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Effect of Mtb-Ag-activated γδT cells on the expression of CD69.

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Abstract

Objective: Activating and amplifying γδT cells with Mycobacterium tuberculosis low molecular peptide antigen (Mtb-Ag), in order to investigate the expression of CD69 molecules on γδT cellular surface.
Methods: Activate health human peripheral blood mononuclear cells (PBMC) separately obtained, PBMCs were stimulated with Mtb-Ag and further isolate positive cells by immuno-magnetic beads selection, measure the proportion of γδT cells in the PBMCs by fluorescent monoclonal TCR γδT-PE staining and flow cytometry. Then, measure the expression of CD69 molecules in first stimulation and re-stimulation of γδT cells by γδ-PE/CD69FITC double staining.
Results: The proportion of γδT cells were 4.9% in freshly isolated from PBMC, 69.2% after 10 days of Mtb-Ag activation, and 99.3% after immuno-magnetic beads selection. After 24 hours, the expression of CD69 molecules in γδT cells with initial Mtb-Ag stimulation arrived at peak at 75.2%. 6 hours later, in the second stimulation, it peaked at 72.0%.
Conclusion: Mtb-Ag can specifically stimulate the proliferation of γδT cells in the PBMC. Both its initial and the second stimulation can specifically activate γδT cells.

Keywords: γδT cells, Mtb-Ag, CD69 molecules.

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Introduction

γδT cells are a subgroup of T cells identified in 1986, mainly distributed in mucosa and subcutaneous tissues, such as 10-18% in human intraepithelial lymphocytes (IEL),and 25%-37% in human large intestinal IEL, 50% in mice IEL, accounting for only 0.5% to 5.0% of the total number of lymphocytes in adult peripheral blood [1]. Mucosa and epithelial tissues are the first line of defense against pathogen invasion and are also the frequent occurrence of tumors. The high proportion of γδT cells in mucosa and epithelial tissues suggests that γδT cells are crucial in resistant to microorganisms and parasites, anti-tumor and immune regulation. Since the recognition of antigen by γδT cells is not restricted by major histocompatibility complex (MHC), and antigen-presenting cells are not required to treat and present antigens, so γδT cells are more efficient and more extensive than α, β, T cells. For this reason, γδT cells have received increasing attention [2-5]. In the past, the use of flow cytometry or magnetic cell separation techniques to separate γδT cells requires not only a large amount of peripheral blood, but also expensive equipment and complicating operations. The authors used Mtb-Ag to specifically stimulate the proliferation of γδT cells. The obtained cells were positively sorted by magnetic beads selection to obtain a large number of high-purity γδT cells, and the expression of CD69 molecules in Mtb-Ag primary stimulation and re-stimulation of γδT cells was observed. Now introduced as follows [6].

Materials and Methods

Main instruments and reagents

Ordinary optical microscope (Olympus BH, model BH2-MA-2, Japan); inverted microscope (Dawning WJ12-50, XSB-14, China); CO_2 incubator (Harris hw0301T-VBA, USA); flow Cytometry (Coulter EPICSR XL-MCL, Beckman-counter, USA); cell culture plates (Falcon, USA); fully automated microplate reader (SLT-II, Austria); magnetic cell sorter (Miltenyi Biotec, Midi) -MACS, Germany). Mtb-Ag (a gift from Dr. Henry Boom, Department of Medicine, Case Western Reserve University, USA); lymphocyte separation solution (Institute of Hematology, Chinese Academy of Medical Sciences, batch number: 20000408); RPMIMedia1640 (RPMI1640) cell culture medium (Gibico, US). mouse anti-human fluorescent monoclonal antibody TCRγδ-PE (Becton Dickinson, USA, product number: 3437907); activation-inducing molecule CD69 antibody (anti-CD69FITC, Ancell,

USA, product number: 819010); recombinant human leukocytes Recombinant Human Interleukin-2 (rhIL-2, PTK, Korea); TCR γδ magnetic bead kit (Miltenyi Biotec, USA).

Methods

Cell preparation: Taking out the peripheral venous blood of 5 healthy adults and make heparin anticoagulated. PBMC was isolated by routine separation with lymphocyte separation solution, and the cell concentration was adjusted to 1.5 × 10^6/ml with RPMI1640 complete culture solution [5] for spare.

Mtb-Ag activated γδT cell proliferation and γδT cell isolation and purification: Activated γδT cells by Mtb-Ag, then separates and purifies the γδT cells. Take 1.5 × 10^6/ml PBMC suspension in 24-well culture plate, 1ml/well, add Mtb-Ag 5 μg/well for culture. Add the rIL-2 50 μ once each three days to keep cells growing. After 10 days, collect the γδT cells activated by Mtb-Ag. The γδT cells were sorted by immunomagnetic beads positive sorting method. Strictly follow instructions, and by PI single staining way, measure the freshly isolated PBMC, before sorting after culturing cells and after sorting cells proportion of γδT cells in the PBMCs with flow cytometry.

Detection of CD69 expression in γδT cells stimulated by Mtb-Ag for the first time: Draw 24 well cell culture plates, add the above prepared PBMC suspension to 24 wells in culture plate, 1 ml/well, and add Mtb-Ag (5 μg/hole). Then, cells were harvested at 37°C, 5% CO_2 incubator for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h. Use the direct immunofluorescence staining measure the expression of the CD69 in γδT cells by CD3PE/CD69FITC, γδPE/CD69FITC cell double staining. On the flow cytometer (Coulter EPICS XL), the argon ion laser wavelength was 488 nm as the excitation light, and the FSC/SSC (three-color flow cytometry) two-dimensional dot pattern was set. The gates were tested in lymphocyte populations and the resulting data files were analyzed using MinMDI 2.8 software.

Mtb-Ag re-stimulation of Mtb-AT cells induced re-expression of CD69 molecules in γδT cells: PBMC was stimulated by Mtb-Ag stimulation and cultured for 10 days, then Mtb-Ag (5 μg/well) was added again. The cells were harvested at 37°C, 5% CO_2 incubator for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h. The detection method was the same as before.

Results

Mtb-Ag-induced lymphocyte expansion

The proliferation of lymphocytes was observed by active quantitative method. After stimulation with Mtb-Ag, PBMC was cultured with rIL-2. The cells proliferated slowly in the first few days. After about 4 days, the proliferation accelerated, reaching a peak at 12 days, and the number of cells increased by nearly 40 times.

Proportion of γδT cells in lymphocytes

Freshly isolated PBMCs and PBMCs cultured for 10 days after stimulation with Mtb-Ag were detected by flow cytometry. As a result, γδT cells in freshly isolated PBMCs accounted for only 4.9%, while Mtb After 10 days of Ag-stimulated culture, the proportion of γδT cells can be as high as 69.2%, and then by immunomagnetic beads positive sorting, the proportion of γδT cells can be as high as 99.3%, as shown in Figure 1.

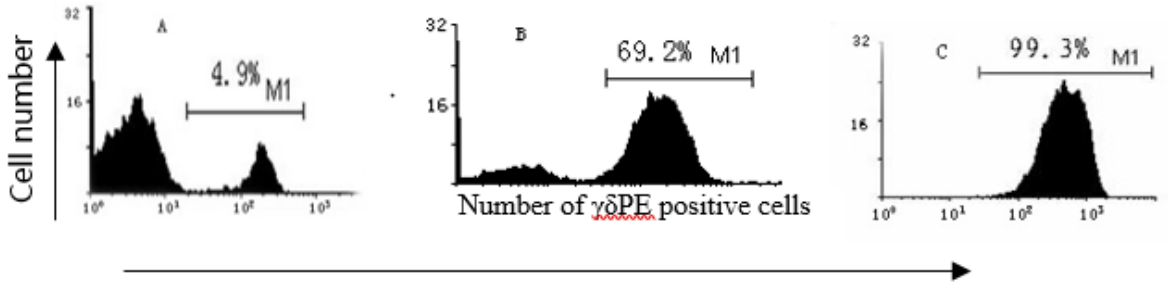

Figure 1. *The proportion of γδT cells. (A) PBMC; (B) Mtb-AT before sorting; (C) Mtb-AT after sorting.*

Mtb-Ag firstly stimulated the expression of CD69 in γδT cells

After 0 h, 6 h, 12 h, 24 h, 48 h and 72 h, the expression of CD69 molecules in Mtb-Ag was stimulated by 0.9 ± 0.22%, 15.1 ± 2.59, 35.2 ± 3.12%, 75.2 ± 6.29%, 59.4 ± 5.51%, and 50 ± 4.97% (Figure 2).

Mtb-Ag re-stimulated Mtb-AT cells induced re-expression of CD69 molecules in γδT cells

PBMC was stimulated by Mtb-Ag stimulation and cultured for 10 days, then stimulated with Mtb-Ag for 0 h, 6 h, 12 h, After 24 h, 48 h and 72 h, the expression of CD69 in γδT cells was 1.7 ± 0.46%, 72.3 ± 6.12%, 73.5 ± 6.45%, 50.3 ± 5.11%, 45.6 ± 4.84%, and 41.7 ± 4.49% respectively (Figure 3).

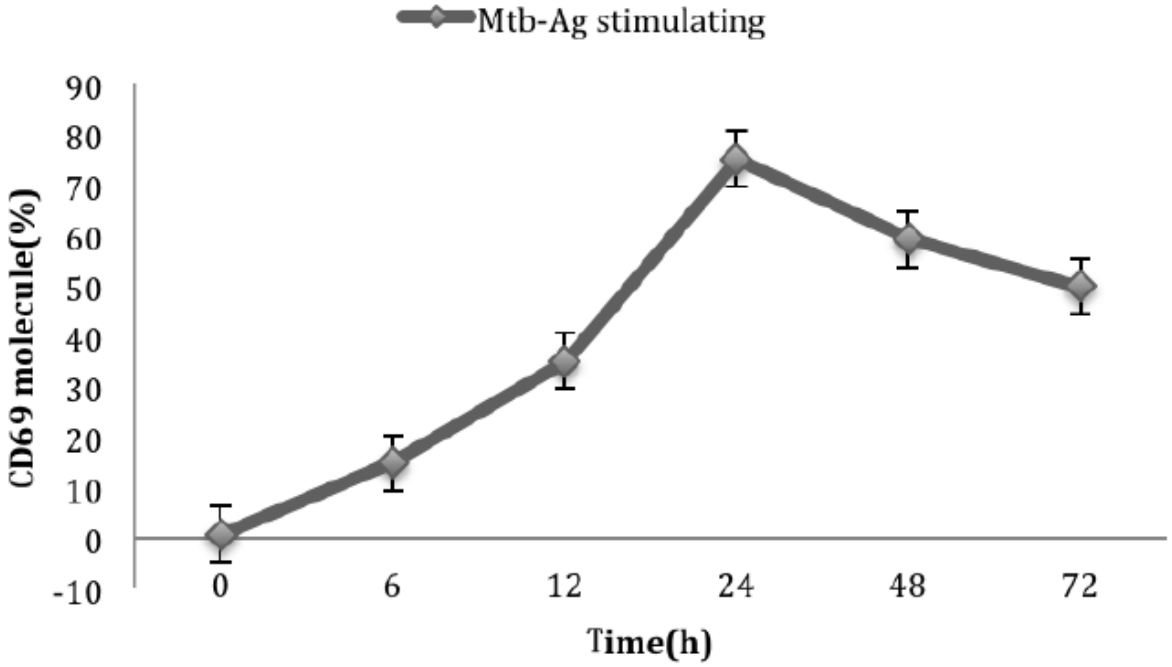

Figure 2. *The expression of CD69 molecule for the firstly stimulated by Mtb-Ag.*

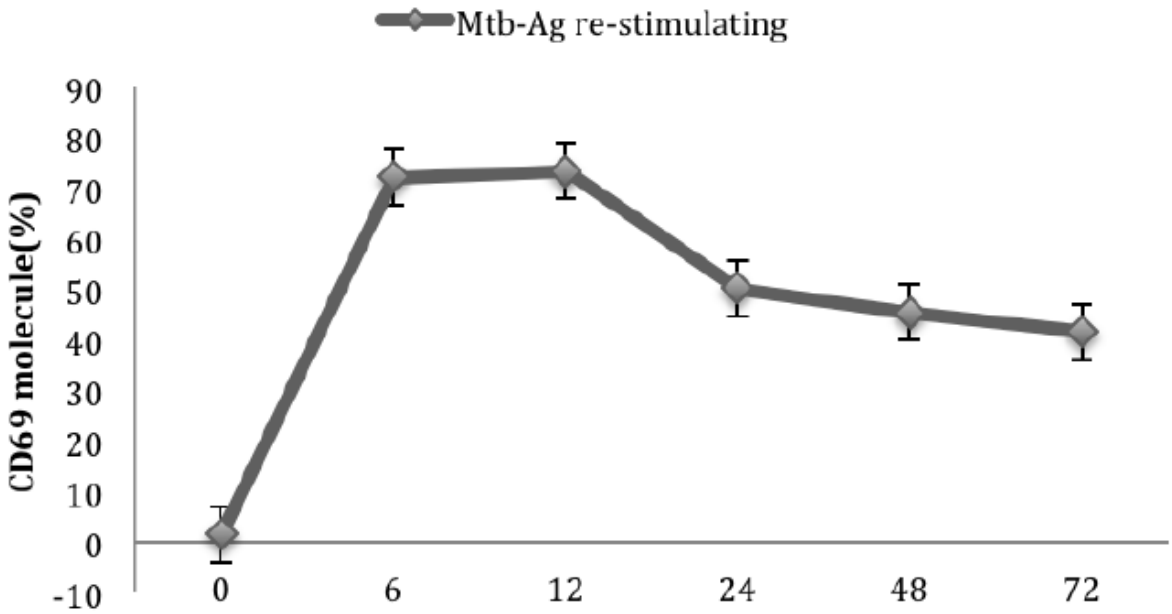

Figure 3. *The re-expression of CD69 molecule induced by Mtb-Ag re-stimulates Mtb-AT.*

Discussion

The results of this experiment showed that the ratio of γδT cells in freshly isolated peripheral PBMC was 4.9 ± 1.85%, and that Mtb-Ag stimulated PBMC up to 69.2 ± 6.57% after 10 days of culture; It is the same as the results of previous experiments in our laboratory and other researchers [7,8], indicating that Mtb-Ag has the characteristics of preferentially activating and amplifying γδT cells. Therefore, Mtb-Ag stimulates PBMC, interleukin-2 (IL-2) maintains cell proliferation and culture, and then positively sorted by immunomagnetic beads, a large number of γδT cells can be obtained, which can be used as a simple Rapid γδT cell amplification acquisition method. The method has the advantages of low blood volume, high specificity, short cycle, no special equipment, and the like, and can provide a source for the research on the biological characteristics of γδT cells and the immunotherapy of clinical diseases. Of course,

domestic stimulants have also been used to amplify γδT cells, such as Han et al. [9] After stimulating PBMC with zoledronic acid for 10 days, γδT cells increased from 4.21% to 70.35% before amplification; After Gui [10] sheep culturing human PBMC with isopentenyl pyrophosphate and rhIL-2 for 10 days, γδT cells increased from 4.34% to 55.65% before amplification; Xi et al. found that isopentenyl pyrophosphate (IPP) and ammonia Disodium hydroxy diphosphate (PAM) has similar effects on stimulating γδT cells at 14 days of action [11]; heat extracted by anti-γδT cell receptor, IL-2, human hepatoma cell SMMC-7721, etc. Shock protein 70 (HSP70) and their different combinations induced the production of γδT cells in human peripheral blood. As a result, γδT cell receptor (0.4 μg/ml) induced a large amount of γδT cells, reaching 61.5%, at 0.8. When it was increased at μg/ml, it reached 75.7% [12]. When it was combined with IL-2, the yield did not change significantly. When used in combination with HSP70, the yield increased significantly, reaching 71.1% and 85.6%, respectively. HSP70 and IL-2 combined use can also produce a large number of γδT cells, up to 75.6%; Ding [13] and other use of Mycobacterium tuberculosis heat-resistant antigen (Mtb-HAg) and butenyl diphosphate stimulated human PBMC, while IL-2 (50 μ/ml) was administered to maintain cell proliferation, and a control group supplemented with IL-2 was also established. As a result, after 10 days of culture, the rhIL-2 group proliferated. The ratio of γδT cells was (13.61 ± 4.14%), the ratio of γδT cells in the Mtb-HAg-stimulated group was (50.71 ± 7.49%), and the ratio of γδT cells in the butenyl diphosphate-stimulated group was (67.39 ± 6.40%). These stimulation methods can also obtain a large number of γδT cells, but no immunomagnetic beads positive sorting method is used to obtain higher purity γδT cells.

For the two stimulations of Mtb-Ag, the activation of γδT cells was very different. The expression of CD69 molecules in the first stimulation of γδT cells reached a peak at about 24 h (75.2%), then decreased rapidly, and decreased to 1.7% on the 10th day. about. At this time, Mtb-Ag re-stimulation can re-express CD69 molecules in γδT cells in Mtb-AT. Unlike the initial stimulation, the number of CD69-positive cells reached the peak (72%) after 6 h of Mtb-Ag stimulation. By 12 h (73%), it decreased to 24 h (50%), and decreased to 41% at 72 h. When the polypeptide purified from Mtb-Ag (C-main peptide) reported by Chen [14] stimulated γδT cells again, it can significantly express CD69 molecules, and same as the results of γδT have significant proliferative activity. It lays a methodological basis for seeking the rapid activation of γδT cells and the rapid expression of CD69 molecules, crucial to further exploring the signaling pathways involved in γδT cells activation.

Conclusion

Mtb-Ag can specifically stimulate the proliferation of γδT cells in the PBMC. Both its initial and the second stimulation can specifically activate γδT cells.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Funding

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Ethical Approval and Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. The study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the affiliated hospital of Taishan University. Written informed consents were obtained from all the subjects recruited into our study.

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