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The source of Mycobacterium tuberculosis-specific IFN-γ production in peripheral blood mononuclear cells of TB patients

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ABSTRACT

Mycobacterium tuberculosis (Mtb)-specific IFN-γ secretion plays important roles in anti-tuberculosis (TB) immunity. Mtb-specific IFN-γ response can be induced in HIV/TB co-infected patients with a low CD4 lymphocyte count; this suggests that the source of Mtb-specific IFN-γ production is not limited in $CD4^+$ T lymphocytes. Currently, the major sources of Mtb-specific IFN-γ production and the function and phenotype of Mtb-specific IFN-γ-producing cells still remain unclear. Thirty-nine participants (24 active TB patients, 10 HIV/TB co-infected patients, and 5 healthy volunteers) were recruited according to conventional tests and Mtb-specific IFN-γ ELISPOT assay. Multicolor flow cytometry was used to investigate the production of intracellular IFN-γ in peripheral blood mononuclear cells (PBMCs) after Mtb-specific antigen stimulation. Our results showed that $CD4^+$, $CD8^+$ T cells and NK cells are all major sources of Mtb-specific IFN-γ production in PBMCs of TB patients. Moreover, $CD8^+$ T cells are the highest number of Mtb-specific IFN-γ-producing cells in HIV/TB co-infected patients. Although the activity of NK cells is significantly reduced in TB patients when compared with healthy controls, Mtb-specific antigen stimulation induces a significant increase in NK cell activity. We also showed that CD45RO is the characteristic marker of Mtb-specific IFN-γ-producing T cells but not that of Mtb-specific IFN-γ-producing NK cells in peripheral blood. High expression of CD11a may be the characteristic feature of Mtb-specific IFN-γ-producing NK cells. This study put forward a new insight on the source of antigen-specific IFN-γ-production in PBMCs of TB patients.

1. Introduction

Tuberculosis (TB) remains the most hazardous bacterial infection worldwide. One-third of the world's population is infected with Mycobacterium tuberculosis (Mtb) [1]. Antigen-specific IFN-γ secretion plays important roles in protection against Mtb infection through recruitment of monocytes and granulocytes and activating the antimicrobial activity of macrophages [2,3]. Some studies have underscored that $CD4^+$ and $CD8^+$ T cells are the Mtb-specific IFN-γ-producing cells after antigen stimulation [4–6]. Additionally, NKT cells also participate in the local immune response to Mtb through the secretion of IFN-γ [7]. However, which cells are the most important producers of Mtb-specific IFN-γ remain unclear.

Interferon-gamma release assays (IGRAs) as classic method to detect Mtb-specific IFN-γ in vitro, are based on IFN-γ secretion by T cells exposed to Mtb-specific antigens (TBAg), such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) [8–10]. Current studies have shown that IGRAs are less influenced by immunosuppression in HIV-infected patients with a low CD4 count [11–14]. This also indicates that Mtb-specific IFN-γ secretion is not limited in $CD4^+$ T cells and that there are other important Mtb-specific IFN-γ producers.

In this study, we used multicolor flow cytometry to determine the source and phenotype of Mtb-specific IFN-γ-producing cells. We found that $CD4^+$, $CD8^+$ T cells and NK cells are all major sources of Mtb-specific IFN-γ production in peripheral blood mononuclear cells (PBMCs) of TB patients. Moreover, Mtb-specific IFN-γ-producing T cells express memory marker CD45RO, and high expression of CD11a may be the characteristic feature of Mtb-specific IFN-γ-producing NK cells.

2. Materials and methods

2.1. Study groups

This study was carried out from January to July 2014 at Tongji Hospital (TJH), the largest hospital in central region of China. Participants were recruited based on Mtb-specific IFN-γ ELISPOT responses routinely performed for the diagnosis of Mtb infection at TJH. Besides positive IFN-γ ELISPOT responses (ESAT-6 and/or CFP-10 spot-forming cells (SFCs) > 100), patients with active TB had a diagnosis based on laboratory isolation of Mtb in mycobacterial culture from sputum, broncho

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alveolar lavage fluid or plerual effusion, and/or acid-fast staining (AFS), and/or PCR. The final diagnosis was given by a clinician after validation of positive culture results associated with typical clinical symptoms and radiographic presentations. The active TB patients (IFN-γ ELISPOT responses: ESAT-6 and/or CFP-10 SFCs > 50) co-infected with HIV were also recruited in this study. HIV infection was confirmed by clinical records, routine serum tests (competitive ELISA and Western blotting confirmation). Healthy volunteers were defined as those with a negative IFN-γ ELISPOT response and without any clinical symptoms or signs of diseases. This study was approved by the ethical committee of Tongji hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All participants gave written consent to the study.

2.2. IFN-γ ELISPOT assay

PBMCs were isolated from heparinized blood of participants by using Ficoll–Hypaque density gradients (Sigma-Aldrich, St Louis, MO). Mtb-specific IFN-γ ELISPOT assay was performed according to the instruction of T-SPOT.TB kit (Oxford Immunotec, Abingdon, UK).

2.3. Cell preparation and activation

For cell stimulation studies, the cultured PBMCs were stimulated with IL-12 (100 U/mL, BioLegend) or TBAg (ESAT-6 and CFP-10 complex, each at 10 μg/mL) in the presence of 2 μM monensin (eBioscience) for 24 h. In some experiments, either ESAT-6 or CFP-10 was used to stimulate PBMCs for getting better intracellular IFN-γ staining results. PBMCs cultured with medium alone served as negative control.

2.4. Flow cytometric analysis

Cell surface staining was performed on PBMCs after 24-h stimulation. The following monoclonal antibodies were added to the cell suspensions: anti-CD3-PerCP cy5.5 (SK7), anti-CD8-PerCP (SK1), anti-CD56-PE (CMSSB), and anti-CD69-FITC (FN50) (eBioscience); anti-CD107a-FITC (H4A3), anti-CD11a-FITC (HI111), anti-CD62L-FITC (DREG-56), and anti-CCR7-FITC (G043H7) (BioLegend); anti-CD4-PE (RPA-T4), anti-CD25-FITC (M-A251), and anti-CD45RO-FITC (UCHL1) (BD Pharmingen). Isotype controls with irrelevant specificities were included as negative controls. All of these cell suspensions were incubated for 30 min on ice. For intracellular staining, the cells were fixed and permeabilized with Fixation and Permeabilization Buffer (BD Pharmingen) and stained with APC-labeled anti-IFN-γ antibody (4S.B3) (eBioscience). After washing, the pellets were resuspended in 500 μL cold staining buffer, followed by analysis with FACSCalibur cytometer (Becton Dickinson). Data analysis was performed using FlowJo version 9 software (TreeStar).

2.5. CD4 count

The CD4 cell count was estimated in blood samples of participants by flow cytometry as previously described [12]. In brief, one hundred microliters of whole blood was labeled with anti-CD3 and anti-CD4 antibodies. After 30 min incubation on ice, the red blood cells were lysed using FACS lysing solution (BD Biosciences) and then analyzed with Flow cytometry. The percentages of CD3 and CD4 positive cells among the total lymphocytes were obtained using FlowJo Software. The absolute $CD3^+CD4^+$ cell counts were calculated by multiplying the percentage with the total lymphocyte count.

2.6. Statistical analysis

Flow cytometry data were analyzed using FlowJo software. GraphPad Prism software (version 6.0) was used for statistical analysis. Statistical analysis differences between groups were analyzed using the Mann–Whitney U test. Statistical significance was determined as $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Study participants

On the basis of conventional tests and T-SPOT.TB responses, 24 TB patients, 10 HIV/TB co-infected patients, and 5 healthy volunteers were recruited in this study. Among 24 active TB patients, 13 (54%) subjects were female, and the mean age was 40 years (range, 20–68 years). The median absolute $CD4^+$ T-cell count was 772 cells/μL (range, 489–1009 cells/μL). Among 10 HIV/TB co-infected patients, 4 (40%) were female, and the mean age was 44 years (range, 38–55 years). The median absolute $CD4^+$ T-cell count was 361 cells/μL (range, 258–569 cells/μL). The demographic characteristics and clinical presentations of the subjects included in the study are shown in Supplemental Table 1.

3.2. The source of Mtb-specific IFN-γ in PBMCs of TB patients

We tried to determine which cells were the main producers of Mtb-specific IFN-γ in PBMCs. Our results showed that intracellular IFN-γ could be detected in $CD4^+$ T cells, $CD8^+$ T cells, NK cells and NKT cells after stimulation with TBAg in vitro (Fig. 1A). Although the percentage of $CD4^+$ T cells in PBMCs was relatively higher, the percentage of Mtb-specific IFN-γ-producing $CD4^+$ T cells was significantly lower than that of Mtb-specific IFN-γ-producing NK and NKT cells (Fig. 1B). We also calculated the absolute number of IFN-γ-producing cells, and we found that the total numbers of Mtb-specific IFN-γ-producing cells had no statistical difference in $CD4^+$, $CD8^+$ T cells and NK cells. A significant difference was found only when the above cells were compared with NKT cells (Fig. 1C). The pie charts show the proportions of Mtb-specific IFN-γ-producing cells in PBMCs of six representative patients (Fig. 1D), which suggested that the Mtb-specific IFN-γ producers were heterogeneous in different TB patients. These data suggested that $CD4^+$, $CD8^+$ T cells and NK cells are all major sources of Mtb-specific IFN-γ production in PBMCs of TB patients.

3.3. The source of Mtb-specific IFN-γ in PBMCs of HIV/TB co-infected patients

To further confirm that $CD4^+$ T cells are not the only source of Mtb-specific IFN-γ in PBMCs, another 10 HIV/TB co-infected patients were included in the study. Although the percentage of $CD4^+$ T cells were significantly lower than that of $CD8^+$ T cells in these patients, the Mtb-specific IFN-γ could still be detected in $CD4^+$ T cells (Fig. 2A and B). Differently, the absolute number of IFN-γ-producing $CD8^+$ T cells was the highest and significantly higher than that of IFN-γ-producing $CD4^+$ T cells (Fig. 2C). Furthermore, the mean number of IFN-γ-producing NK cells was also higher than that of $CD4^+$ T cells, but there was no significant difference between these two groups (Fig. 2C). Our results showed that $CD8^+$ T cells are the highest number of Mtb-specific IFN-γ-producing cells in HIV/TB co-infected patients. These data indicated that besides $CD4^+$ T cells, $CD8^+$ T cells and NK cells are also the important producers of Mtb-specific IFN-γ in peripheral blood. We also analyzed the source of Mtb-specific IFN-γ production in PBMCs of patients with low level of IFN-γ ELISPOT response (TB: SFCs < 100, HIV/TB: SFCs < 50). However, no intracellular IFN-γ was detected with the flow cytometry assay in most of these patients.

3.4. The activity of NK cells after different stimulation conditions

Our above results have shown that there are Mtb-specific IFN-γ-producing NK cells in the peripheral blood of TB patients. However, with the exception of cytokine-producing capability, little is known