# The Role of Innate Immune Cells in the Response of Heat-Treated *Mycobacterium tuberculosis (M.tb)* Antigens Stimulating PBMCs

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**The proliferation response of γδT cells to the antigen from heat-treated *Mycobacterium tuberculosis* H37Ra (*M.tb* Ag) was used as a good model in γδT cell research. From preliminary research it is found that activated NK cells positively elevated γδT cells proliferation after simulating PBMCs with *M.tb* Ag. To investigate different behaviors of NK cells, γδNKT cells, γδT cells and relationships between these cell subsets, activation and proliferation of different cell subsets of PBMCs in response to *M.tb* Ag were analyzed. We demonstrated that NK cells, γδNKT cells and γδT cells could be activated after stimulation with *M.tb* Ag. γδNKT cells and γδT cells proliferated while the number of NK cells decreased after 11 day-simulation with *M.tb* Ag. Meanwhile, at the early time of stimulation the cytotoxicity of PBMCs was enhanced. *Cellular & Molecular Immunology*. 2004;1(6):467-470.**

**Key Words: tuberculosis, *M.tb* Ag, NK cell, NKT cell, γδT cell**

## Introduction

Natural killer cells are multifunctional cells which are not only the key effectors in the innate immune response to various infectious and transformed cells, but also the effective regulators in innate and adaptive immune responses. NK cells are involved in the maturation of $CD8^+$ cytotoxic T cells and the activation of B cells (1). Natural killer T (NKT) cells are also multifunctional cells and play an important role in innate immunity. As a special subset of T cells, NKT cells can quickly secrete different kinds of cytokines, such as IL-4, IFN-γ, IL-10 and IL-13. NKT cells can also play cytotoxic functions through Fas-FasL interactions (2, 3).

Tuberculosis is a major world disease estimated to kill over 2 million people annually. Fortunately, *Mycobacterium tuberculosis* (*M.tb*) can be controlled by cellular immunity and γδT cells play an important role in the immune response to *M.tb* (4-6). It has been reported that the active pulmonary tuberculosis patients had lower NK cell activity and were lack of γδT cells. But as a whole system, there are several cell subsets in PBMCs which play important roles in innate immunity, including NK cells, NKT cells (αβNKT and γδNKT) and γδT cells (2, 3). Under the stimulation of *M.tb*, different behaviors of these cell subsets and relationships between these cell subsets still remain unclear. To investigate the possible functional cell subsets in this process, we used the supernatant from heat-treated (SHT) H37Ra (*M.tb* Ag) to expand the number of γδT cells *in vitro* (7-9).

## Materials and Methods

*Preparation of M.tb antigen*

According to the methods of previous report, we inoculated H37Ra to Sauton culture medium and cultivated for 1 month at 37°C. The *M.tb* were collected and washed three times with PBS and one time with $ddH_2O$, resuspended with double volumes of $ddH_2O$ and then heated to 120°C for 30 min. The supernatants from heat-treated *M.tb* were harvested and filtrated with 0.22 μm pore-size filter (7, 10-12). The heat-treated antigen preparations were concentrated to 2 mg/ml in $H_2O$ and injected into Sephacryl S-100 HR. A bioactive fraction of 10-14 kD was collected and analyzed by SDS-PAGE (7).

*Cell isolation and culture*

PBMC samples from healthy subjects of blood donors were obtained from the Blood Institute of Anhui Province (Hefei, Anhui, China). Informed consent was obtained from each healthy volunteer. PBMCs were isolated from freshly heparinized venous blood by Ficoll-Hypaque (Pharmacia). Cells were maintained in RPMI 1640 medium (GIBCO BRL Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone), 100 U/ml

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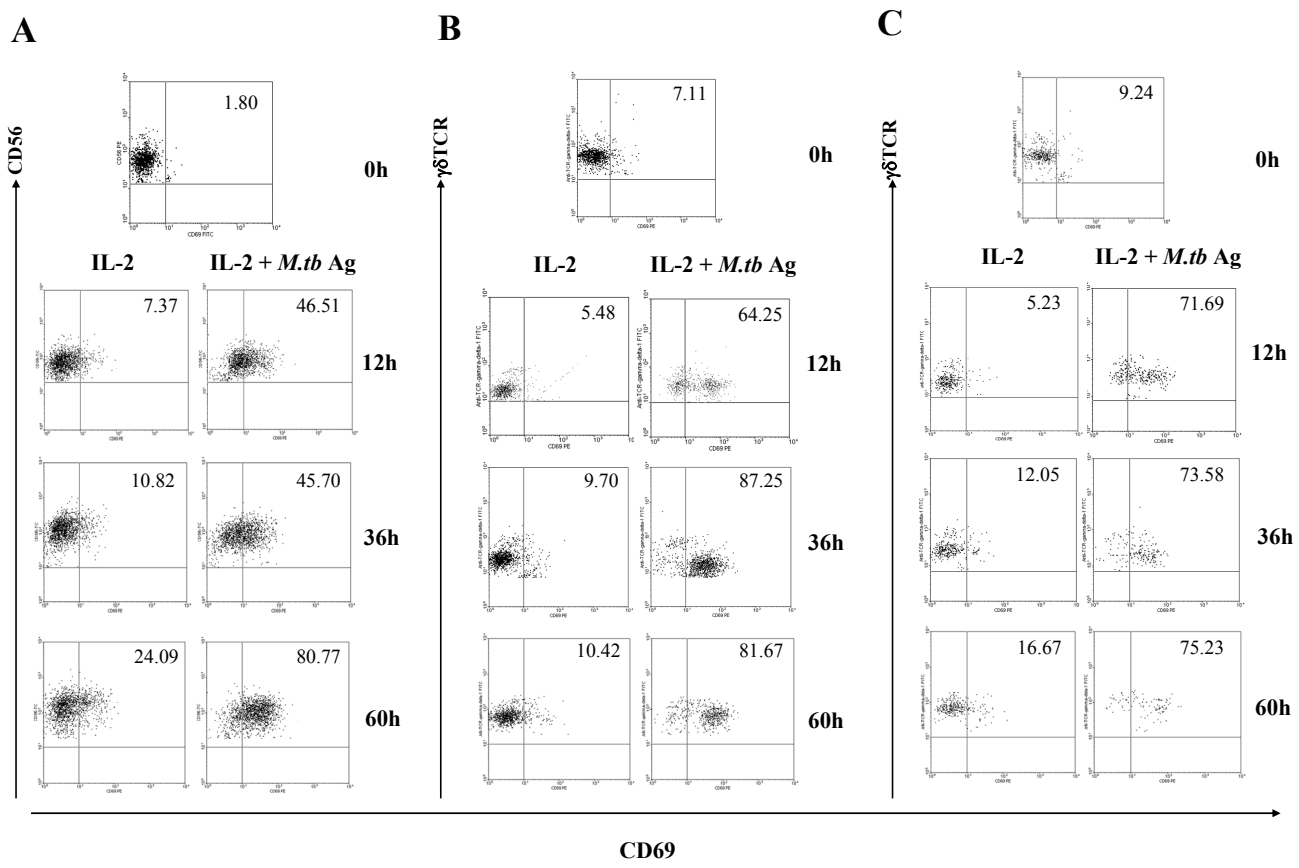


**Figure 1.** NK cells, γδT cells and γδNKT cells were activated by *M.tb* Ag. Freshly isolated PBMCs were cultured in the presence of 20 U/ml IL-2 with or without 5 μg/ml *M.tb* Ag. The cells were harvested at 0 h, 12 h, 36 h and 60 h. Expression of CD69 on NK cells (A, $CD56^+CD3^-$ cells were gated), γδT cells (B, $\gamma\delta TCR^+CD3^+CD56^-$ cells were gated) and γδNKT cells (C, $\gamma\delta TCR^+CD3^+CD56^+$ cells were gated) were analyzed by flow cytometry.

penicillin and 100 μg/ml streptomycin at 37°C in 5% $CO_2$. PBMCs were cultured at $1.5 \times 10^6$ cells/well in 24-well cell culture plate. Recombinant human rhIL-2 (20 U/ml, from PeproTech) with or without 5 μg/ml *M.tb* Ag was added into the medium. Every 3-4 days, half of the medium was discarded and replenished by fresh medium and cytokines.

*Flow cytometric analysis*

PBMCs were washed twice in washing buffer (PBS with 5% FBS) and resuspended in 100 μl staining buffer (PBS with 0.5% BSA and 0.1% $NaN_3$) at $10^6$ cells/ml. Cells were blocked with mouse serum for 30 min at 4°C and then were stained with following fluorochrome-conjugated Abs for 1 h at 4°C. The mAbs, including CD56-FITC, CD56-PE, CD56-TC, CD3-TC, CD69-FITC, CD69-PE, γδTCR-FITC, αβTCR-PE were purchased from BD PharMingen. Then PBMCs were washed three times with wash buffer and analyzed with FACScalibur (Becton Dickinson) and CellQuest software.

*Cytotoxicity assay*

Cytotoxic activity of NK cells in PBMCs was assessed against $^{51}Cr$-labeled K562 cells with a standard 4 h $^{51}Cr$-release assay (13). K562 cells ($1 \times 10^6$) were labeled with 200 μCi $Na^{51}CrO_4$ (Amersham Pharmacia Biotech) for 1 h at 37°C and washed three times. Then effector cells (PBMCs) and target cells (K562) were incubated for 4 h at 37°C and 5% $CO_2$ with the E:T from 5:1 to 100:1. Supernatants were harvested and radioactivity was determined using a γ counter. The percentage of specific $^{51}Cr$ release was calculated from the formula $(A - B)/(C - B) \times 100\%$, where $A$ is $^{51}Cr$ release in the presence of effector cells, $B$ is the spontaneous release in the absence of effector cells, and $C$ is the total $^{51}Cr$ release from K562 incubated with 1% Triton X-100. Spontaneous release did not exceed 10% of the maximum release.

## Results

*NK cells, γδNKT cells, γδT cells were activated quickly under the stimulation of M.tb Ag*

CD69 is the activation marker of lymphocytes. Expression of CD69 on PBMCs indicated that PBMCs were activated. To investigate the functions of different cell populations in innate immune system in response to the heat-treated antigens of *M.tb*, we detected the expression of CD69 on PBMCs from 0 to 60 h under the stimulation of *M.tb* Ag. Freshly isolated PBMCs were cultured with 20 U/ml IL-2 in the presence or absence of 5 μg/ml *M.tb* Ag. At 0 h, 12 h, 36 h and 60 h, the expression of CD69 were detected using

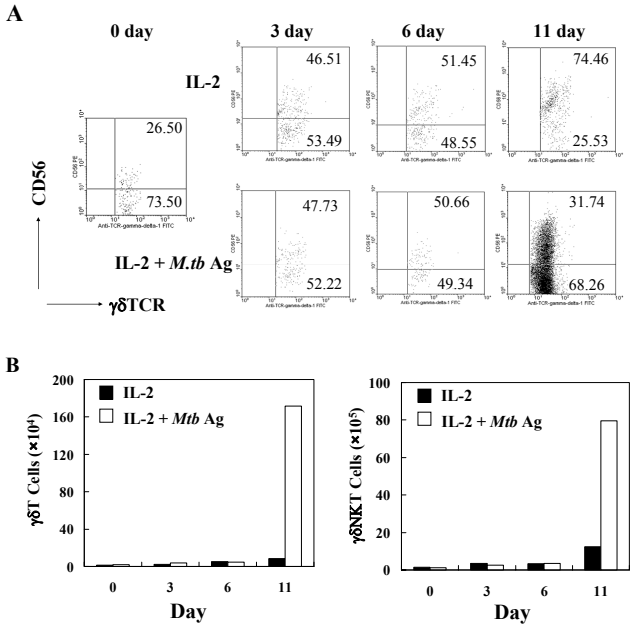


**Figure 2.** *M.tb* Ag induced the proliferation of γδT and γδNKT cells. PBMCs ($1.5 \times 10^6$ cells/well) were cultured in the presence 20 U/ml IL-2 with or without 5 μg/ml *M.tb* Ag. The cells were harvested on days 0, 3, 6 and 11. The percent (A) and numbers (B) of γδT and γδNKT cells were analyzed, respectively.

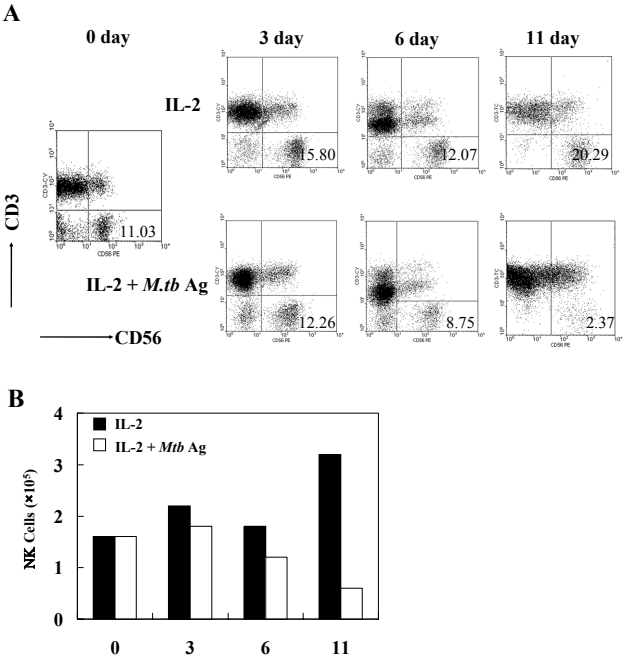


**Figure 3.** The NK cells decreased under the stimulation of *M.tb* Ag. PBMCs ($1.5 \times 10^6$ cells/well) were cultured in the presence of 20 U/ml IL-2 with or without 5 μg/ml *M.tb* Ag. The cells were harvested on days 0, 3, 6 and 11. The percent (A) and numbers (B) of NK cells were analyzed, respectively.

flow cytometry. Compared with 0 h, the expression of CD69 on NK cells, γδT cells and γδNKT cells increased to high levels. There were significant differences between experiment groups (*M.tb* Ag + IL-2) and control groups (IL-2). These results suggest that not only γδT cells, but also $CD56^+$ cells-NK cells and γδNKT cells take part in the immune response induced by tuberculosis (Figure 1).

*$\gamma\delta TCR^+$ cells obviously proliferated after long time culture but the amount of NK cells decreased*

It is generally accepted that the cytotoxicities of NK cells, NKT cells and γδT cells play important roles in innate immunity (3, 4). The proliferation situations of these cell subsets are very important for us to understand the defense mechanisms of human body against tuberculosis, whereas activation is prerequisite to proliferation. At the beginning, $1.5 \times 10^6$ cells/well were cultured in the presence of IL-2 (control group) or *M.tb* Ag plus IL-2 (experimental group) for 12 days, both with IL-2 added every 3-4 days. On days 0, 3, 6 and 11, cell count and flow cytometric analysis were used to measure the absolute number of different cell subsets (Figures 2A and 3A). From the results of day 11, we found that $\gamma\delta TCR^+$ cells, including γδT and γδNKT cells, proliferated obviously and the absolute number amplified about 80 folds, while the amount of NK cells decreased to half of its initial number (Figures 2B and 3B). These results suggest that γδT and γδNKT may be the main effectors in long time denfense against tuberculosis.

*Cytotoxicity of PBMCs was upregulated under the stimulation of M.tb Ag*

The feature of innate immunity is that it can clean pathogen in a short time. NK cells could be activated in the early stage of immune response to *M.tb* Ag, and in this process activated NK cells must play some roles, so the cytotoxicity of NK cells was measured with $^{51}Cr$-release assay. Freshly isolated PBMCs were cultured in the presence *M.tb* Ag plus IL-2 or IL-2 alone for 4 h (Figure 4A) or 24 h (Figure 4B). Then the cytotoxicity of PBMCs against K562 cells was detected respectively. The data displayed that the cytotoxicity of PBMCs was upregulated when NK cells were activated.

## Discussion

The behavior and responses of functional innate immunity cell subsets in response to *M.tb* Ag were shown in this paper. Previous research demonstrated that γδT cells played an important role in immune response to *M. tuberculosis* and proliferated vigorously to *M.tb* Ag *in vitro* (14, 15). The specific response of PBMCs to *M.tb* Ag is a good model employed in the research of innate immunity (7-9). We investigated the possible important innate immunity cell subsets' responses under the stimulation of *M.tb* Ag and found that all the $\gamma\delta TCR^+$ cells (γδT cells and γδNKT cells) and most $CD56^+$ cells (NK cells and γδNKT cells) were activated. Other cell subsets, such as αβT cells and αβNKT cells, did not have any special response to *M.tb* Ag (data not shown). It is known that some important functions of lymphocytes act out only under the status of activation. So in the early response to *M.tb* Ag, $CD56^+$ and $\gamma\delta TCR^+$ cells must participate and play important roles. $CD56^+$ cell subsets (NK and NKT) are paid more and more

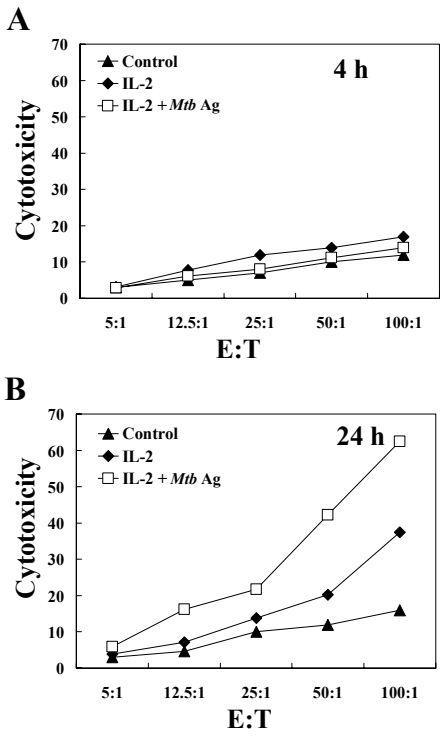


**Figure 4.** The cytotoxicity of PBMCs was upregulated under the simulation of *M.tb* Ag. PBMCs were incubated in the presence of 20 U/ml IL-2 with or without 5 μg/ml *M.tb* Ag. for 4 h (A) and 24 h (B). Then the cytotoxicity of PBMCs was determined by 4 h $^{51}$Cr-release assay.

attention in recent years. Their functions in innate immunity are emphasized because they may be the key element in many innate immune responses (2, 3). We also noticed that almost all the γδTCR$^+$ cells were activated but αβTCR$^+$ cells remained unchanged. So there may be some recognition mechanism between γδTCR and *M.tb* Ag.

To evaluate the functions of CD56$^+$ cells and γδTCR$^+$ cells, we detected the cytotoxicity of PBMCs in the early time of stimulation and measured the proliferation of NK cells, γδNKT cells and γδT cells after long time culture. Cytotoxicity and immunological regulation are two main functions of lymphocytes. The proliferation of one cell subset in some way means that it may play some roles in clearing the pathogens. We found that at the early stage of *M.tb* Ag simulation, the cytotoxicity of PBMCs was obviously upregulated. So the early activated CD56$^+$ cells at least partly play some killing roles. But after 11 days' culture, only γδT cells and γδNKT cells proliferated. The decrease of NK cells meant that NK cells only played some functions in the early stage of stimulation. The proliferation of γδNKT is not reported yet. It has a high proliferation with amplifying to more than 80 folds. But the mechanism is still unclear and need to be investigated further. The cytotoxicity may become the main function of γδNKT.

Our results roundly evaluate the behaviors of all the possible cell subsets of human immune system in response to tuberculosis. In summary, CD56$^+$ cells and γδTCR$^+$ cells were activated in the early stage of stimulation but only γδTCR$^+$ cells proliferated after long time culture. These results are useful to consequently deep research on the response and the relationship between CD56$^+$ cells and γδTCR$^+$ cells. They are also useful to the therapy of tuberculosis by activated NK cells and NKT cells.

## References

1. Moffett-King A, Entrican G, Ellis S, Hutchinson J, Bainbridge D. Natural killer cells and reproduction. Trends Immunol. 2002;23:332-333.
2. Loza MJ, Metelitsa LS, Perussia B. NKT and T cells: coordinate regulation of NK-like phenotype and cytokine production. Eur J Immunol. 2002;32:3453-3462.
3. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Vα14 NKT cells in innate and acquired immune response. Annu Rev Immunol. 2003;21:483-513.
4. Augustin A, Kubo RT, Sim GK. Resident pulmonary lymphocytes expressing the γδT-cell receptor. Nature. 1989; 340:239-241.
5. Kabelitz D, Bender A, Schondelmaier S, Schoel B, Kaufmann SH. A large fraction of human peripheral blood γδ$^+$ T cells is activated by Mycobacterium tuberculosis but not by its 65-kD heat shock protein. J Exp Med. 1990;171:667-679.
6. Kabelitz D, Bender A, Prospero T, Wesselborg S, Janssen O, Pechhold K. The primary response of human γδ$^+$ T cells to Mycobacterium tuberculosis is restricted to Vγ9-bearing cells. J Exp Med. 1991;173:1331-1338.
7. Boom WH, Balaji KN, Nayak R, Tsukaguchi K, Chervenak KA. Characterization of a 10- to 14-kilodalton protease-sensitive Mycobacterium tuberculosis H37Ra antigen that stimulates human-γδT cells. Infect Immun. 1994;62:5511-5518.
8. Chen Y, Lu HZ, Hu JG, Li BQ. Characterization of Mycobacterium tuberculosis polypeptide antigen capable of stimulating the proliferation of human γδT cells. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2003;19:121-123.
9. Chen Y, Lu HZ, Hu JG, et al. Purification by FPLC and characterization of Mycobacterium tuberculosis heat-resistant peptide antigen simulating proliferation of human γδ$^+$ T cell. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2003;19:86-89.
10. Li B, Rossman MD, Imir T, et al. Disease-specific changes in γδT cell repertoire and function in patients with pulmonary tuberculosis. J Immunol. 1996;157:4222-4229.
11. Li B, Bassiri H, Rossman MD, et al. Involvement of the Fas/Fas ligand pathway in activation-induced cell death of mycobacteria-reactive human γδ T cells: a mechanism for the loss of γδT cells in patients with pulmonary tuberculosis. J Immunol. 1998;161:1558-1567.
12. Tsukaguchi K, Balaji KN, Boom WH. CD4$^+$ αβ T cell and γδ T cell responses to Mycobacterium tuberculosis. Similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production. J Immunol. 1995;154:1786-1796.
13. Bol SJ, Rosdorff HJ, Ronteltap CP, Hennen LA. Cellular cytotoxicity assessed by the $^{51}$Cr release assay. Biological interpretation of mathematical parameters. J Immunol Methods. 1986;90:15-23.
14. Carding SR, Egan PJ. γδT cells: functional plasticity and heterogeneity. Nat Rev Immunol. 2002;2:336-345.
15. Hayday AC. γδ cells: a right time and a right place for a conserved third way of protection. Ann Rev Immunol. 2000;18:975-1026.