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Original Article

Inhibitory effect of the mitogen activated protein kinase specific inhibitor PD98059 on Mtb-Ag-activated γδT cells

Keqiang Wang[1], Yanqiang Hou[2], Chenxing Gu[3], Dapeng Zhao[1], Yanchao Duan[4], Zhangshen Ran[5], Qinghua Li[6], Xiangqi Li[7]

Departments of [1]Clinical Laboratory, [4]Hematology, [5]Medical Examination Center, [6]Burn and Plastic, [7]Breast Surgery, The Affiliated Hospital of Taishan Medical University, Tai'an, Shandong Province, China; [2]Department of Clinical Laboratory, The First People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China; [3]Department of Cardiology, The First Affiliated Hospital of General Hospital of Chinese PLA, Beijing, China

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Abstract: Objective: This study aimed to observe the inhibitory effects of mitogen activated protein kinase (MAPK/ERK) specific inhibitor PD98059 on Mtb-Ag activated γδT cells and to investigate the role of MAPK in MAPK/ERK pathway of γδT cells activated by Mtb-Ag. Methods: Healthy human peripheral blood mononuclear cell (PBMC) was isolated from the peripheral blood, and then stimulated by phorbol esters (PMA), ionomycin (IM) and Mtb-Ag. PBMC of the experimental group was pretreated by PD98059 of 0, 1, 10 or 100 μmol/L while the control group was given no pretreatment. Flow cytometry was applied to detect the expression of CD69 in γδT cells at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h after treatment respectively. The influence of PD98059 on the amplification of γδT cells was also detected. Results: After stimulated by PMA and IM for 6 hours, the expression of CD69 in γδT reached the peak of (99.3±1.09)%, while in the Mtb-Ag stimulation group, it reached the highest level of 75.2% at 24 hours. The level of CD69 in two group was significant difference between the two groups at each time respectively ($P<0.05$). Pretreated with PD98059 of 0, 1, 10, 100 μmol/L, the level of CD 69 expression in Mtb-Ag stimulated γδT was 79±0.8%, 75±0.7%, 54±0.5% and 17±0.2% respectively; In PMA+IM stimulated group, CD69 expression were all more than 98% in different concentration of PD98059. After treated with PD98059 of 0, 1, 10, 100 μmol/L, total cell number increased from 1.5×10^6 to $(10.3\pm2.5)\times10^6$, $(9.5\pm2.1)\times10^6$, $(5.8\pm1.8)\times10^6$ and $(2.1\pm0.5)\times10^6$ respectively. Number of γδT cells reached to $(6.2\pm0.9)\times10^6$, $(5.02\pm0.8)\times10^6$, $(2.05\pm0.5)\times10^6$ and $(0.41\pm0.1)\times10^6$ respectively. Conclusion: γδT cells were specifically activated by Mtb-Ag, and the activation was depended on MAPK/ERK pathway. The activation effects of Mtb-Ag on γδT cells can be obviously inhibited by PD98059.

Keywords: γδT cells, CD69 molecules, PD98059, CD3mAb, PMA, IM, Mtb-Ag, mitogen-activated protein kinase (MAPK)

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. γδT cells are a subpopulation of T lymphocytes discovered in 1986, accounting for about 0.5-5% of the whole peripheral lymphocytes of adults. T cells chiefly distributed in mucosa and subcutaneous tissues: such as in human intra-epithelial lymphocyte (IEL) of 10-18%, human large intestine IEL of 25-37% and small mice IEL of 50%. Mucosa and subcutaneous tissues are the first defense for the prevention of pathogens invasion, and tumors are often observed in these tissues. The high portion of distribution of T cells in mucosa and subcutaneous tissues indicated that the probable important role of T cells in anti-microbiology and anti-tumors. There have been many studies on the immunological functions of cells, but the investigations on the mechanism by which cells develop the immunological functions are remained few, especially in 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 for Mtb-Ag [1]. Mitogen-activated protein kinase (MAPK) is a type of intra-cellular serine/threonine protein kinase. MAPK signal transduction pathway was proved to be existed in most cells and played an important role in transduction of extracellular stimulating signals to intra cells, and initiation of cellular biological reactions, such as proliferation, differentiation, transformation and apoptosis. MAPK signal transduction pathway included extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, (JNK)/SAPK, and p38MAPK signal transduction pathways [2]. ERK pathways included Ras-Raf-MEK-Erk [3]. PD98059 is a specific inhibitor for MEK pathway [4], and the combination of PD98059 and MEK prevent the phosphorylation and activation of MEK by -Raf, and then ERK pathway was inhibited. In this study, we aimed to study the effects of the mitogen activated protein kinase (MAPK/ERK) specific inhibitor PD98059 on Mtb-Ag activated γδT cells, and to investigate the role of MAPK in MAPK/ERK pathway of γδT cells activated by Mtb-Ag.

Materials and methods

Reagents and instruments

All the following reagents were obtained from commercial source respectively. 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 Zhi-gang, Shandong Academy of Medical Sciences), Anti-TCRγδPE (Becton Dickinson Company, USA, SN3437907), Anti-CD69-FITC (Ancell Company, USA, SN819010), anti-CD3PE (Ancell Company, USA, SN9734008), anti-IL-2FITC (Ancell Company, USA, SN6150-15), PD98059 (Germany Calbiochem, 513000).

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), 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 PD98059 on the CD69 expression in γδT cells

PBMC was added into 24-well culture plate, followed by the addition of different concentrations of specific signal inhibitor PD98059 (0 μM, 0.4 μM, 2 μM and 10 μM). After being cultured for 30 min, Mtb-Ag (5 μg/ml) was added into γδT cells. IL-2 50 u/ml was added into cells every 3 days. Cells were collected after 10 days and counted the numbers of all cells. γδPE single stain was performed to observe the proportion of γδT cells and the absolute number of γδT cells were counted.

Effects of PD98059 on the proliferation of γδT cells induced by Mtb-Ag

One milliliter cell cultures of each of the five were added into 24-well culture plate respectively. The different concentrations of PD98059 (0 μM, 1 μM, 10 μM and 100 μM) were also added into 24-well plate. After being cultured for 30 min in 37°C, Mtb-Ag (5 mg/L) was added for further culturing, then IL-2 with concentration of 50 u/ml was added into the former cultures. Cells were collected after 10 days followed by the calculation of cell numbers. The proportion of γδT cells was analyzed by γδPE single staining, and the absolute number of γδT cells was also counted.

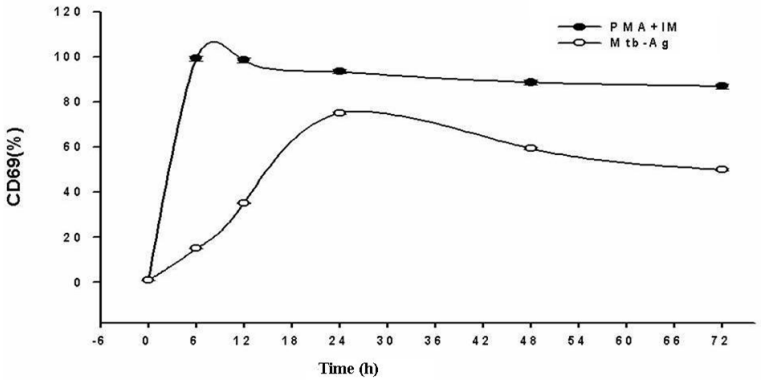

Figure 1. The expression of CD69 in γδT cells by activating of PMA+IM and Mtb-Ag ($\bar{x}\pm s$)% (n=5).

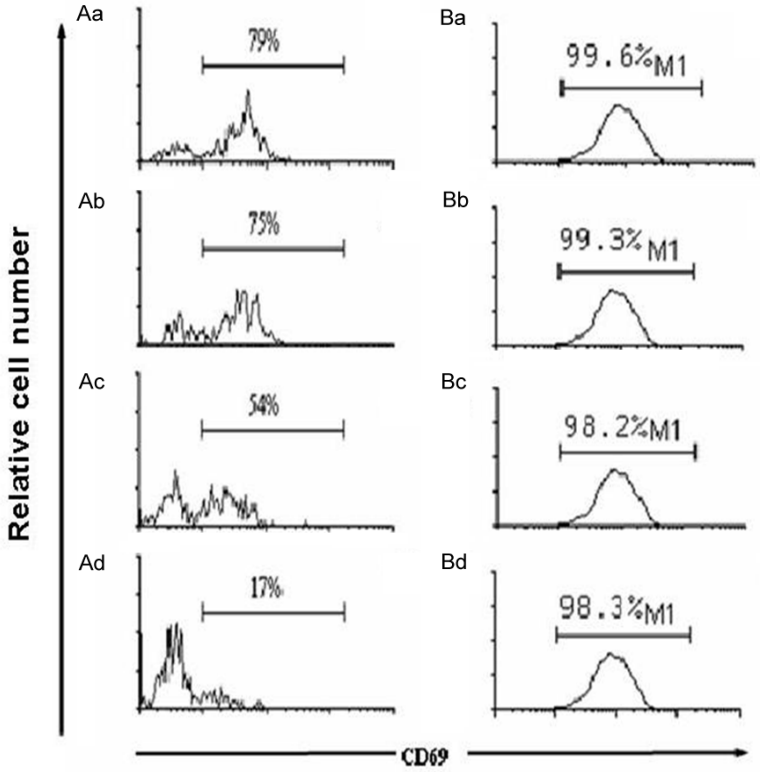

Figure 2. The effects of PD98059 on the expression of CD69 in the activation of γδT cells induced by Mtb-Ag and PMA+IM. Mtb-Ag: (Aa-d); PMA+IM: (Ba-d). PD98059 (μmol/L): 0 (Aa, Ba); 1 (Ab, Bb); 10 (Ac, Bc); 100 (Ad, Bd).

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 software 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 expressions in γδT cells of PBMC

CD69 expression almost could not be observed without PBMC stimulation With the average level about (0.91± 0.06)% of γδT cells. CD69 expression in γδT cells after different extracellular stimulations for 6 to 72 hours of PBMC were displayed in **Figure 1**. γδT cells rapidly activated after being stimulated by PMA and IM., and CD69 expression reached the peak less than 6 hours. Activation changes of γδT cells stimulated by Mtb-Ag was slower than stimulating by PMA and IM, and CD69 expression reached the peak after about 24 hours stimulated by Mtb-Ag.

Effects of PD98059 on the expression of CD69 in the activation of γδT cells induced by Mtb-Ag

After the treatment by different concentrations of PD98059 (0 μM, 1 μM, 10 μM and 100 μM), CD69 expressions in γδT cells was up to (79±0.8)%, (75±0.7)%, (54±0.5)% and (17± 0.2)% respectively (**Figure 2**), while PD98059 showed no inhibitory function in PMA+IM group with CD69 expression was more than 98%.

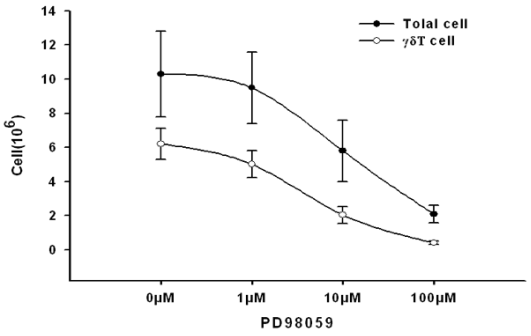

Figure 3. The inhibitory effects of PD98059 on the proliferation of γδT cells induced by Mtb-Ag.

Effects of PD98059 on the activation of γδT cells induced by Mtb-Ag

After treatment by PD98059 (0 μM, 1 μM, 10 μM and 100 μM), the total number of cells reached to (10.3±2.5)×10^6, (9.5±2.1)×10^6, (5.8±1.8)×10^6 and (2.1±0.5)×10^6 respectively. The proportion of γδT cells was (60.2±0.5)%, (52.8±0.4)%, (35.2±0.2)% and (19.6±0.1)%. Total number of γδT cells reached (6.2±0.9)×10^6, (5.02±0.8)×10^6, (2.05±0.5)×10^6 and (0.41±0.1)×10^6 respectively. PD98059 significantly inhibited the activation of γδT cells induced by Mtb-Ag (**Figure 3**).

Discussion

γδT cells were identified to be subsets of T lymphocytes The proportion of γδT cells in newly isolated blood PBMC is 4.5-4.9% in our former studies. 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%, and reached to 99.3% after immunomagnetic positive sorting [5]. The results showed that Mtb-Ag had the characteristics of typically activating and proliferating the γδT cells. And then, Mtb-Ag stimulating PBMC and IL-2 maintaining cell proliferation could make lots of γδT cells. It could be an easy and rapid method for γδT cells proliferation. CD69 also called activating induced molecules. CD69 can be detected after 2 hours of T cells activation, but cannot be detected in silencing T cells. There was a big difference between total T cells and γδT cells after activation by PBMC through Mtb-Ag. CD69 expression is low (69%) in total T cells, but high (75.2%) in γδT cells. CD69 expression reached the peak in both total T cells and γδT cells [6]. Mtb-Ag is a specific activator for γδT cells, and the highest peak of CD69 expression is about 24 hours after stimulating.

Currently, three MAPK signal transduction pathways were proved to be existed in mammalian cells, including ERK, JNK/SAPK and p38MAPK pathways. ERK pathway was firstly reported in 1986 by Sturgill et al [2]. In mammalian cells, intracellular signal transduction pathway related to ERK was considered as the classic MAPK pathways [2]. ERK pathway is an important part of MAPK pathways, and plays an important role in the procession of extracellular signals transduction into intracellular signals [3]. It has been proved that receptor tyrosine kinase, G protein coupled receptor and partial cytokine receptor can all activate ERK pathway. The binding of growth factors and cell membrane receptor allowed the receptor to form dimers, and dimerization of the receptor activated its tyrosine kinase. Tyrosine phosphorylation of the receptor and the growth factor receptor combined with protein 2 (Grb2) on the membrane of the SH2 domain. The SH3 domain of Grb2 is combined with the SOS (of Sevenless Son) of the guanine nucleotide exchange factor. The latter makes the GDP dissociation of small molecule guanine nucleotide binding protein GTP and combined with Ras and activated Ras. The activation of Ras was further combined with the amino terminal of serine/threonine protein kinase Raf-1, and activated Raf-1 by some unknown mechanism. Raf-1 can phosphorylate the two regulatory serine of MEK1/MEK2 (MAP kinase/ERK kinase), and then activate Meks. MEKs is a dual specificity kinase. MEK can phosphorylate serine/threonine tyrosine. Finally it can selectively activate ERK1 and ERK2 (p44MAPK and p42MAPK). ERKs is a proline directed serine/threonine kinase, and can phosphorylate proline adjacent the serine/threonine. In mitogen stimulation, ERKs cascade upstream signal can be translocated into the nucleus. ERKs not only can phosphorylate cytosolic protein, but also can phosphorylate in some nuclear transcription factors such as c-fos, c-Jun, Elk-1, c-myc and ATF2. and then it can be involved in the regulation of cell proliferation and differentiation [7]. ERK can also phosphorylate upstream proteins of ERKs pathways such as NGF receptor, SOS, Raf-1, MEK, etc., and then negative feedback regulate the

pathway. Studies have also found that ERKs can phosphorylate the cytoplasm of cytoskeletal components, such as microtubule associated proteins MAP-1, MAP-2 and MAP-4, involving in the regulation of cell morphology and the redistribution of the cytoskeleton [7].

ERK pathways included Ras-Raf-MEK-Erk [3]. PD98059 is a specific inhibitor for MEK pathway [4], and the combination of PD98059 and MEK prevent the phosphorylation and activation of MEK by -Raf. And then ERK pathway was inhibited. PD98059 has no inhibitory effects for JNK/SAPK and p38MAPK signal pathways. Our studies showed that PD98059 inhibited the activation of MEK, ERK pathways of γδT cells induced by Mtb-Ag, and then the activation of γδT cells. CD69 expression in γδT cells decreased accompanied by the increase of PD98059, from (79±0.8)% to (17±0.2)%. There has no inhibitory effect in the activation of PMA+IM. The reason is that PMA+IM passed through TCR pathways, and activated MAPK/ERK signal transduction by the downstream of signal transduction. Mtb-Ag activated signal transduction pathway from the TCR-CD3 complex, and the rate of cell activation was slow. PD98059 also exhibited obvious inhibition for γδT cells proliferation, from $(6.2+0.9)\times10^6$ to $(0.41+0.1)\times10^6$. Due to the inhibition of γδT cells by PD98059, the proliferation will decrease. Our studies showed that the activation of γδT cells induced by Mtb-Ag passed through MAPK/ERK signal transduction pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiangqi Li, Department of Breast Surgery, Affiliated Hospital of Taishan Medical University, 706 Taishan Road, Tai'an 271000, China. Tel: +86-538-6230822; Fax: +86-538-8420042; E-mail: drlixqi@hotmail.com

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