Indian J Med Res 158, July 2023, pp 40-46
DOI: 10.4103/ijmr.ijmr_2143_21

Quick Response Code:

Bacterial dissemination in *Mycobacterium tuberculosis* by CD+ T-cells & proinflammatory cytokines

Kusuma Sai Davuluri[1,2], Shoor Vir Singh[2] & D.S. Chauhan[1]

[1]Department of Microbiology & Molecular Biology, ICMR-National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra & [2]Department Biotechnology, GLA University, Mathura, Uttar Pradesh, India

Received July 8, 2021

***Background & objectives*: As CD4+ and CD8+ T lymphocyte numbers decline, the conventional, localized forms of tuberculosis shift to the atypical, disseminated forms. Variations in lymphocyte and immune cell expression levels affect how tuberculosis manifests in disseminated forms. Understanding the relationship between lymphocyte counts (CD4+ and CD8+) and pro-inflammatory cytokines such as tumour necrosis factor-alpha, interleukin-12 and interferon, we may therefore be able to shed light on how infections spread and suggest potential biomarkers for these immune factors.**

***Methods*: In this study, 15 guinea pigs were infected with *Mycobacterium tuberculosis (M.tb)* H37Rv strain and grouped into three groups of five each for further investigation. Serum samples and bronchoalveolar lavage (BAL) fluid were examined for the expression of pro-inflammatory cytokines and T-cell subsets in guinea pigs infected with pulmonary tuberculosis and disseminated tuberculosis.**

***Results*: We found that *M.tb* escapes macrophages due to pro-inflammatory cytokine dysregulation. Despite the protective immunity created by T-cells and cytokines, *M.tb* bacilli may spread to other organs due to inflammation induced by these immune components. A high number of T-cells and stimulated cytokine production are involved in triggering inflammation after necrotic tissue develops and tuberculosis spreads.**

***Interpretation & conclusions*: Our findings imply that increased bacilli in the spleen at the 8th wk of infection may be caused by the overexpression of CD4+ T-cell lymphocyte subsets and cytokines that generated inflammation during the 4th wk of infection. This is a pilot study with a small sample size and less assertive inference. Larger studies would be helpful to validate the results of the present investigation.**

Key words Bronchoalveolar lavage - CD4+ T cells - CD8+ T cells - chronic stage - dissemination - early stage-inflammation - proinflammatory cytokines

It was estimated that over 4100 individuals would die from tuberculosis (TB) every day in 2021 and close to 28,000 more would contract the disease[1]. In 2021, of the registered TB cases, 75.2 per cent were extrapulmonary cases and 24.8 per cent were pulmonary ones[2]. Due to recurrent exposure to the same antigen, T-cells develop exponentially in several of these stages[3]. Even before exhaustion or cell death, the inflammatory processes exert a major impact on T-lymphocyte differentiation from naive T-lymphocytes[4]. After being triggered by a cognate antigen, resting T-lymphocytes become activated

in the presence of an antigen-presenting cell and co-stimulatory signals[5]. A lack of proliferative/ survival cytokines, particularly IL-2, can lead to cycling T-cells dying if there are CD4+CD25+ regulatory T (Treg) cells present[6]. On the other hand, if they are frequently subjected to low-level antigenic stimulation, the cells will survive but undergo a transcriptional reprogramming process known as T-cell exhaustion[7], which causes them to become unresponsive.

Histopathological lesions and other clinical signs can be used to diagnose a disseminated infection[8]. From an extrapulmonary form, disseminated tuberculosis may develop, infecting two or more organs, such as the brain and liver[9]. Particularly vulnerable to the miliary form of *Mycobacterium tuberculosis* (*M.tb*) infection is infants[10,11]. Interferon-gamma (IFN-γ) production and *M.tb* control are highly dependent on CD4 T-cells[12]. The CD4-/- mice's CD8 T-cells are able to compensate for the lack of CD4 lymphocytes by producing adequate IFN-γ[13]. Numerous studies show that pulmonary tuberculosis (PTB) patients have significantly higher CD4/CD8 ratios; however, the lowering ratio that occurs after chemotherapy is still debatable. Before immunity develops, the bacilli are carried and dispersed throughout the body from the lung or the closest lymph node once they are inhaled and enter primary infectious site[14]. Patients with disseminated TB who tested negative for HIV-1 had significantly decreased T-cell counts[15]. Potential pathways for the activation or deactivation of T-cells in disseminated TB were questioned. In the present investigation, we used guinea pigs, which are highly sensitive to *M.tb* infection[16]. Cellular activation in patients with disseminated tuberculosis has been reported to be significantly higher than in patients with localized disease[17]. The study was aimed to understand the relationship between lymphocyte counts, pro-inflammatory cytokines, and the dissemination of tuberculosis, potentially leading to improved diagnostic markers and therapeutic approaches for the disease.

Material & Methods

The study was carried out on guinea pigs (n=15) in the department of Microbiology & Molecular Biology, ICMR-National JALMA Institute for leprosy (NJIL) & other mycobacterial diseases (OMD), Agra, Uttar Pradesh, India, with the approval from the Institutes Animal Ethics Committee (NJIL and OMD-3-IAEC/2019-08). All protocols (animal experimentation) were carried out in accordance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (*https://arriveguidelines.org/*). Hartley strain, male guinea pigs weighing around 350 g were purchased from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India.

Experimental Mycobacterium tuberculosis (M.tb) infection in guinea pigs: H37Rv strain of *M.tb* was procured from the institute's repository and cultured in Middlebrook 7H9 medium with 10 per cent albumin dextrose catalase (ADC; Difco Laboratory, USA) at 37°C. Before usage, cultures were diluted in sterile normal saline, with the bacterial concentration being set at 1×10^6 cfu/ml as per the protocol described earlier[18].

Whole body inhalation exposure device (Glas-Col LLC., IN, USA) calibrated to deliver ~100 colony forming unit (CFU) to the lung[19], guinea pigs were infected through aerosol with the *M.tb* H37Rv strain; n=15 animals were divided into three groups randomly having five in each group.

Collection of samples:

Serum: Blood (2-3 ml) was drawn from the jugular vein of the guinea pigs in vials without anticoagulant; serum was extracted from blood by centrifugation at 3000 g for 15 min at room temperature.

Bronchoalveolar lavage (BAL) samples: The spleen was surgically removed aseptically from the guinea pigs. After the trachea was dissected, phosphate-buffered saline containing three per cent foetal bovine serum (Invitrogen, USA) was injected into the lungs through the trachea using a 20 ml syringe and an 18G needle. To remove the media, the fluid was sucked back into the syringe. Each animal received a total of 3 ml washings[20].

Histopathology: An *M.tb* infected guinea pig's lung and spleen tissue samples were taken in the 8th wk. First, 10 per cent neutral buffered formalin was gradually administered into the right caudal lung lobe through the large arteries at the hilus. This was done to aid in morphometry and prevents alveolar collapse. The lobe was then submerged in formalin.

Determination of bacterial counts: At the 4th and 8th wk following infection, the guinea pigs' bacterial loads in the lungs and spleen were assessed. Organ homogenates were serially diluted ten times and placed on Middlebrook 7H10 (Difco Laboratory, USA) agar plates with 10 per

cent OADC. The plates were then incubated at 37°C for 21 days. For the purpose of calculating the bacterial burden, colonies on plates were counted.

Immunohistochemistry (IHC): Following the manufacturer's instructions with a minor modification[21], the dewaxed tissue sections were subjected to IHC staining using streptavidin peroxidase and antibodies. Anti-CD4 antibody (Vector labs, USA) was applied to the tissues and incubated for an hour. After washing with phosphate-buffered saline (PBS) for 30 min at room temperature, the secondary detection antibody, rabbit anti-mouse conjugated to horseradish peroxidase (HRP), was added. The chromogen used to visualize the final product was 3',3-diaminobenzidine tetrahydrochloride-dihydrate (DAB)/hydrogen peroxide (H_2O_2; Sigma Aldrich, USA).

Evaluation of inflammatory cytokines and CD4+ T lymphocytes in BAL and serum samples: The expression of CD4 markers were determined through enzyme-linked immunosorbent assay (ELISA) using Anti-CD4 antibody (Vector laboratories, USA) by separating the macrophages from the BAL and serum samples. The expression of tumour necrosis factor-alpha (TNF-α), IFN-γ and Interleukin (IL-12) were determined though ELISA by separating the macrophages from BAL samples. Anti-TGFB1, anti-IL-12A and anti-IFNGR2 produced in rabbits (Sigma Aldrich, USA) were used to perform ELISA; 75 µl of the primary antibody was added to each well and incubated at 4°C for overnight. Following the washing, 300 µl of blocking buffer was added to each well. The plate was incubated, and blocker was discarded. After washing 1:1000 ratio of enzyme conjugate HRP, anti-goat IgG secondary antibody was added to each well and incubated. o-Phenylenediamine dihydrochloride (OPD) was added to each well and developed at room temperature for 30 min, later to stop the reaction, 50 µl of 70 per cent H_2SO_4 was added. Quantification was performed by calculating the concentration/ml through the OD values at 490 nm.

Statistical analysis: Data was analyzed using GraphPad Prism 9 software (GraphPad Software Inc, CA, USA). Fractal analysis of granulomas was measured using Magvision software (Magnus Opto Systems India Pvt. Ltd.) and significance was calculated using area covered in same panel 500 µm, $P<0.01$ was considered significant. For ELISA, two-way ANOVA test was used to evaluate significance on concentrations; $P<0.01$ was considered significant.

Results

Splenomegaly: There was a decrease in the nodules at all lungs at the 8th wk of infection, but splenomegaly was seen in all *M.tb* infected guinea pigs. The host's immune response was efficient to decrease the bacterial count in the lung. Due to the dissemination of bacteria to the spleen, enlargement and damage to the spleen was seen (Fig. 1).

Bacterial dissemination from lungs to spleen: During the 8th wk, host immunity could mediate protection against the *M.tb* infection, but it could not stop the spread of the infection. In the *M.tb* infected control group, there was an increase in bacterial severity at peripheral liver and lymph node sites. We generated evidence that bacteria in lung macrophages migrated to the liver, spleen and lymph nodes. The right caudal lung lobe was removed at appropriate time intervals to quantify the bacterial severity. The $\log_{10}$ mean number of viable bacteria was used to express the data, and the standard error of the mean was calculated; $P\leq0.05$ was considered significant (Fig. 2).

Increased inflammation due to the excessive migration of CD4 T-cells and inflammatory cytokines: Multiple and confluent granulomatous lesions (mean=700 µm in diameter) with central necrosis, mean of infiltration fraction mononuclear cells increased up to 65 per cent and number of lesions were observed in the infected group at 4th wk infection and less granulomas. At 4th wk of infection, round lesions with similar distribution and necrosis were seen. During the 8th wk infection, fibrous web enveloped with macrophages was seen (Fig. 3).

Stimulation of T-lymphocytes subsets during the early pulmonary infection and reduced T-cells in guinea pigs with disseminated tuberculosis: A lower concentration of circulating CD4+ T cells was noted during the 8th wk of infection compared with that in the 4th wk of infection. We also found reduced percentages of cytokines responsible for immune cell signalling. Nodules at lungs during the advanced stage of infection were reduced, indicating the possibility that CD4+ T-cells played a key role during the early stage of infection but induced inflammation. Circulating CD4+ T-cells in BAL samples showed a significant decline during the 8th wk of infection, which revealed the disseminated infection following reduced T-cell counts (Fig. 4 and Table Ia and b).

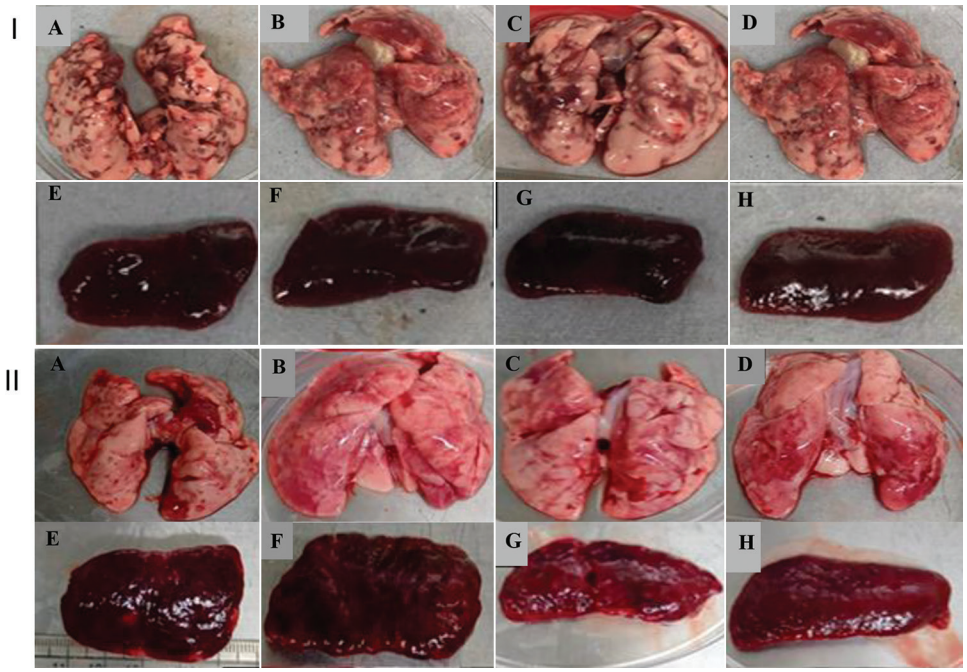

Fig. 1. Efficient immune in guinea pig following aerosol infection with *M.tb* H37Rv. **I**: (**A-D**) At the 4th wk, *M.tb*-infected lungs revealed considerable increased size and lesion progression. (**E-H**) At the end of the 4th wk, the *M.tb*-infected spleen had a low bacilli count and no growth. **II:** (**A-D**) Reduced bacilli in *M.tb*-infected lungs in the 8th wk indicated that the bacilli present in the necrotic core, where they are most concentrated, easily escape and spread to other organs, (**E-H**) *M.tb*-infected spleen at the 8th wk showing splenomegaly. *M.tb*, *Mycobacterium tuberculosis.*

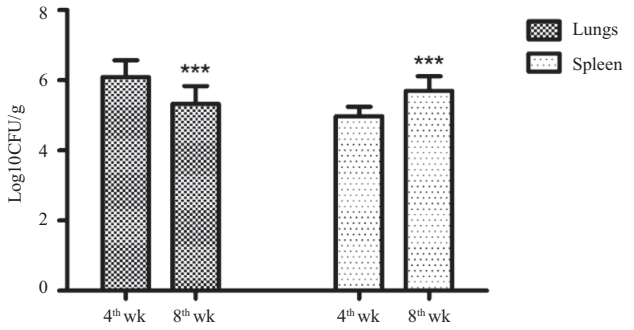

Fig. 2. Bar plots showing the log difference in colony forming units (CFU) in *M.tb* infected guinea pigs lung and spleen tissue at different time periods. *M.tb, Mycobacterium tuberculosis*

Evidence of increased proinflammatory cytokine expression: Throughout the animal experimentation, cellular infiltrates and inflammation were seen. Three proinflammatory cytokines were measured using ELISA to assess the immune responses induced in *M.tb* infected guinea pigs during PTB and disseminated tuberculosis. While disseminated TB was present at the 8th wk after *M.tb* infection, the proinflammatory cytokine levels were much lower. Table II shows the mean levels of TNF-α, IL-12A and INF-γ in healthy guinea pigs, *M.tb* infected guinea pigs at four weeks and *M.tb* infected guinea pigs at eight weeks (Fig. 5 and Table II).

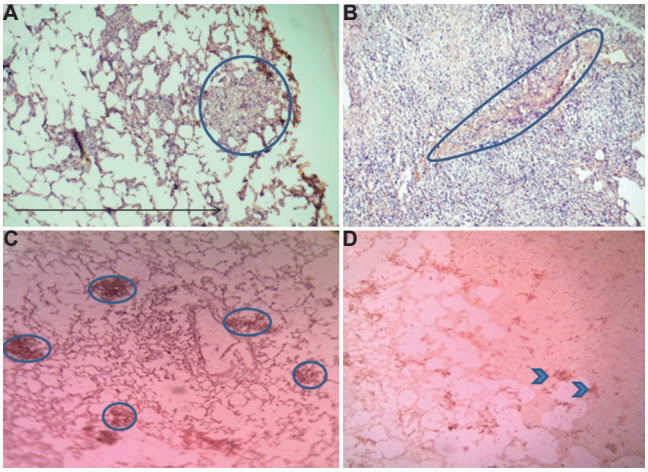

Fig. 3. (**A**) Histopathological section with the granuloma formation in the *M.tb* infected guinea pig during the 4th wk infection with no signs of inflammation and necrosis showing the constructed granuloma. (**B**) The deconstructed granuloma leaving the inflammation and large necrotic areas. (**C**) The stimulated expression of CD4+T cells in IHC stained lung tissue section during the 4th wk. (**D**) Reduced expression of CD4+T cells after the dissemination of infection.

Discussion

More data on lymphocytes could be useful in considering them as biomarkers in clinical evaluation of patients with disseminated tuberculosis. After

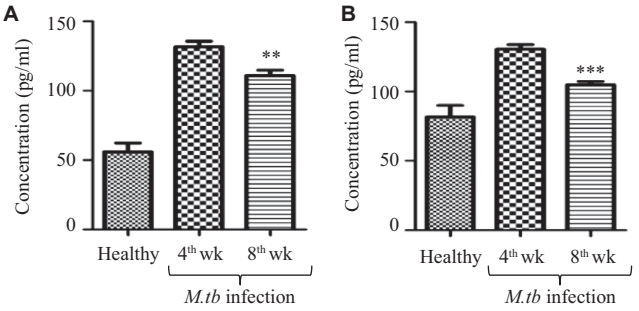

Fig. 4. Bar graph showing the concentration of cells with CD4+ marker among the study groups (**A**) BAL samples, (**B**) Serum samples. P **<0.01, ***<0.001. BAL, bronchoalveolar lavage

Table Ia. Quantification of CD4+ T cell expression/unit area in various groups through immunohistochemistry

Number of cells/2 mm (x 100 magnification)		
M. tuberculosis infection 4th wk	*M. tuberculosis* infection 8th wk	Healthy
214	127	83.325
251	131	96.844
231	179	80.774
158	103	82.505
148	162	77.83

M. tuberculosis, *Mycobacterium tuberculosis*

treatment, the CD4/CD8 ratio falls. Many studies have been conducted to investigate the role of CD4+ and CD8+ lymphocytes in antimicrobial immunity, but studies on the changes in the CD4+/CD8+ ratio in pulmonary tuberculosis (PTB) and extrapulmonary cases have been sparse[22]. The majority of clinical reports of tuberculosis infection are based on the findings in peripheral blood and BAL, but the results in both samples vary[23,24]. Analysis of T-lymphocyte involvement in cell-mediated immune response is crucial because adaptation of the anti-mycobacterial defence in the lungs is caused by immune T-cells activating macrophages[25]. Recently, a more comprehensive picture of the functional and phenotypic profiles of exhausted T-cells has emerged[26]. T-cell exhaustion has been defined in a variety of experimental and clinical contexts. When previously primed effector T-cells are exposed to *M.tb* specific antigens, they produce cytokines[27]. Proinflammatory cytokines are expressed at varying levels in tuberculosis patients, according to the majority of the studies. We must first comprehend the immunopathogenesis brought on by *M.tb* to look into the causes of transmission and reactivation of tuberculosis infection. It may be possible to initiate right life-saving treatment by analyzing the IFN/IL-12 axis in PTB[28]. The CD8+ T cell response is triggered by CD4 cells and during the early stages of *M.tb* infection, a cell-mediated immune response is completely established[29]. Central necrosis develops within the granuloma as a result of lysis of infected macrophages by CD4+ T-helper cells and CD8+ suppressor T-cells[30]. T-lymphocytes migrate to the granuloma site and use cytolytic action to control the infection. Our findings support the idea of previous studies showing decreased cytokine production in extrapulmonary tuberculosis patients. Early T-lymphocyte migration in guinea pigs correlated with the early initiation of antimycobacterial action[29]. We entertained the hypothesis that the formation of inflammatory lesions due to excess cellular immune response might provide access to bacteria to disseminate through the lymphatics. During the early stage of TB infection, the stimulation caused significant changes in the migration of CD4+ T-cells to the granuloma site, inducing inflammation and limiting the bacilli. This descriptive analysis showed a positive association of CD4+ T-cells immune staining at the infection site consisting of bacterial load, number of lesions (Figs 1 and 2) and number of affected cells in *M.tb* infected guinea pigs after the 4th wk of post infection. In contrast to CD4 depletion, pulmonary *M.tb* infection in CD4-depleted macaques unexpectedly caused extremely early extrapulmonary *M.tb* dissemination[31]. At the conclusion of more severe TB, CD4-depleted macaques showed no or few pulmonary T effector cells expressing IFN-γ, TNF-α, IL-17, IL-22 and perforin, but they did show pulmonary IL-4+ T-cells[32]. Despite the existence of IL-17+ and IL-4+ cells, TB granulomas in CD4-depleted macaques contained less IL-22+ and perforin+ cells. The host's defence against *M.tb* includes CD8+ cytotoxic cells[33]. Anti-IFN-γ therapy successfully treated diffused lesions in extrapulmonary tuberculosis. A defect in IL-12 and IFN-γ production has been linked to *M.tb* infection susceptibility and *M. avium* complex infection spread[34]. Our study demonstrated that the mean CD4+ cell count was lower in the guinea pigs with disseminated infection, compared to those with higher counts in PTB guinea pigs, making it less likely for them to be AFB positive due to the frequency of extrapulmonary involvement. The reduced CD4+ cell concentration was observed in the guinea pigs having a higher frequency of extrapulmonary involvement, which differed from early infection guinea pigs. Circulating levels of T-lymphocyte subsets were found to be less in serum samples compared with the

Table Ib. Quantification of CD4+ T-cells in bronchoalveolar lavage (BAL) samples and serum samples through ELISA

Concentration (pg/ml)					
M. tuberculosis infection 4th wk		*M. tuberculosis* infection 8th wk		Healthy	
BAL	Serum	BAL	Serum	BAL	Serum
116.645	140.975	115.975	105.273	98.056	35.12
130.08	132.762	117.761	103.161	64.39	95.927
141.683	130.173	116.016	104.292	63.497	62.5
136.389	127.348	107.71	113.459	58.497	60.06
132.762	121.339	97.003	98.056	62.136	91.471

M. tuberculosis, *Mycobacterium tuberculosis*; ELISA, enzyme linked immunosorbent assay

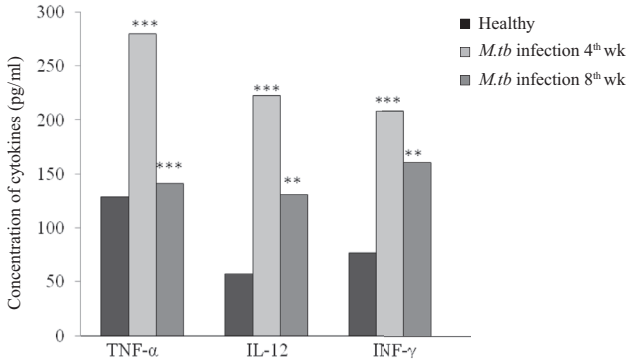

Fig. 5. Bar graph showing the levels of TNF-α, IFN-γ, and IL-12A during 4th and 8th wk of *M.tb* infected guinea pigs. *M.tb, Mycobacterium tuberculosis*; TNF-α, tumour necrosis factor-alpha; IL-12, interleukin-12; IFN-γ, interferon-gamma

bronchoalveolar lavage. We observed an increase in the expression of CD4+ and CD8+ cells at an early stage (during 4th wk) of infection that limits the bacilli count at the infection site but leaves the inflammation. Showing effective immune response in lungs, reduced bacilli count directs to the reduced migration of CD4+ and CD8+ cells at the chronic stage of infection following dissemination of infection to the spleen.

This study examined whether depressed T-cell responses during the late stage of infection or elevated T-cell responses during the early stage of infection could contribute to the spread of infection. The number of T-cells were found to increase during the initial stages of infection, leading to reduced bacilli and elevated inflammation. At this stage, bacilli spread due to increased inflammation triggered by the increase in T-cells at an early stage. We should not ignore the decreased levels of T-cell lymphocytes during the 8th wk of infection following reduced bacterial severity in lungs. This pilot study underlines the need for larger studies to identify the aetiology and the involvement of immune responses in miliary tuberculosis, which are often ignored.

Table II. Quantification of cytokines in serum through ELISA

Cytokines	Healthy	*M. tuberculosis* infection 4th wk	*M. tuberculosis* infection 8th wk
TNF-alpha	116.265	125.39	311.59
	150.39	144.421	242.52
	119.96	154.36	284.96
	128.87	141.39	279.69
	69.261	170.965	209.516
IL-12	57.792	124.686	235.557
	66.299	128.94	210.361
	48.96	139.26	222.352
	57.68	130.962	222.75
	148.89	159.49	227.352
IFN-γ	47.86	222.352	279.516
	27.39	208.361	452.648
	35.86	351.52	286.646
	37.03	260.74	339.60
	55.96	105.686	227.352

TNF, tumour necrosis factor; IL-12, interleukin-12; IFN-γ, interferon-gamma

***Financial support and sponsorship*:** None.

***Conflicts of Interest*:** None.

References

1. World Health Organization. *Global tuberculosis report 2022*. Available from: *https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2022*, accessed on July 1, 2022.
2. National Tuberculosis Elimination Programme. National Health Mission. Ministry of Health and Family Welfare, Government of India. *India TB report 2022: Coming together to end TB altogether*. Available from: *https://tbcindia.gov.in/WriteReadData/IndiaTBReport2022/TBAnnaulReport2022.pdf*, accessed on July 1, 2022 .

3. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: From T cell basic science to clinical practice. *Nat Rev Immunol* 2020; *20* : 651-68.
4. Kaufmann SH. Protection against tuberculosis: Cytokines, T cells, and macrophages. *Ann Rheum Dis* 2002; *61* (Suppl 2) : ii54-8.
5. Ross R. The role of T lymphocytes in inflammation. *Proc Natl Acad Sci U S A* 1994; *91* : 2879.
6. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and chemokines in *Mycobacterium tuberculosis* infection. *Microbiol Spectr* 2016. Doi: 10.1128/microbiolspec.TBTB2-0018-2016.
7. Khan N, Vidyarthi A, Amir M, Mushtaq K, Agrewala JN. T-cell exhaustion in tuberculosis: Pitfalls and prospects. *Crit Rev Microbiol* 2017; *43* : 133-41.
8. Djannah F, Massi MN, Hatta M, Bukhari A, Hasanah I. Profile and histopathology features of top three cases of Extra Pulmonary Tuberculosis (EPTB) in West Nusa Tenggara: A retrospective cross-sectional study. *Ann Med Surg (Lond)* 2022; *75* : 103318.
9. Lee JY. Diagnosis and treatment of extrapulmonary tuberculosis. *Tuberc Respir Dis (Seoul)* 2015; *78* : 47-55.
10. Marais BJ, Schaaf HS. Tuberculosis in children. *Cold Spring Harb Perspect Med* 2014; *4* : a017855.
11. Correa AG. Unique aspects of tuberculosis in the pediatric population. *Clin Chest Med* 1997; *18* : 89-98.
12. Lin PL, Flynn JL. CD8 T cells and *Mycobacterium tuberculosis* infection. *Semin Immunopathol* 2015; *37* : 239-49.
13. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol* 1999; *162* : 5407-16.
14. Ganchua SKC, White AG, Klein EC, Flynn JL. Lymph nodes-the neglected battlefield in tuberculosis. *PLoS Pathog* 2020; *16* : e1008632.
15. Kony SJ, Hane AA, Larouzé B, Samb A, Cissoko S, Sow PS, *et al*. Tuberculosis-associated severe CD4+ T-lymphocytopenia in HIV-seronegative patients from Dakar. SIDAK Research Group. *J Infect* 2000; *41* : 167-71.
16. Singh AK, Gupta UD. Animal models of tuberculosis: Lesson learnt. *Indian J Med Res* 2018; *147* : 456-63.
17. Rodrigues DS, Salomão R, Kallas EG. Profound peripheral T-lymphocyte depletion and activation in disseminated tuberculosis. *Braz J Infect Dis* 2006; *10* : 59-61.
18. Davuluri KS, Singh AK, Kumar V, Singh SV, Singh AV, Kumar S, *et al*. Stimulated expression of ELR+ chemokines, VEGFA and TNF-AIP3 promote mycobacterial dissemination in extrapulmonary tuberculosis patients and *Cavia porcellus* model of tuberculosis. *Tuberculosis (Edinb)* 2022; *135* : 102224.
19. Klinkenberg LG, Lee JH, Bishai WR, Karakousis PC. The stringent response is required for full virulence of *Mycobacterium tuberculosis* in guinea pigs. *J Infect Dis* 2010; *202* : 1397-404.
20. Tremblay G, Gagnon L, Cormier Y. Sequential bronchoalveolar lavages by endotracheal intubation in guineapigs. *Lab Anim* 1990; *24* : 63-7.
21. Larsson LI. *Immunocytochemistry: Theory and practice*. Boca Raton: CRC Press; 1988. p. 147-70.
22. de Almeida AS, Fiske CT, Sterling TR, Kalams SA. Increased frequency of regulatory T cells and T lymphocyte activation in persons with previously treated extrapulmonary tuberculosis. *Clin Vaccine Immunol* 2012; *19* : 45-52.
23. Radha S, Afroz T, Prasad S, Ravindra N. Diagnostic utility of bronchoalveolar lavage. *J Cytol* 2014; *31* : 136-8.
24. Liu X, Hou XF, Gao L, Deng GF, Zhang MX, Deng QY, *et al*. Indicators for prediction of *Mycobacterium tuberculosis* positivity detected with bronchoalveolar lavage fluid. *Infect Dis Poverty* 2018; *7* : 22.
25. Khan FY. Review of literature on disseminated tuberculosis with emphasis on the focused diagnostic workup. *J Fam Community Med* 2019; *26* : 83-91.
26. Wherry EJ. T cell exhaustion. *Nat Immunol* 2011; *12* : 492-9.
27. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, *et al*. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; *439* : 682-7.
28. 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* : 1194-7.
29. Winslow GM, Cooper A, Reiley W, Chatterjee M, Woodland DL. Early T-cell responses in tuberculosis immunity. *Immunol Rev* 2008; *225* : 284-99.
30. Walker NF, Meintjes G, Wilkinson RJ. HIV-1 and the immune response to TB. *Future Virol* 2013; *8* : 57-80.
31. Lin PL, Rutledge T, Green AM, Bigbee M, Fuhrman C, Klein E, *et al*. CD4 T cell depletion exacerbates acute *Mycobacterium tuberculosis* while reactivation of latent infection is dependent on severity of tissue depletion in cynomolgus macaques. *AIDS Res Hum Retroviruses* 2012; *28* : 1693-702.
32. Yao S, Huang D, Chen CY, Halliday L, Wang RC, Chen ZW. CD4+ T cells contain early extrapulmonary tuberculosis (TB) dissemination and rapid TB progression and sustain multieffector functions of CD8+ T and CD3- lymphocytes: Mechanisms of CD4+ T cell immunity. *J Immunol* 2014; *192* : 2120-32.
33. Seneviratne SL, Doffinger R, Macfarlane J, Ceron-Gutierrez L, Amel Kashipaz MR, Robbins A, *et al*. Disseminated *Mycobacterium tuberculosis* infection due to interferon gamma deficiency. Response to replacement therapy. *Thorax* 2007; *62* : 97-9.
34. Frucht DM, Holland SM. Defective monocyte costimulation for IFN-gamma production in familial disseminated *Mycobacterium avium* complex infection: Abnormal IL-12 regulation. *J Immunol* 1996; *157* : 411-6.

For correspondence: Dr D. S. Chauhan, Department of Microbiology & Molecular Biology, National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Tajganj, Agra 282 001, Uttar Pradesh, India
e-mail: devchauhan01@yahoo.co.in