CYTOKINE PRODUCTION IN NK AND NKT CELLS FROM *MYCOBACTERIUM TUBERCULOSIS* INFECTED PATIENTS

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Abstract. Tuberculosis, a major health problem in developing countries, has re-emerged in recent years in many countries. While it is accepted that various lymphocyte subsets are important responses to mycobacterial infection, the roles of NK and NKT cells in producing cytokines are still unclear. Thus we have evaluated, in *Mycobacterium tuberculosis* infection, the frequency of cytokine producing cells by flow cytometry. Of 30 individuals examined, 17 had clinical evidence of pulmonary tuberculosis while the rest showed no evidence of infection. Patients had a significantly higher number of IFN-γ and IL-4-producing T cells compared to control subjects, but the ratio of IFN-γ to IL-4-producing T cells was similar in both groups. There were no differences between cytokine profiles of NK cells in patients and control subjects. A significant increase in the number of NKT cells was observed in patients. A striking finding was the higher frequency of IL-4-producing NKT cells compared to IFN-γ-producing cells. Moreover, individual NKT cell produced both IFN-γ and IL-4. The preferential type of Th1 or Th2 cells is due to mycobacterial strain, type of antigen presenting cells and stage of disease, all of which can lead to different patterns of cytokine production by variety of lymphocyte subsets.

INTRODUCTION

Tuberculosis is a contagious disease of enormous importance to global public health. The largest number of new TB cases in 2004 occurred in Southeast Asian region, which accounted for 33% of cases globally (WHO, 2004). In spite of this devastating impact on human population, how the human immune system is capable of providing effective protection against this infection remains unclear. The majority of immunocompetent people infected by mycobacteria do not develop signs of serious illness (Ulrichs *et al*, 2003). In some cases, mycobacteria can successfully invade host tissues, but extracellular mycobacteria is generally cleared rapidly by macrophages. Pathogenic mycobacteria persist for a long period within host tissues, likely within infected phagosome of host macrophage (Saxena *et al*, 2002). Intracellular growth and persistence account for the ability of mycobacteria to produce chronic diseases. Tuberculosis may be reactivated in the setting of malnutrition, aging or acquired immunodeficiency.

Cellular immunity to mycobacteria is crucial to the successful host response to infection, and antigen-specific Th1 T-cells that activate cell-mediated immunity by IFN-γ production have been shown experimentally to mediate mycobacterial clearance in infected

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animals (Flynn *et al*, 1993; Moody *et al*, 2000). Most studies of T-cell activation by mycobacteria have emphasized the role of MHC class II or class I-peptide antigen presentation systems. In the past decade, evidences have shown that CD1 molecules that bind lipids and present these to specific T-cells are involved in the generation of cell-mediated immune responses to mycobacterial pathogen (Ulrichs–*et al*, 2003). The discovery of the CD1 antigen presentation system offers an alternative and complementary mechanism by which mycobacterial glycolipids specifically activate T- cells or NKT cells (Moody *et al,* 2000). NKT cells recognize and respond to mycobacterial phosphatidylinositol tetramannoside in conjunction with CD1d (Fischer *et al*, 2004). The recognition of glycolipid antigens in association with CD1d means that NKT cells recognize a class of antigens ignored by conventional T-cells. The complex of glycolipids plus CD1d binds the T-cell receptor (TCR) of NKT cell, leading to activation of these cells in both mice and humans (Moody *et al*, 2000). When activated, NKT cells respond with vigorous cytokine production within 1-2 hours of TCR ligation (Godfrey *et al*, 2000). NKT cells are capable of secreting large amounts of cytokines, including IL-4 and IFN-γ (Campos-Martin *et al*, 2004).

However, the mechanisms that determine the cytokine polarity of the NKT cell response are not well understood. In support of the hypothesis of lipid antigen recognition by NKT cells, we investigated the responsiveness of NKT cells from *Mycobacterium* infected patients prior to treatment by analyzing the frequency and type of cytokine-producing NKT cells.

MATERIALS AND METHODS

Clinical samples

After obtaining informed consent, blood samples for PBMC isolation were obtained from *Mycobacterium tuberculosis*-infected patients and healthy volunteers. Blood samples from 17 of pulmonary TB patients (6 females and 11 males, mean age = 43.7 years) were collected prior to initiation of anti-TB chemotherapy. These patients presented at the chest clinic of HRH Maha Chakri Sirindhorn Medical Center in Nakhon Nayok Province, Thailand. The diagnosis of TB was confirmed by culture of *M. tuberculosis*. Thirteen control subjects (8 females and 5 males; mean age = 36.6 years) with no history of tuberculosis exposure were recruited from laboratory and hospital personnel. At the time of blood collection, none of them had any known history of TB infection. Other inclusion criteria for the two study groups were absence of a positive human immunodeficiency virus test and any other known concurrent infection. This study had been reviewed and approved by the Institutional Ethical Review Board, Faculty of Medicine, Srinakharinwirot University.

Antigen preparation

Antigen of *M. tuberculosis* (strain H37RV KK11-20) was prepared by suspending 10 mg of dried bacilli in one ml of ice-cold phosphate-buffered saline (PBS) and sonicating with a probe sonicator (Sonic Vitra Cell™). This *M. tuberculosis* sonicate represents a suspension of all of the antigens of the bacillus (Ulrichs *et al,* 2003).

Peripheral blood mononuclear cell preparation

Blood samples were collected and peripheral blood mononuclear cells (PBMC) were isolated by using Isoprep (Robbin Scientific Corporation Sunnyvale, CA.) within 2-4 hours. Isolated PBMC were suspended in complete medium [RPMI 1640, 10 mM HEPES buffer, 200 mM L-glutamine, 5 U of streptomycin-penicillin/ml (all from Gibco-BRL, Rockville, MD)] supplemented with 10% bovine serum albumin (Sigma, St Louis, MO.) and were cultured in plastic tissue culture tube for 3 hours in the presence of 20 μl of TB antigen and 10

μl of Belfidine (BD Biosciences, San Jose, CA). After incubating in 5% CO_2 incubator, cells were removed and washed in PBS. After washing, the number of PBMCs was determined with a blood cell counter, and the cell suspension was adjusted to a concentration of $2x10^6$ cells/ml PBS. Activated cells were subsequently aliquoted for staining.

Surface marker and intracellular cytokine staining

The following monoclonal antibodies (mAbs) were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-CD161 (Serotec, Oxford, UK); Tricolor - conjugated anti-CD3, R-phycoerythrin conjugated anti-human IL-4, R-phycoerythrin conjugated anti-human IFN-γ and isotype control $\gamma_1\gamma_2$ mAb (all from Caltag Laboratories, Burlingame, CA).

PBMC ($2x10^5$ cells) were surface-stained with FITC-conjugated anti-CD161 mAb and Tricolor - conjugated anti-CD3 at room temperature for 20 minutes in the dark. For isotype control, PBMC of each sample were stained with isotype control $\gamma_1\gamma_2$ mAb in parallel. After two washes in PBS, cells in all tubes including isotype control were fixed and permeablized by using Fix&perm® (Caltag Laboratories, Burlingame, CA) according to manufacturer's protocol. Then R-phycoerythrin conjugated anti-human IFN-γ and anti-human IL-4 were added to separate aliquots of membrane-stained and permeabilized cells. Cells in both tubes were further incubated for 30 minutes at room temperature in the dark. Sample tubes were washed 2 times with PBS and resuspended in 2% paraformaldehyde and then stored at 4ºC until analyzing by FACS Calibur instrument (BD Biosciences, San Jose, CA) with CELLQUEST software (BD Biosciences).

Statistical analysis

Nonparametric methods were used since the data were not normally distributed. The data from the two groups (cells from TB patients and cells from control subjects) were compared by using Mann-Whitney *U*-test (SPSS 11.5). In determining the significant difference between IFN-γ and IL-4-producing cells in mycobacterium infected patients, Wilcoxson–rank tests (SPSS 11.5) were carried out. The level of significance was set at $p<0.05$. Data were given as median and interquartile range (percentile 25 - percentile 75).

RESULTS

Lymphocyte subpopulation from tuberculosis-patients and control subjects

Using three-color immunophenotypic data, cell surface markers of T- lymphocyte were analyzed for 10,000 lymphocytes. The median and interquartile range of $CD3^+$ (T-lymphocyte), $CD161^+$ (NK cell) and $CD3^+/CD161^+$ (NKT cell) from tuberculosis- patients was 72.1 (53.7-76.4), 3.5 (3.0-4.35) and 1.5 (1.25-1.85), respectively. The median and interquartile range of $CD3^+$ (T-lymphocyte), $CD161^+$ (NK cell) and $CD3^+/CD161^+$ (NKT cell) from control subjects was 73.1 (64.75-76.95), 3.4 (2.85-3.85) and 1.1 (0.9-1.4), respectively. Significant difference was observed only in the number of NKT cells ($p = 0.012$).

Comparison of cytokine producing cells between tuberculosis patients and control subjects

Since non-stimulated T-cells usually do not produce measurable amounts of cytokines, *in vitro* stimulation is required. Mycobacterium antigen under the influence of Brefeldin A was used to stimulate production and intracellular accumulation of cytokines, respectively. The frequencies of cytokine producing cells were determined. In TB infected patients, the frequency (median and interquartile) of IFN-γ-producing T cells 1.5 (1.2-1.85) and IL-4-producing T cells 1.1 (0.9-1.45) were higher ($p<0.01$) than those from control subjects, 0.6 (0.5-0.65) and 0.5 (0.2-0.8), respectively. Similarly, the frequency of IFN-γ-producing NKT cells from tuberculosis patients, 0.9 (0.71-1.2) was greater ($p<0.01$)

than those in control, 0.31 (0.24-0.41), as well as IL-4- producing NKT cells from tuberculosis patients, 1.2 (0.9-1.38), compared with control subjects, 0.36 (0.27-0.46) ($p<0.01$). In contrast, frequency of IFN-γ and IL-4-producing NK cells did not show any significant difference between patients and controls ($p=0.3$ and $p=0.12$, respectively).

In tuberculosis patients the frequency (median and interquartile range) of IFN-γ-producing T cells, 1.5 (1.2-1.85), is not significantly different from that of IL-4-producing T cells, 1.1 (0.9-1.45). Similarly, there is no significant difference between frequency of IFN-γ-producing [0.4 (0.2-1.0)] and IL-4-secreting NK cells [0.5 (0.25-1.1)]. However, the percentage of IFN-γ and IL-4-producing NKT cells in infected patients was 57.4% and 70.4%, indicating that some of these cells secreted both types of cytokines. This phenomenon was observed only in infected patients.

DISCUSSION

In this study, we observed no significant difference in percentage of T-lymphocyte subpopulations between tuberculosis patients and control subjects. A similar observation has been reported by others (Bhattacharyya *et al*, 1999). This study did not detect a quantitative difference in the number of CD161+ (NK cells) in tuberculosis patients compared with control subjects. This result is consistent with the notion that local factors at the lesional site appear to critically influence the migration, sensitization, and activation of these NK cells (Carbone *et al*, 2000). Activated NK cells migrate and often be found in the spleen and liver (Ortaldo *et al*, 2004). However there is a significant increased in NKT cells in TB infected patients. The recruitment of NKT cells into circulation might be promoted by TB infection since these cells recognize lipid antigens (usually glycolipid antigens) in association with CD1d. Increase in NKT cells may contribute to host resistance against *M. tuberculosis*.

Although the cytokine synthesis potential of individual cell is largely unknown, it has been accepted that response with a predominance of IFN-γ and IL-4- producing T- cells is termed “Th1” or “Th2” response, respectively (Picker *et al*, 1995). In this study, cytokine-producing T cells are significantly higher in untreated TB patients than those in control subjects. Activation of cellular immune response by mycobacterium may increase various types of lymphocyte subpopulations, in particular T-lymphocytes. However, the ratio of IFN-γ and IL-4-producing T cells was similar in patients and control subjects. The dominant cytokine pattern was a combination of Th1 and Th2 cytokines. Earlier studies in patients with pulmonary TB have revealed a higher production of Th1 cell type in moderate TB, with predominant Th2-like response in advanced disease (Dlugovitzky *et al*, 2000). Mycobacterium-reactive T-cell clones from healthy tuberculin reactors exhibit a Th1-like pattern of cytokine production, characterized by high concentration of IFN-γ but low concentration of IL-4 and IL-5 (Barnes *et al*, 1993). Our results indicated no significantly higher frequency of IFN-γ-producing T cells from TB patients compared to control subjects. There are evidences suggesting that suppression of IFN-γ, along with the maintenance of the steady-state of IL-4 production, could be responsible for the persistence and progression of tuberculosis in susceptible individuals (Bhattacharyya *et al*, 1999). Differences in cytokine patterns of T-cells derived from different laboratories may result from variability in antigen specificity, as distinct microbial antigens can favor preferential development of Th1 or Th2 (Jardim *et al*, 1990; Liew *et al*, 1990).

Although the role of NK cells in mycobacterium infection is unclear, it has been postulated that NK cells could participate in the early innate immune response to mycobacterium infection (Junqueira-Kipnis *et al*, 2003). The

primary function of NK cells is their capacity to act as cytolytic cells, so they are not believed to be essential for protection (Scharton and Scott, 1993). Nevertheless, other studies have shown that they can also produce either IFN-γ or IL-4 in response to *M. tuberculosis* infection (Junqueira-Kipnis *et al*, 2003; Olsen *et al*, 2005). Our results demonstrate a balance between IFN-γ and IL-4-producing NK cells. Based on previous studies, NK cells responded to extracellular BCG with proliferation, IFN-γ production, and cytotoxicity (Vankayalapati *et al*, 2004).

A hallmark of NKT cells is their capacity to quickly produce large amounts of cytokines. When activated, these cells release Th1-type cytokines, including IFN-γ and TNF, as well as Th2-type cytokines, including IL-4 and IL-13. Individual NKT cells were able to make both Th1-and Th2-type cytokines following stimulation, in agreement with previous studies (Zeng *et al*, 1997; Chackerian *et al*, 2002; Godfrey and Kronenberg, 2004). There was a higher frequency of IL-4 producing than IFN-γ-producing cells. The role for NKT cell-derived IL-4, a mediator of humoral immunity, is suggested by studies demonstrating that NKT cells are necessary for the formation of granuloma-like lesions in mice exposed to mycobacterial cell walls (Schmieg *et al*, 2005). In contrast, the predominance of IFN-γ-producing NKT cells had been reported in mice following *M. tuberculosis* infection (Chackerian *et al*, 2002). NKT cell-derived IFN-γ might be particularly beneficial in the setting of mycobacterium infection because the virulence of *M. tuberculosis* strains from human clinical isolates varies inversely with the ability to induce a Th1 response (Schmieg *et al*, 2005). Difference in cytokine patterns might be due to the duration of TCR stimulation (Oki *et al*, 2004) and variation in antigen specificity, leading to differential cytokine production by NKT cells (Barnes *et al*, 1993). Overall, it appears that activated NKT cells can initiate both Th1 and Th2 immune response that can contribute to host defense against *M. tuberculosis* infection (Gansert *et al,* 2003). However, the mechanism that determines cytokine polarity of NKT cell response and the influence of the NKT cell response are not well understood. These problems present a key challenge in the field of NKT cell research.

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