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IMMUNOLOGICAL ASPECTS

Mycobacterium tuberculosis-specific polyfunctional cytotoxic CD8$^+$ T cells express CD69

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SUMMARY

Increasing evidences in animals and humans suggest that CD8$^+$ T cells contribute significantly to immune defenses against *Mycobacterium tuberculosis* (Mtb). In the present study, we found that without any stimulation, CD8$^+$ T cells in pleural fluid cells (PFCs) expressed significantly higher levels of CD69 than PBMCs from patients with tuberculous pleurisy (TBP). CD8$^+$CD69$^+$ T cells expressed significantly higher levels of CD45RO and HLA-DR and lower levels of CD45RA than CD8$^+$CD69$^-$ T cells, demonstrating that CD8$^+$CD69$^+$ T cells were activated memory cells. Furthermore, we found higher expression of CCR6 and lower expression of CCR7 and CD62L on CD8$^+$CD69$^+$ T cells compared with CD8$^+$CD69$^-$ T cells, suggesting that the expression of CCR6 and reduced expression of CCR7 and CD62L might facilitate the migration of circulating CD8$^+$CD69$^+$ T cells into tuberculous pleural space. Importantly, following stimulation with culture filtrate protein of 10 kDa (CFP10) peptides, CD8$^+$CD69$^+$ T cells but not CD8$^+$CD69$^-$ T cells expressed CD107a/b, IFN-γ and TNF-α, demonstrating that CD8$^+$CD69$^+$ T cells were MTB-specific cells. In addition, the majority of CD8$^+$CD69$^+$ T cells were dominated by polyfunctional T cells. In summary, we demonstrated that CD69 as a useful marker for MTB-specific CD8$^+$ T cells in PFCs from patients with TBP enabled a direct *ex vivo* estimation of the quantity, as well as the quality, of MTB-specific CD8$^+$ responses.

1. Introduction

Tuberculosis (TB) is a leading cause of infectious mortality worldwide, accounting for over 8 million new cases and 2.9 million deaths annually [1–3]. Increasing experimental evidence in the mouse tuberculosis (TB) model has suggested a protective role for CD8$^+$ T cells in the host defense [4–6]. For example, adoptive transfer or *in vivo* depletion of CD8$^+$ cells showed that this population could confer protection against subsequent challenge. β2-microglobulin- deficient mice are more susceptible to Mtb and to large doses of Bacille Calmette Gue'rin infection than their wild-type littermates [5]. In murine experimental models, CD4$^+$ T cells are essential for control of acute TB infection and CD8$^+$ T cells are essential to maintain and control the chronic phase of TB [7]. In humans, Mtb-specific CD8$^+$ T cells have been identified in Mtb-infected individuals [8,9].

It has been demonstrated that T-lymphocytes in the lungs, both in normal individuals and in those with granulomatous disease, are almost entirely CD45RO$^+$ memory T cells and express both early- and late-activation markers, such as CD69 and CD29 respectively [10]. CD69 is a membrane molecule transiently expressed on activated lymphocytes, and its selective expression in inflammatory infiltrates probably suggests that it plays a role in the pathogenesis of inflammatory diseases [11]. We have previously found a significant increase in CD69 expression on CD4$^+$ T cells in PFCs and demonstrated that most of these CD4$^+$CD69$^+$ T cells were MTB-specific Th1 cells [12]. Moreover, we have demonstrated that CFP10-specific CD8$^+$ T cells were present in patients with TBP [13]. In the present study, we found that without any stimulation, CD8$^+$ T cells in PFCs expressed significantly higher levels of CD69 than PBMCs from patients with TBP. Further phenotypic analysis indicated that CD8$^+$CD69$^+$ T cells were activated memory T cells. In order to further characterize the biological functions of CD8$^+$CD69$^+$ T cells, we stimulated PFCs with CFP10 peptides and assessed the expression of cytotoxic molecules and cytokines as well. By using the protein transport inhibitor, Brefeldin A (BFA), we could block the transport of newly synthesized CD69 molecule to the cell

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surface. We could determine the function of $CD8^+CD69^+$ T cells which were induced *in vivo*. Our results indicated that cytotoxic and cytokine-producing $CD8^+$ T cells were mostly enriched within $CD8^+CD69^+$ T cells, suggesting that CFP10-specific $CD8^+$ T cells primarily expressed CD69 *in vivo*. CD69 could be used as a marker for the enrichment and isolation of MTB-specific $CD8^+$ T cells for further study.

2. Methods

2.1. Study participants

A total of thirty-three patients with tuberculous pleurisy (12 females and 21 males, range 20–65 years of age) were recruited from the Chest Hospital of Guangzhou, China. Diagnosis of pleural effusion from TB etiology was based on one of the following criteria: (i) demonstration of MTB on pleural fluid smear (by the Ziehl-Neelsen method); (ii) pleural fluid or pleural biopsy specimens growing *Mycobacterium tuberculosis* on Lowenstein–Jensen medium; or (iii) histological evidence of caseating granuloma on biopsy specimens of pleural tissue with positive staining for *M. tuberculosis*. Patients who had been previously diagnosed with HIV, HBV, or HCV or with a history of autoimmune diseases were excluded from the study. None of the patients was receiving MTB-related treatments at the time of the study. The study was approved by the Zhongshan School of Medicine Review Board (Guangzhou, China).

2.2. Peptides, reagents and mAbs

Twenty-six 13-15mer peptides that overlapped by 11 aa and spanned the CFP10 protein were synthesized. All of the peptides were synthesized by Sangon Biotech (Shanghai)Co.,Ltd. Peptide purity was >95%, as assayed by HPLC, and their composition was verified by mass spectrometry. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, aliquoted, and stored at −80 °C. Purified anti-CD28 (clone CD28.2) and anti-CD49d (clone 9F10) mAbs were purchased from BD Biosciences (San Jose, CA). The following mAbs were used for phenotypic and intracellular cytokine analysis and were purchased from BD Biosciences (San Jose, CA, USA): CD3-APC-Cy7 (SK7), CD3-Pacific Blue (UCHT1), CD8-FITC (RPA-T8), CD8-APC (RPA-T8), CD8-eFluor450 (RPA-T8), CD69-PE (FN50), CD69-Pecy7 (FN50), CCR4-PE (1G1), CCR6-PE (11A9), CCR7-PE (3D12), CXCR3-APC (1C6), CXCR4-PE (12G5), CD62L-PE (Dreg56), CD45RA-FITC (L48), CD45RO-FITC (UCHL1), CD127-APC (hIL-7R-M21), CD25-FITC (M-A251), HLA-DR-PE (G46-6), CD107a-FITC (H4A3), CD107b-FITC (H4B4), IFN-γ-APC (clone B27), TNF-α-PE-Cy7 (MAb11), IL-2-APC (MQ1-17H12), and isotype-matched control antibodies. CD27-APC (O323) was obtained from Biolegend (San Diego, CA). IFN-γ-FITC (45.15) was purchased from Beckman Coulter (Fullerton, CA).

2.3. Preparation of PFCs and PBMCs

PFCs were isolated by lysing erythrocytes using ammonium chloride solution and resuspended to a final concentration of 2×10^6 cells/mL in complete RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation of heparinized venous blood obtained from patients with tuberculous pleurisy.

2.4. Flow cytometry

For surface staining, PFCs or PBMCs were washed with PBS buffer containing 0.1% BSA and 0.05% sodium azide and incubated with the respective mAbs for 30 min. The cells were thereafter washed twice and resuspended in PBS. For the detection of intracellular cytokines, cells were incubated at a concentration of 2×10^6 cells/mL with 1 μg/mL peptides plus 1 μg/mL anti-CD28 and 1 μg/mL anti-CD49d for 8 h in the presence of brefeldin A (BFA, 10 μg/mL; Sigma–Aldrich, St Louis, MO). For the detection of CD107a/b, PFCs were stimulated with CFP10 peptides plus CD107a-FITC and CD107b-FITC. One hour later, Brefeldin A and Monensin (1 μL/mL, BD Biosciences Pharmingen) were added and the plates were incubated for another 5 h. After stimulation, cells were washed with PBS containing 0.1% BSA and 0.05% sodium azide. Cells were incubated with antibodies for surface staining and then fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% saponin and stained for intracellular cytokines. Flow cytometry was performed using BD FACSCalibur (BD Biosciences) or FACSAria II (BD Biosciences) and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

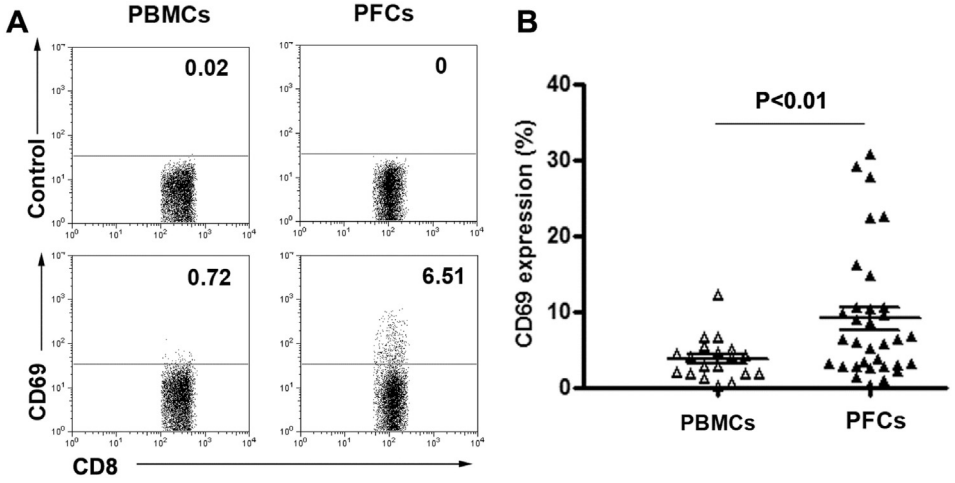

Figure 1. The expression of CD69 on $CD8^+$ T cells in PBMCs and PFCs from patients with TBP without any stimulation. (A) Representative expression of CD69 on $CD8^+$ T cells is determined by FACS in PBMCs and pleural fluid cells (PFCs) from patients with TBP. The numbers in each quadrant represent the percentages of CD69 expression in gated $CD8^+$ T cells. (B) Summary data of CD69 expression on PBMCs ($n = 19$) and PFCs ($n = 33$). Each symbol represents a value from a single donor. Differences between groups are assessed by the Student's *t* test. Horizontal lines represent means ± SEM.