

Tanaffos (2007) 6(1), 59-65

©2007 NRITLD, National Research Institute of Tuberculosis and Lung Disease, Iran

Immune Responses of CD₄⁺ T-Cells of MDR-TB Patients to *M. Tuberculosis* Total Lipid Antigens

Ali Shams Shahemabadi¹, Ahmad Zavaran Hosseini¹, Majid Rayani², Majid Valiollahpour Amiri^{2,3}, Mohammad Reza Masjedi⁴

¹ Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, ² Department of Infectious Diseases, ³ Mycobacteriology Research Center, ⁴ Department of Pulmonary Medicine, NRITLD, Shaheed Beheshti University of Medical Sciences and Health Services, TEHRAN-IRAN.

ABSTRACT

Background: CD₄⁺ T-cell have a central role in protective immune responses to *Mycobacterium tuberculosis* (*M. tuberculosis*) protein antigens, but function of these cells in response to *M. tuberculosis* total lipid antigens has remained unclear. The present study was undertaken to determine role of CD₄⁺ T cells in the MDR-TB patients against *M. tuberculosis* total lipid antigens.

Materials and Methods: CD₄⁺ T-cells were isolated from MDR-TB patients and stimulated with *M. tuberculosis* total lipid antigens. Proliferative responses and cytokine secretion were assessed by MTT and ELISA, respectively.

Results: Our study results showed that proliferative responses of stimulated CD₄⁺ T-cells to *M. tuberculosis* total lipid antigens and IFN- γ production in MDR-TB patients were significantly lower than those of the PPD-positive subjects ($P < 0.05$) whereas, IL-4 production in the MDR-TB patients was elevated ($P < 0.05$).

Conclusion: Responses of CD₄⁺ T-cells of MDR-TB patients to total lipid antigens was significantly lower than that of PPD-positive healthy subjects. Therefore, it seems that *M. tuberculosis* lipid antigens, as protein antigens, have an important role in specific immune response. (Tanaffos 2007; 6(1): 59-65)

Keywords: *M. tuberculosis*, Multidrug-resistant tuberculosis patients, Lipid antigens, IFN- γ , IL-4

INTRODUCTION

Appearance of strains of *M. tuberculosis* resistant to the current antibiotics is a developing problem in many countries (1, 2). The strains of *M. tuberculosis* resistance to both isoniazid and rifampicin with or

without resistance to other drugs have been termed as multidrug-resistant (MDR) strains. In addition, co-infection of HIV-1 and *M. tuberculosis* in the MDR-TB patients due to suppressed immune system is concerned (3).

Numerous studies have shown that antigen-specific T cells protein and "Major Histocompatibility Complex" (MHC) systems play a major role in developing and maintaining immunity

Correspondence to: Zavaran Hosseini A

Address: Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Jalale-Ale-Ahmad Ave, Tehran, IRAN.

P.O.BOX: 14115-331

Email address: zavarana@modares.ac.ir

Received: 9 December 2006

Accepted: 8 February 2007

against *M. tuberculosis* (4). However, in recent years it has been clear that CD1 molecules are also involved in the generation of cell-mediated immune responses to mycobacterial pathogens (5). The human CD1 markers are a family of antigen presenting molecules that bind to lipids and present them to T-cells. The molecules are expressed constitutively on professional antigen-presenting cells and can be induced by immature dendritic cells derived from peripheral blood monocytes by treatment with granulocyte-macrophage-Colony Stimulating Factor (GM-CSF) and IL-4 (6). CD1-restricted T-cells can contribute to protective immunity by the production of high levels of IFN- γ and IL-4 (7). Administration of *M. tuberculosis* lipid vaccine to guinea-pig created considerable immune responses (8). It has recently been revealed that type of lipids contributes to the severity of *M. tuberculosis* strain infection (9).

The exact role and relative importance of this novel pathway for antigen recognition in generating protective immunity against *M. tuberculosis* especially in TB and MDR-TB patients remain poorly understood. The present study was undertaken to determine the role of CD4⁺ T-cells in MDR-TB patients against *M. tuberculosis* total lipid antigens.

MATERIALS AND METHODS

Preparation and evaluation of *M. tuberculosis* antigens:

M. tuberculosis strain H37Rv total sonicate antigens were prepared by using the previously described methods (8). Briefly, the suspension of *M. tuberculosis* (20 μ g/ml in RPMI-1640) was sonicated and centrifuged. The supernatant was filtered and stored at -20 °C as the total sonicate antigens.

The Folch procedure with some modifications was used to produce *M. tuberculosis* total lipid antigens as previously described (10). Briefly, the dried bacteria were resuspended in chloroform:methanol (2:1) and sonicated. The suspension was centrifuged and the supernatant was removed and

dried. The extracted lipid antigens were weighed and a concentration of 20 μ g/ml was prepared in chloroform:methanol (2:1) and stored at -20 °C.

To evaluate the possible contamination of protein in the extracted lipid solution, the Lamelli SDS-PAGE procedure was carried out on the dried lipid and silver staining method was used according to the procedure described previously (11). The extracted lipid was also analyzed by Thin-Layer Chromatography (TLC) as described (12).

Human subjects

There were 115 individuals in this study; out of which 30 were healthy tuberculin skin test positive (PPD-positive) donors (14 females, 16 males, mean age: 35.7 years), 30 were healthy tuberculin skin test negative (PPD-negative) subjects (13 females, 17 males, mean age: 33.5 years), 30 were new smear positive TB patients (18 males, 12 females, mean age: 47.7 years), and 25 were MDR-TB patients (12 males, 13 females, mean age: 44.2 years). All patients and healthy individuals were selected from Masih Daneshvari Hospital donors and personnel or patients' companions, respectively. Positive skin test would be confirmed if the diameter of induration at the site of injection was >10 mm. New smear positive TB patients had positive results for smear and culture examination. MDR-TB cases had the following inclusion criteria: they had a history of at least one course of TB treatment under the centers for direct observation (6 months documentation), two positive sputum smear tests and a positive sputum culture. Their susceptibility testing showed resistance to isoniazid and rifampin, and their chest X-ray and clinical symptoms were compatible with pulmonary tuberculosis. All patients and healthy volunteers consented to take part in this study and 15-20 ml of heparinized peripheral blood samples were collected from the subjects.

Preparation of cells and magnetic cell sorting:

Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphodex (Inno-Train) density centrifugation. The cells were resuspended in

complete RPMI-1640 medium (10 mM HEPES buffer, 200 mM L-glutamine, 50 U of streptomycin-penicillin /ml , all from Gibco) and cultured for 2 h in 37°C to allow firm adherence of monocytes. After adherence of monocytes, nonadherent PBMCs were isolated and monocytes were cultured in the complete medium supplemented with 10% AB serum (Sigma Co.) and 200 u/ml IL-4 (R&D Minneapolis MN) and 400 u/ml GM-CSF (Roche, Germany). On the third day, the same doses of IL-4 and GM-CSF were added and in the fifth day, the immature dendritic cells were detached and irritated with 5000 rads. For the enrichment of CD4⁺ cells, non-adherent PBMCs were incubated with anti-CD4 magnetic microbeads (Miltenyi-Biotec) according to the protocol. The separated cells were analyzed for CD4 marker using anti-CD4 and anti-CD3 antibodies (DakoCytomation, Denmark) by flow cytometry.

MTT and cytokine assay:

Immature dendritic cells were cultured in a flat-bottom micro titer plate at 30,000 cells/well in 0.20 ml of complete medium and were stimulated by *M. tuberculosis* total sonicate and total lipid antigens in 20 µg/ml concentration and incubated for 24 h. Autologous CD4⁺T-cells with density of 100,000 cells/well were added and incubated for 72 h. The supernatants of CD4⁺T- cells were collected for IL-4 and IFN-γ ELISA measurements. Cytokine concentrations in pg/ml were measured with ELISA Quantikine human IL-4 and IFN-γ immunoassay kits (R&D) as duplicate according to the manufacturers' instructions. MTT assay was carried out as described on the remaining cells (13).

Statistical methods

Statistical analysis was performed by nonparametric analysis and Mann-Whitney U test. A value of $p < 0.05$ was considered as significant.

RESULTS

Cell analysis and proliferation responses:

Flow-cytometric analysis showed that more than

95% of separated cells expressed CD3⁺CD4⁺ cells (Figure 1).

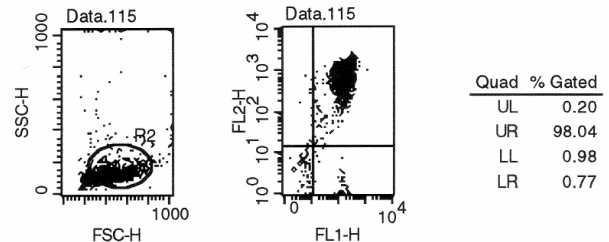


Figure 1. Analysis of separated CD3⁺CD4⁺ T-cells: Nonadherent PBMCs were separated to CD3⁺CD4⁺ T cells by magnetic cell sorting according to the methods described. Purity of the CD4⁺T-cells according to flow cytometric analysis was more than 98%. (FL1-H= anti-CD3-FITC, FL2-H= anti-CD4-PE)

Proliferative responses of the CD4⁺T-cells to the total sonicate antigens in PPD positive (OD= 0.501±0.071) and PPD negative (OD= 0.453±0.074) healthy donors were greater than those of TB (OD= 0.408±0.076) and MDR-TB (OD= 0.393±0.060) patients ($p=0.002$). On the other hand, these responses were not different in TB and MDR-TB patients (Figure 2).

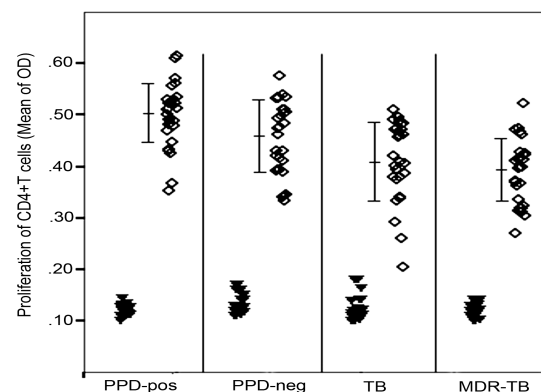


Figure 2. Proliferative responses of CD4⁺T cells to *M. tuberculosis* total sonicate antigens. The responses in the PPD-positive and PPD-negative subjects were higher than those of TB and MDR-TB patients ($p=0.00$).

According to figure 3, proliferative responses of activated CD4⁺T-cells to the total lipid antigens in the PPD-positive donors (OD= 0.417±0.060) showed a significant increase compared to PPD-negative subjects (OD= 0.228±0.066), TB (OD= 0.190±0.051) and MDR-TB patients (OD= 0.197±0.042) (p=0.001).

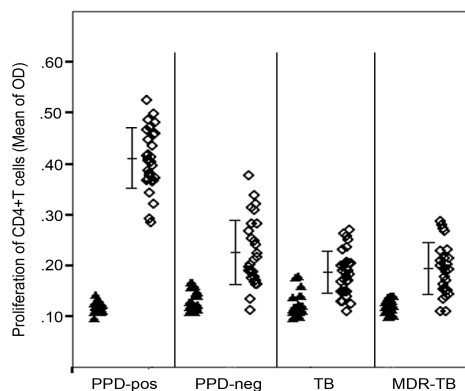


Figure 3. Proliferative responses of CD4⁺T-cells to *M. tuberculosis* total lipid antigens. Black symbols represent control cells (without antigenic stimulation) and white symbols represent stimulated cells. The responses in the PPD-positive donors were greater than in the PPD-negative subjects, TB and MDR-TB patients (p=0.00).

Cytokine production:

IFN- γ concentration mean in each group was compared with that of the other groups by Mann-Whitney U test. The results showed that, IFN- γ concentration mean in MDR-TB and TB patients was significantly lower than that of PPD-positive healthy donors (p=0.004) (Fig 4). In addition, IFN- γ levels in PPD-negative donors were lower than those in PPD-positive subjects (p=0.063).

Analysis of IFN- γ production by the CD4⁺T cells in response to the total lipid antigens showed that the IFN- γ concentration mean in PPD-positive subjects was significantly higher than in the PPD-negative healthy donors (p=0.003)(Fig 4). Furthermore, in TB and MDR-TB patients, the IFN- γ concentration means were lower than in the healthy PPD-negative and PPD-positive donors (p=0.00) (Figure 4).

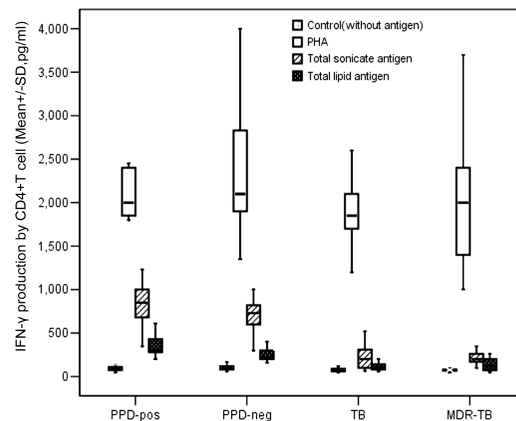


Figure 4. IFN- γ production by CD4⁺T-cells. IFN- γ production in response to the total lipid and total sonicate antigens in TB and MDR-TB patients was lower than that in the healthy donors (p=0.00).

In MDR-TB and TB patients, production of IL-4 by CD4⁺T-cells in response to total sonicate antigens was significantly greater than its corresponding values in PPD-positive and PPD-negative healthy donors (p=0.004) (Fig 5). However, this value was similar between PPD-positive and PPD-negative subjects. In response to lipid antigens, IL-4 concentration in the TB and MDR-TB cases was higher than that of healthy donors (p=0.00), while this rate was similar in PPD-positive and PPD-negative subjects. (p=0.233) (Figure 5)

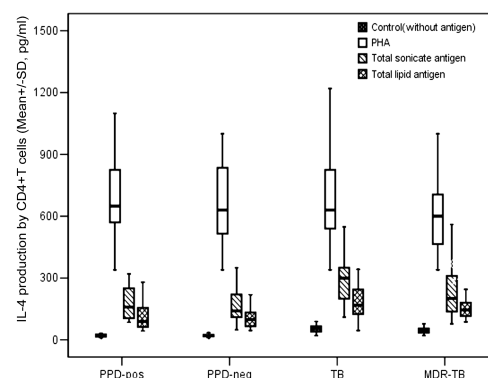


Figure 5. IL-4 production by CD4⁺T cells. Mean of IL-4 concentration in response to the total sonicate antigens in TB and MDR-TB patients was significantly greater than that in the healthy donors (p=0.004). IL-4 production in response to the lipid antigens in TB and MDR-TB patients was higher than that in the other groups (p=0.00).

DISCUSSION

Although several studies have investigated the role of T cells stimulated by *M. tuberculosis* protein antigens (14), the function of CD4⁺T- lymphocytes against *M. tuberculosis* lipid antigens in TB and MDR-TB patients is less investigated. For the first time in the present study, the functions of CD4⁺ T-cells of MDR-TB patients against *M. tuberculosis* lipid antigens were assessed.

In agreement with the previous studies (7, 15), our results showed that proliferative responses of CD4⁺ T cells of PPD-positive subjects to *M. tuberculosis* total lipid antigens significantly increased in comparison to the MDR-TB patients. It seems that in PPD-positive donors, *M. tuberculosis* total lipid antigens recruit T-cells memory immune responses. It is possible that in TB and MDR-TB patients, specific-lipid T cells are accumulated in the inflammatory tissues of lung. Stenger et al. showed that expression of CD1 molecules on monocytes infected with *M. tuberculosis* was decreased (16). Also, our data demonstrated that *M. tuberculosis* total lipid antigens up regulated the production of IL-10 in monocytes of MDR-TB patients (data not shown). These findings could be related to the suppression of immune responses against *M. tuberculosis* lipid antigens in the TB and MDR-TB patients. Proliferative responses to *M. tuberculosis* lipid were also observed in PPD-negative subjects. It seems that there was cross-reactivity between the *M. tuberculosis* lipid antigens and the lipid antigens of other bacteria (7).

Cytokines analysis showed that secretion of IFN- γ CD4⁺T cells of PPD-positive donors in response to total sonicate and total lipid antigens significantly increased in comparison to that of other groups. Other researches have shown that CD4⁺ T -cells activated by *M. tuberculosis* total lipid antigens in the PPD-positive donors produced more IFN- γ than those of PPD-negative donors (7). According to other studies, production of IFN- γ by the PBMCs in

response to the 30kDal *M. tuberculosis* antigen in MDR-TB and TB patients was suppressed (15, 17). Similar studies have confirmed that decrease of IFN- γ production in MDR-TB and TB patients is not due to the increase of IL-10 (18). In addition, high frequency of T cell apoptosis, decrease in IL-12R β 1 and IL-12R β 2 expression and up regulation of TGF- β 1 and IL-10 simultaneously in mycobacterium infection, could be the cause of IFN- γ down regulation in CD4⁺T cells of TB and MDR-TB patients (19,20,21). But the obvious reason for the suppression of IFN- γ production by CD4⁺T- cells against *M. tuberculosis* total lipid antigens in the MDR-TB patients is not well known.

According to our results, CD4⁺T- cells of TB and MDR-TB patients activated by total sonicate antigens produced high levels of IL-4 compared with the PPD-positive donors. This study showed that production of IL-4 by T cells infected with live *M. tuberculosis* was similar in TB patients and PPD-positive donors (21). Bai et al. showed that expression of IL-10 and IL-4 on the biopsies of granulomatous tissues from a pulmonary tuberculosis subject was reduced compared to the PPD- positive subjects (22). This result might be related to local responses to *M. tuberculosis*. Based on the majority of studies, expression of IL-4 was elevated in T cells stimulated by *M. tuberculosis* protein antigens (23). It seems that *M. tuberculosis* protein and lipid antigens in this way have similar behavior in stimulation of CD4⁺T-cells. According to other studies, IL-4 expression could sensitize the lymphocytes apoptosis (24). Therefore, IL-4 can suppress T-cell mediated immunity in TB and MDR-TB patients. On the other hand, IL-4 knockout mice was more susceptible to *M. tuberculosis* infection (25). These data show that in human immune responses, IL-4 may also have a beneficial effect on prevention of lung tissue destruction induced by Th1

cells. Accordingly, investigation on the exact role of IL-4 in the pathogenesis of TB and MDR-TB patients is strongly suggested.

CONCLUSION

CD4⁺T-cell proliferative responses and IFN- γ production to *M. tuberculosis* lipid antigens in PPD-positive healthy donors were significantly higher than those in MDR-TB patients. Furthermore, the lipid antigens in TB and MDR-TB patients promote IL-4 expression in CD4⁺T-cells. Therefore, it seems that *M. tuberculosis* lipid antigens, as protein antigens, have an important role in specific immune response.

Acknowledgments

The authors would like to thank NRITLD for its financial support and Dr. Farnia for the courtesy of *M. tuberculosis* H37Rv.

REFERENCES

1. Dye C. Global epidemiology of tuberculosis. *Lancet* 2006; 367 (9514): 938- 40.
2. World Health Organization. Guidelines for the programmatic management of drug-resistant tuberculosis. Available from URL: [http:// www. who. int/ gtb/ publications/ factsheet/ index.htm](http://www.who.int/gtb/publications/factsheet/index.htm). 2006.
3. Sharma SK, Mohan A. Multidrug-resistant tuberculosis. *Indian J Med Res* 2004; 120 (4): 354- 76.
4. Flynn JL, Ernst JD. Immune responses in tuberculosis. *Curr Opin Immunol* 2000; 12 (4): 432- 6.
5. Gumperz JE, Brenner MB. CD1-specific T cells in microbial immunity. *Curr Opin Immunol* 2001; 13 (4): 471- 8.
6. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994; 179 (4): 1109- 18.
7. Ulrichs T, Moody DB, Grant E, Kaufmann SH, Porcelli SA. T-cell responses to CD1-presented lipid antigens in humans with Mycobacterium tuberculosis infection. *Infect Immun* 2003; 71 (6): 3076- 87.
8. Dascher CC, Hiromatsu K, Xiong X, Morehouse C, Watts G, Liu G, et al. Immunization with a mycobacterial lipid vaccine improves pulmonary pathology in the guinea pig model of tuberculosis. *Int Immunol* 2003; 15 (8): 915- 25.
9. Manca C, Reed MB, Freeman S, Mathema B, Kreiswirth B, Barry CE 3rd, Kaplan G. Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis. *Infect Immun* 2004; 72 (9): 5511- 4.
10. FOLCH J, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957; 226 (1): 497- 509.
11. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 1987; 8: 93.
12. Moody DB, Guy MR, Grant E, Cheng TY, Brenner MB, Besra GS, et al. CD1b-mediated T cell recognition of a glycolipid antigen generated from mycobacterial lipid and host carbohydrate during infection. *J Exp Med* 2000; 192 (7): 965- 76.
13. van de Loosdrecht AA, Nennie E, Ossenkoppele GJ, Beelen RH, Langenhuijsen MM. Cell mediated cytotoxicity against U 937 cells by human monocytes and macrophages in a modified colorimetric MTT assay. A methodological study. *J Immunol Methods* 1991; 141 (1): 15- 22.
14. Feng CG, Bean AG, Hooi H, Briscoe H, Britton WJ. Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with Mycobacterium tuberculosis. *Infect Immun* 1999; 67 (7): 3242- 7.
15. Lee JS, Song CH, Kim CH, Kong SJ, Shon MH, Kim HJ, et al. Profiles of IFN-gamma and its regulatory cytokines (IL-12, IL-18 and IL-10) in peripheral blood mononuclear cells from patients with multidrug-resistant tuberculosis. *Clin Exp Immunol* 2002; 128 (3): 516- 24.
16. Stenger S, Niazzi KR, Modlin RL. Down-regulation of CD1 on antigen-presenting cells by infection with Mycobacterium tuberculosis. *J Immunol* 1998; 161 (7): 3582- 8.

17. Song CH, Kim HJ, Park JK, Lim JH, Kim UO, Kim JS, et al. Depressed interleukin-12 (IL-12), but not IL-18, production in response to a 30- or 32-kilodalton mycobacterial antigen in patients with active pulmonary tuberculosis. *Infect Immun* 2000; 68 (8): 4477- 84.
18. Jo EK, Kim HJ, Lim JH, Min D, Song Y, Song CH, et al. Dysregulated production of interferon-gamma, interleukin-4 and interleukin-6 in early tuberculosis patients in response to antigen 85B of *Mycobacterium tuberculosis*. *Scand J Immunol* 2000; 51 (2): 209- 17.
19. Dao DN, Kremer L, Guerardel Y, Molano A, Jacobs WR Jr, Porcelli SA, et al. *Mycobacterium tuberculosis* lipomannan induces apoptosis and interleukin-12 production in macrophages. *Infect Immun* 2004; 72 (4): 2067- 74.
20. Kim J, Uyemura K, Van Dyke MK, Legaspi AJ, Rea TH, Shuai K, et al. A role for IL-12 receptor expression and signal transduction in host defense in leprosy. *J Immunol* 2001; 167 (2): 779- 86.
21. Zhang M, Lin Y, Iyer DV, Gong J, Abrams JS, Barnes PF. T-cell cytokine responses in human infection with *Mycobacterium tuberculosis*. *Infect Immun* 1995; 63 (8): 3231- 4.
22. Bai X, Wilson SE, Chmura K, Feldman NE, Chan ED. Morphometric analysis of Th(1) and Th(2) cytokine expression in human pulmonary tuberculosis. *Tuberculosis (Edinb)* 2004; 84 (6): 375- 85.
23. van Crevel R, Karyadi E, Preyers F, Leenders M, Kullberg BJ, Nelwan RH, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. *J Infect Dis* 2000; 181 (3): 1194- 7.
24. Seah GT, Rook GA. IL-4 influences apoptosis of mycobacterium-reactive lymphocytes in the presence of TNF-alpha. *J Immunol* 2001; 167 (3): 1230- 7.
25. Sugawara I, Yamada H, Mizuno S, Iwakura Y. IL-4 is required for defense against mycobacterial infection. *Microbiol Immunol* 2000; 44 (12): 971- 9.