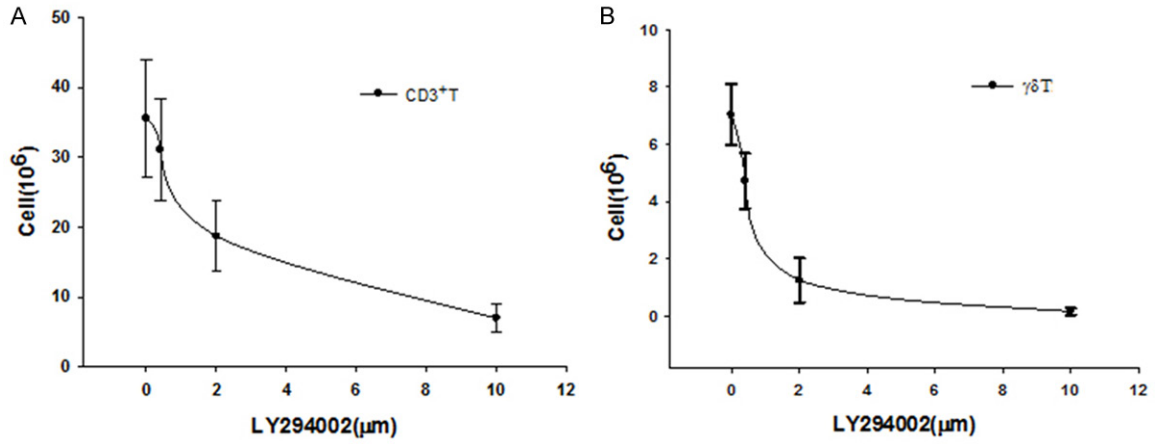

Figure 4. The inhibitory effect of LY294002 on the proliferation of CD3mAb activated T cells (A) and Mtb-Ag activated γδ Tcells (B).

one domain which can combine with p110. P110 has the isogenesis with protein kinase, and has the activity of Ser/Thr kinase and phosphatidyl inositol kinase. PI3K can specifically phosphorylate the three hydroxyl of inositol cycle, and produce the second messenger of 3,4-diphosphate phosphatidyl inositol (PI-3,4-P_2) and 3,4,5-trisphosphate phosphatidyl inositol (PI-3,4,5-P_3). PI3K may act as an important signal transduction molecule because its specific structure and function. In recent studies, PI3K was proved to play critical role in TCR/CD3 activating signal and IL-2/IL-2R signal transduction. PI3K can participate in the activation of γδT cells by the way of activating protein kinase B (PKB), Rac and Ca^{2+} pathways, and mainly participate in the CD28 mediated signal transduction [1]. T-cell co-stimulation through the CD2 and CD28 co-receptors induces distinct signalling responses. Laird RM reported that CD28 molecule could be detected in 40%~60% γδT cells [1]. LY294002 was a selective inhibitor for PI3K, and inactivates the PI3K through competitively preventing the combination of ATP and p110 [3]. In our studies, we used CD3mAb to stimulate T cells, and we found that PI3K was absolutely necessary for T cells activation, IL-2 production and cells proliferation. We also used LY294002 to inhibit the action of PI3K, and we found that T cells activation, CD69 expression, IL-2 production and cells proliferation were all down-regulated by the use of different concentrations of LY294002. After being stimulated by PMA+IM, IL-2 production was not affected because T cells phosphatidyl inositol pathway was activated from the downstream of PI3K pathways. The similar results were discovered in the Mtb-Ag-stimulated γδT cells. CD69 expression and cells proliferation was dependent on the activity of PI3K. Generally speaking, PI3K pathways played an important role in the TCR signal transduction of T cells and γδT cells. PI3K was a key signal molecule for activation of T cells and γδT cells, while the mechanism involved in these actions still need further research.

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Disclosure of conflict of interest

None.

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