Türk Göğüs Kalp Damar Cerrahisi Dergisi 2013;21(2):422-427
doi: 10.5606/tgkdc.dergisi.2013.7104

Original Article / Özgün Makale

The effect of Bacille Calmette-Guerin on the immunopathogenesis of tuberculous pleurisy

Tüberküloz plörezi immünopatogenezine Bacille Calmette Guerin'in etkisi

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Background: This study aims to investigate the role of $CD4^+$ and $CD8^+$ T cells and T helper (Th)1/Th2 lymphocyte cell balance in the immunopathogenesis of tuberculous pleurisy and the immunomodulation resulting from Bacille Calmette-Guerin (BCG) were investigated.

Methods: A total of 26 patients, who were pathologically diagnosed as tuberculous pleurisy, including 10 of whom had no BCG vaccine and 16 of whom had BCG vaccine, were included. Total $CD3^+$, $CD4^+$, $CD8^+$ T cells, total $CD19^+$ B cells and natural killer cell ratio as well as intracellular interferon-gamma (IFN-γ), interleukin (IL)-2, IL-4, IL-5 and IL-10 expression in pleural fluid lymphocytes were analyzed.

Results: In BCG positive group, $CD8^+$ T lymphocytes were significantly increased. Intracellular IL-4 levels of lymphocytes in pleural fluid were significantly reduced in these subjects, compared to BCG negative subjects.

Conclusion: BCG vaccine may suppress Th2 type immune response and also lead to increased levels of $CD8^+$ T cells. $CD8^+$ T cells may play an important role in the immunopathogenesis of tuberculous pleurisy.

Key words: Bacille Calmette Guerin; immunopathogenesis; tuberculous pleurisy.

Amaç: Bu çalışmada tüberküloz plörezi immünopatogenezinde $CD4^+$ ve $CD8^+$ T hücrelerinin rolü ve T helper (Th)1/Th2 lenfosit hücre dengesi ve Bacille Calmette Guerin'in (BCG) neden olduğu immünomodülasyon incelendi.

Çalışma planı: Çalışmaya patolojik olarak tüberküloz plörezi tanısı konulmuş, 10'u BCG aşısız ve 16'sı BCG aşılı olmak üzere toplam 26 hasta dahil edildi. Total $CD3^+$, $CD4^+$, $CD8^+$ T hücreleri, total $CD19^+$ B hücreleri ve doğal katil hücre oranı ve yanı sıra plevra sıvısı lenfositlerinden hücre içi interferon-gama (IFN-γ), interlökin (IL)-2, IL-4, IL-5 ve IL-10 düzeyleri analiz edildi.

Bulgular: BCG pozitif grupta, $CD8^+$ T lenfositler anlamlı düzeyde arttı. Bu kişilerde plevra sıvısındaki lenfositlerin hücre içi IL-4 düzeyleri, BCG negatif kişilerle kıyaslandığında, anlamlı düzeyde azaldı.

Sonuç: BCG aşısı, Th2 tipinde immün yanıtı baskılayabilir ve ayrıca $CD8^+$ T hücre düzeylerinin artmasına neden olabilir. $CD8^+$ T hücreleri, tüberküloz plörezi immünopatogenezinde önemli bir rol oynayabilir.

Anahtar sözcükler: Bacille Calmette Guerin; immünopatogenez; tüberküloz plörezi.

Tuberculosis is the second leading cause of death from an infectious disease worldwide after human immunodeficiency virus (HIV), which caused an estimated 1.8 million deaths in 2008.[1] In 2010, there were an estimated 8.5-9.2 million cases and 1.2-1.5 million deaths associated with tuberculosis, including deaths from patients who were also HIV-positive. Shockingly, one-third of the world's population is infected with tuberculosis.[2] However, only 10% of people who were infected with *Mycobacterium tuberculosis (M. tuberculosis)* develop active tuberculosis, which could be a result of the balance between effector

Available online at
www.tgkdc.dergisi.org
doi: 10.5606/tgkdc.dergisi.2013.7104
QR (Quick Response) Code

Received: May 13, 2012 *Accepted:* August 16, 2012

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immune responses and immunosuppressive reactions. Extrapulmonary tuberculosis makes up 11.3% of all tuberculosis cases,[3] and tuberculous pleurisy is the second most frequent manifestation of this type of tuberculosis after tuberculous lymphadenitis.[4] Tuberculosis pleurisy resolves spontaneously in some patients without anti-tuberculosis treatment, and it is thought to be a good model for studying the protective immune response at the site of infection.[5] In tuberculous pleurisy, the T helper (Th) 1 response is predominant *in vivo*,[6] and Th1 cytokines, such as interferon-gamma (IFN)-γ and interleukin (IL)-2, play key roles in controlling *M. tuberculosis* infection.

Bacille Calmette-Guerin (BCG) is the single most commonly used vaccine for tuberculosis in the world, but its is under suspicion due to potential ineffectiveness. The efficiency of BCG ranged from 0-80% in various series that have been conducted in different countries in the world.[7] The BCG vaccine prevents children from getting tuberculosis, but it is insufficient for preventing adults from getting lung tuberculosis, and the immunity levels decrease with age. It is not totally known what changes BCG causes in the course of the disease, and developed countries, such as the United States no longer use it to vaccinate children against tuberculosis; however, but it is still routinely used for immigrant children, people working in hospitals or laboratories, and those having a high risk for tuberculosis.[8] Detecting the cause of a positive tuberculin skin test in people who were vaccinated with BCG is impossible since the result could either be caused by a virulent mycobacteria or the vaccine itself.[9] Because of this, tuberculin skin tests (TSTs) are limited in their ability to diagnose tuberculosis in people that have received this vaccine.

The aim of this study was to detect the contribution of T cells, B cells, and natural killer (NK) cells along with the balance of Th1/Th2 lymphocyte subsets as they relate to the immunopathogenesis of tuberculous pleurisy and the immunomodulation caused by BCG.

PATIENTS AND METHODS

Study population

This study was a prospective multicenter clinical study that involved a total of 26 patients who had been pathologically diagnosed with tuberculosis pleurisy. Ten of the patients had not received the BCG vaccine while 16 had been vaccinated. Those who had chronic diseases affecting immunity, such as diabetes mellitus (DM), hypertension, congestive heart failure, immunological diseases or disorders, or malignancy, were excluded. In addition, all patients were HIV-negative, and none were receiving antituberculous treatment. Pleural biopsies were done after thoracentesis, and the diagnosis was made histopathologically. Any scars on the left shoulders of the patients related to the use of BCG were noted. The demographic characteristics of the BCG (+) and BCG (-) pleural tuberculosis patients are summarized in Table 1. This study was conducted according to the principles expressed in the Declaration of Helsinki, and ethical approval was obtained from the (Gülhane Military Medical Faculty (GATA) ethics committee. All patients provided their written informed consent for the collection of samples and subsequent analysis.

Cell preparation

The pleural fluid samples were collected in heparinized tubes, and the pleural fluid mononuclear cells (PFMCs) were separated by Ficoll-Hypaque (Sigma-Aldrich Co., St. Louis, Missouri, USA) density gradient centrifugation. The interfaced cells were then harvested and washed in phosphate buffer saline (PBS) and resuspended in a complete RPMI-1640 (Sigma-Aldrich Co., St. Louis, Missouri, USA) medium containing 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml), gentamicin (50 mg/ml), and 50 µM 2-mercaptoethanol at a concentration of $2x10^6$ cells/ml.

Determination of T Lymphocyte Surface Phenotypes

After purification of the PFMCs, immunofluorescent staining for flow cytometric analyses were employed,

Table 1. Study population

	Bacille Calmette-Guerin vaccination							
	Positive				Negative			
	n	%	Mean±SD	Median	n	%	Mean±SD	Median
Age			28±9.1	25.5			26.6±15.6	21
Gender								
Female	1	6.3			–	–		
Male	15	93. 8			10	100.0		

SD: Standard deviation.

and two-color flow cytometry was performed to determine the phenotypes in the T lymphocytes in the pleural fluid. The monoclonal antibodies (mAbs) used for this study included anti-human CD45-fluoroscein isothiocyanate (FITC)/anti-CD14-phycoerythrin (PE), anti-CD4-FITC/CD8-PE, anti-CD3-FITC/CD19-PE, anti-CD3-FITC/CD16+56-PE, and anti-CD5-FITC/CD19-PE, and appropriate PE- or FITC-conjugated isotype control (IC) mAbs (BD Biosciences, San Jose, California, USA). Next, $5x10^5$ cells were incubated in the dark at room temperature for 30 minutes with the mAbs at the concentrations recommended by the manufacturer and washed once in the PBS. Then the stained cells were fixed in 1% paraformaldehyde (Sigma-Aldrich Co., St. Louis, Missouri, USA). Acquisition was performed on a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, California, USA), and $3x10^4$ events were collected for each sample. Analysis was performed using BD CELLQuest™ (BD Biosciences, San Jose, California, USA) on list-mode data, and the lymphocyte gate was defined by forward/side scatter characteristics.

Intracytoplasmic cytokine staining of the pleural fluid lymphocytes

The purified PFMCs were washed and $1x10^6$ cells/ml were stimulated for cytokine production. The cells were incubated with a combination of phorbol ester phorbol 12-myristate 13-acetate (PMA) 50 ng/ml and 250 ng/ml ionomycin (a calcium ionophore agent) (Sigma-Aldrich Co., St. Louis, Missouri, USA) for 18 hours. Brefeldin A (BFA) (Sigma-Aldrich Co., St. Louis, Missouri, USA) was added at a final concentration of 10 µg/mL during the last three hours of the culture since it significantly increases the ability to detect cytokine-producing cells by immunofluorescent staining. After incubation, the PFMCs were washed with a PBS solution and then fixed and permeabilized with a Fix & Perm cell permeabilization kit (Caltag Laboratories, Burlingame, California, USA) that contained a paraformaldehyde/saponin solution. After washing, the cells were stained with PE or FITC-conjugated IC (IgG1), anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-10, and anti-IFN-γ (Caltag Laboratories, Burlingame, California, USA) mAbs for 30 minutes at room temperature. After washing, the cells were resuspended in the 1% paraformaldehyde at +4 °C and analyzed by the FACSCalibur flow cytometer. Cell debris was excluded by threshold, and the results were presented as mean values of percentages and standard deviation.

Statistical analysis

The data was expressed as mean ± standard deviation, and statistical analysis was performed by a chi-square test or the Mann-Whitney U test using the SPSS (SPSS Inc., Chicago, Illinois, USA) version 11.5 for Windows software program.

RESULTS

Increased expression of CD8⁺ T-cell subsets from the pleural fluid from patients vaccinated with Bacille Calmette-Guerin

Cellular components from the pleural fluid were analyzed by flow cytometry. After isolating the PFMCs, the cells were stained with mAbs against T cells, B cells, and NK cells and also analyzed by flow cytometry. After the analyses, the expression of activated $CD8^+$ cytotoxic T cells was significantly higher in the tuberculosis pleurisy patients who were vaccinated with BCG than in those who had not been vaccinated ($p=0.021$). However, no differences were detected between the two groups concerning the helper T lymphocytes, B lymphocytes, NK cells, and CD45 molecule expression (Figure 1).

Th1 and Th2 type cytokine secretion of lymphocytes from the pleural fluid

Differentiation of $CD4^+$ T cells is based on their profile of cytokine secretion. Th1 cells produce IFN-γ, IL-2, and tumor necrosis factor-beta (TNF-β) which

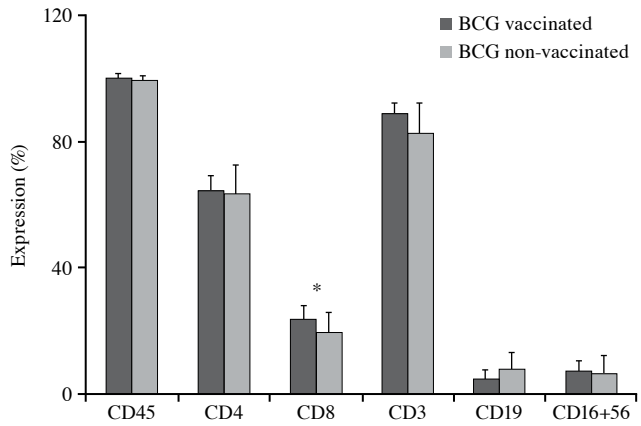

Figure 1. Flow cytometric analysis of pleural fluid lymphocyte subsets in tuberculosis pleurisy patients. After isolating the pleural fluid mononuclear cells from the peripheral blood, the cells were stained with fluorescent-conjugated monoclonal antibodies specific to lymphocyte subpopulations and analyzed by flow cytometry. The results of the lymphocyte markers are shown as mean ± standard deviation for 16 tuberculosis patients who were vaccinated with Bacille Calmette-Guerin (BCG) and 10 patients who were not.

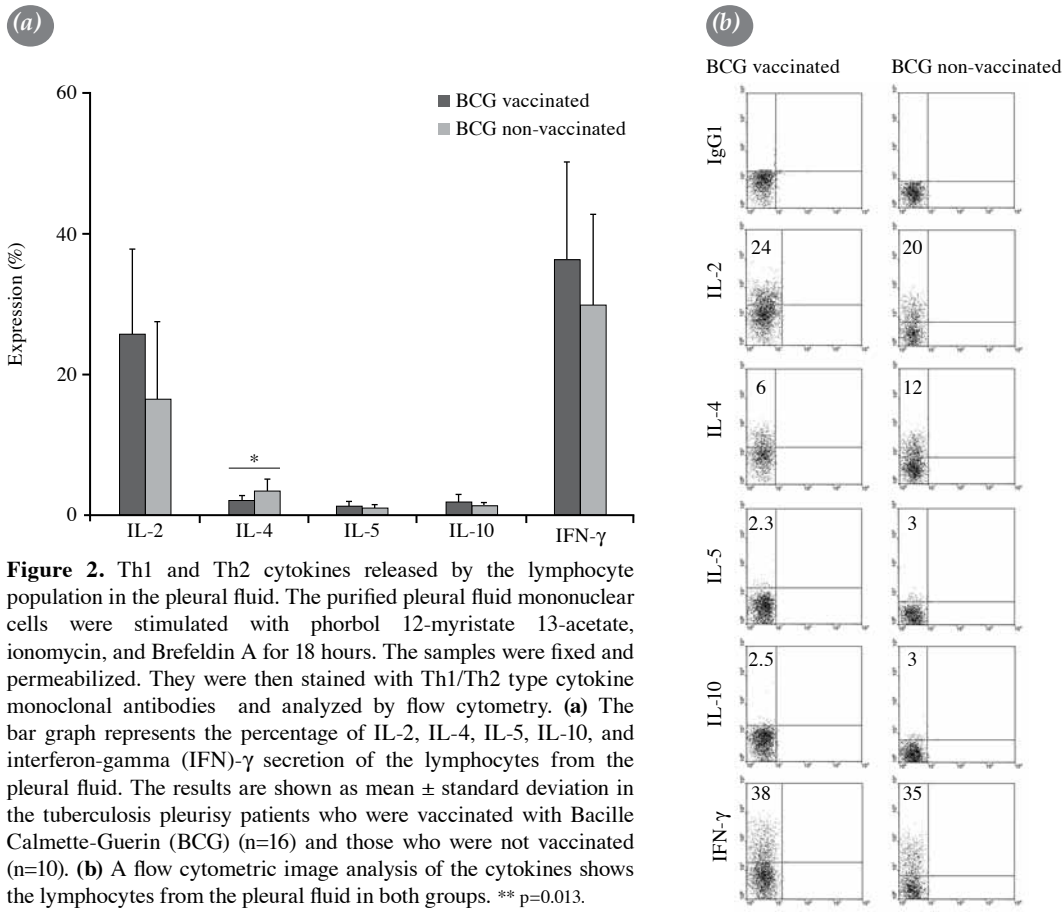

Figure 2. Th1 and Th2 cytokines released by the lymphocyte population in the pleural fluid. The purified pleural fluid mononuclear cells were stimulated with phorbol 12-myristate 13-acetate, ionomycin, and Brefeldin A for 18 hours. The samples were fixed and permeabilized. They were then stained with Th1/Th2 type cytokine monoclonal antibodies and analyzed by flow cytometry. **(a)** The bar graph represents the percentage of IL-2, IL-4, IL-5, IL-10, and interferon-gamma (IFN)-γ secretion of the lymphocytes from the pleural fluid. The results are shown as mean ± standard deviation in the tuberculosis pleurisy patients who were vaccinated with Bacille Calmette-Guerin (BCG) (n=16) and those who were not vaccinated (n=10). **(b)** A flow cytometric image analysis of the cytokines shows the lymphocytes from the pleural fluid in both groups. ** p=0.013.

activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses.

To analyze Th1 and Th2 type cytokines, the PFMCs were stimulated with a combination of PMA and ionomycin for 18 hours. The cells were stained with IL-2, IL-4, IL-5, IL-10, and IFN-γ mAbs and analyzed by flow cytometry. The expression of the IL-4 level was significantly lower in the tuberculosis patients who had been vaccinated with BCG than in those who received no vaccinations (p=0.013). However, there were no significant differences with respect to IL-2, IL-5, IL-10, or IFN-γ content between the two groups (Figures 2a and 2b).

DISCUSSION

Tuberculosis is still the most common and most deadly infectious disease. One-third of the world's population is infected with *M. tuberculosis*, but more than 90% of the infected people never develop active disease in their lifetime because of an efficient immune system. This demonstrates the importance of immunity in tuberculosis. Although this disease has been known and treated for many years, its immunopathogenesis is still not clearly understood. Because of the limited number of anti-tuberculosis drugs available for treatment and drug resistance, there is a need for new anti-tuberculosis drugs and vaccines for tuberculosis besides BCG, which is now used in many countries.

Tuberculous pleurisy and tuberculosis lymphadenitis account for 18.7% of all tuberculosis cases and make up 60.2% of the cases of extrapulmonary tuberculous.[10] There have been many studies concerning the immunopathogenesis of tuberculous pleurisy, and it is known that its immunological changes resemble the ones found in lung tuberculosis. Therefore, tuberculosis pleurisy is accepted as an *in vivo* model of the protective immune response in tuberculosis immunopathogenesis.[5] It is characterized by antigen-specific IFN-γ production and an increase in the number of $CD4^+$ T lymphocytes.[11] T helper cells have two main groups with antigen specificity, the Th1 and Th2 cells.[12] The Th1 cells produce IL-2 and IFN-γ and affect immunity by the activation of macrophages

and cell-mediated immunity while also causing organ-specific autoimmune diseases. The Th2 cells produce IL-4, IL-5, IL-10, and IL-13 and play a role in humoral immunity. They are efficient in inhibiting the functions of macrophages by producing proinflammatory cytokines.[13]

Tuberculous pleurisy is usually a type of primary tuberculosis, and there is a Th1 type cellular immune response. Miliary tuberculosis is a form of uncontrolled disseminated infection and is usually associated with a Th2 type immune response.[14] The efficacy of the tuberculosis infection and disease control is determined by the predominancy of either the Th1 or Th2 type cytokines.

Interleukin-4 is a pleiotropic cytokine produced by Th2 cells. It regulates immune responses in T cells, B cells, and macrophages and is a key cytokine in driving Th2 differentiation and mediating humoral immunity. In the tuberculosis pleurisy patients who were vaccinated with BCG in our study, the IL-4 secretions in the Th2 type cytokines were decreased. This finding is consistent with evidence from a previous study in which the role of IL-4 in tuberculosis was studied in guinea pigs, a highly relevant model for this disease.[15] The BCG vaccination reduced the expression of IL-4 messenger ribonucleic acid (mRNA) in both the spleen and lung digest cells in the guinea pigs with tuberculosis compared with those with the disease, but the levels of IFN-γ were similar in both groups. The Th2 type immune response may be suppressed by BCG, and IL-4 can undermine the Th1 mediated immune response during tuberculosis and impair antimicrobial immunity. Furthermore, BCG may downregulate the negative regulators of Th1 immunity like IL-4. According to our results, we suggest that there is a Th1 type immune response in patients with tuberculous pleurisy who receive BCG vaccinations; however, the IL-5 and IL-10 levels between the two groups in our study were not statistically significant.

T lymphocytes play the primary role in the cellular immune response against tuberculosis. There were no statistically significant differences in the total lymphocyte and T lymphocyte subsets, including the $CD4^+$ T-cell levels, between the two patient groups in our study. The immune response after *M. tuberculosis* infection is mainly dependent on the $CD4^+$ T cells. The special characteristics of *M. tuberculosis* affect the presentation of antigens along with the ability to process them and determine the protective immunity achieved by the CD4 T cells.[16,17] Interferon-gamma is a key cytokine for protective immunity against tuberculosis. People who are deficient in producing IFN-γ are more prone to getting sustained mycobacterial infections, including tuberculosis.[18] Exogen IFN-γ may be an alternative tuberculosis treatment option in the future.[19] In our study, there were not any statistical differences in IFN-γ and IL-2 levels between the two patient groups. Since both IL-2 and IFN-γ are Th-type cytokines, this result showed that the BCG vaccine did not cause any differences in the Th1 type immune response in our patients.

The $CD8^+$ T lymphocytes may play a role in the regulation of the Th1/Th2 balance by producing IL-4 and IFN-γ in tuberculosis. In our study, $CD8^+$ cytotoxic T lymphocytes were observed to be higher in the patients who were vaccinated with BCG, but there were no differences in the ratios of $CD4^+$ T cells, B cells, and NK cells in the two groups. The $CD8^+$ T lymphocytes may also play a vital role in the immunopathogenesis of tuberculous pleurisy. In addition, they contribute to macrophage activation by producing IFN-γ and are also capable of exhibiting cytolytic functions.[20] It has been shown that serum IL-2, IL-4, and tumor necrosis factor-alpha (TNF-α) levels are diminished in pulmonary tuberculosis, which suggests that $CD8^+$ T cells produce both Th1 and Th2 type cytokines and that these may have an crucial role in the peripheral immune response to mycobacteria.[21]

Natural killer cells can produce IFN-γ and eliminate target cells infected with tuberculosis. Decreased activity of NK cells may help in the fight against multi-drug resistant tuberculosis (MDR-tuberculosis).[22] In our study, there were no statistical differences in the NK cell ratios between the patients vaccinated with BCG or those who were not.

The BCG vaccine has been used for tuberculosis control in the world since 1928. The World Health Organization (WHO) recommends a one-time administration of BCG at birth in endemic countries. The efficacy of BCG vaccinations as measured by case reduction has varied from 0% to greater than 80%. In India, the T-cell responses were evaluated in children who had received BCG vaccinations,[23] and Th1 type immune responses were found in the majority of these children.

Since the BCG vaccine especially protects against disseminated tuberculosis in childhood and is also effective against leprosy, it is widely used throughout the world. However, this vaccine has not caused statistically significant changes in the prevalence of tuberculosis. Nevertheless, providing BCG vaccinations at childhood is still the most reasonable way to fight tuberculosis in countries with a high incidence rate.

Prophylactic treatment strategies and tuberculosis contact examinations are more beneficial and efficient in countries with low rates of tuberculosis. The efficacy of BCG should be increased, especially for pulmonary tuberculosis, and new vaccines that would offer more protection against this type of tuberculosis should be developed in the future. According to our results, the IL-4-secreting pleural lymphocytes decreased, and the number of $CD8^+$ T lymphocytes increased in the patients who received BCG vaccinations, and the vaccine may suppress the Th2 type immune response and also cause increased levels of $CD8^+$ T cells, which may play an important role in the immunopathogenesis of tuberculous pleurisy.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

This study was financially supported by Working Capital of GATA Haydarpaşa Training Hospital.

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