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IMMUNE ASPECTS IN HEALTH CARE WORKERS WHO ARE IN CLOSE CONTACT WITH TUBERCULOSIS PATIENTS

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Abstract. Aim. Health-care workers occupationally exposed to *Mycobacterium tuberculosis* were investigated in order to detect immunological parameters useful in monitoring the progression to the disease. **Material and methods**. We investigated 20 tuberculosis exposed employees, 17 patients with active pulmonary tuberculosis at diagnosis and after 6 months of treatment and 12 healthy controls with no exposure or history of active tuberculosis. We detected flowcytometrically the percentages of circulating lymphocyte subpopulations and Th1/Th2-type cytokines levels. Plasma immunoglobulins were quantified by immune diffusion and chemiluminiscence. **Results and discussion**. B lymphocytes were significantly lower in exposed and patients. Total IgE and interferon gamma (IFNγ) levels were significantly higher in patients compared to exposed or controls. Tumour necrosis alpha (TNFα) was significantly lower in exposed and patients compared to controls. **Conclusions**. A continuous clinical status monitoring, correlated with periodical quantification of the above mentioned immunological parameters in tuberculosis exposed workers will help us decide if these are reliable markers for seizing transition to disease.
Key-words: **tuberculosis occupationally exposed workers, IgE, cytokines, IFNγ, TNFα**

Rezumat. Scopul studiului nostru a fost investigarea personalului medical expus ocupațional la *Mycobacterium tuberculosis* în scopul detectării unor parametri imuni utili în monitorizarea evoluției spre stadiul de boală activă. **Material şi metode**. Au fost investigate 20 de peroane expuse profesional la *M. tuberculosis*, 17 pacienți cu tuberculoză pulmonară activă, la diagnostic şi după 6 luni de tratament, şi 12 persoane sănătoase. Au fost detectate flowcitometric procentele de subpopulații limfocitare şi nivelurile de citokine Th1/Th2 din circulația periferică. Imunoglobulinele plasmatice au fost cuantificate prin imunodifuzie şi chemiluminiscență. **Rezultate si discuții**: Populația de limfocite B a fost semnificativ redusă la persoanele expuse şi la pacienți. Nivelurile de IgE totale şi IFNγ au fost semnificativ crescute la pacienți comparativ cu expuşii sau persoanele sănătoase. **Concluzii**. Continuarea monitorizării clinice a personalului medical expus la *M. tuberculosis* corelată cu determinarea periodică a parametrilor imunologici menționați mai sus vor ajuta la confirmarea utilității acestor markeri în surprinderea evoluției spre starea de boală manifestă.
Cuvinte cheie: muncitori expuşi la tuberculoză, IgE, cytokine, IFNγ, TNFα

INTRODUCTION

Health care workers are exposed to various hazards, with acute or long-term health impairments, such as allergies, infections, reproductive disorders and chronic diseases. Infections may be acquired during clinical and laboratory diagnostic work and represent

the best documented occupational hazard (1).

Respiratory therapists were reported to be at a higher risk of tuberculin conversion than other hospital staff (2).

In Romania, the important surge of new cases of tuberculosis after the 1990s, reaching to 134.6 cases per 100 000 inhabitants in 2004, imposes an increased interest in monitoring health-care workers from tuberculosis wards, who are a high-risk group with regard to infection with *M. tuberculosis* (3). *M. tuberculosis* infection is followed by active tuberculosis in only 10% of cases (4). Most of the infected humans mount an efficient, long-lasting immune response that controls the bacteria, by keeping the infection in a latent form and confers protection even to reinfection (4). Pulmonary tuberculosis in adults usually develops through reactivation of dormant foci of infection, but there are rare situations with high levels of exposure when reinfection is also possible (5, 6).

Protection against *M. tuberculosis* relies on both natural and acquired immunity with the cellular component of the immune response having the main role in the defence.

The first infection generates an inflammatory response of the macro-phages that recognize and phagocytose mycobacteria. Once engulfed by the macrophages *M. tuberculosis* prevents phagosome fusion with lysosomes. This survival strategy allows the bacillus to survive a long time within the macrophages, like in an 'intracellular niche' (7). Thus, the robust, highly hydrophobic cell wall of the bacillus not only confers resistance against complement but also allows mycobacterial replication within phagosomes of resting macrophages, otherwise ones of the most efficacious anti-microbial cellular effectors of a host (5). While inside the macrophages, bacilli secrete antigens that are degraded, processed and MHC (major histocompatibility complex) class II – presented. Bacillar peptides are thus recognized by CD4+ T cells, whose role in protection against tuberculosis is unquestioned (5).

The mechanism of activation of CD8+ T cells during *M. tuberculosis* infection is less understood as long as tubercle bacilli are not supposed to enter the cytoplasm of the macrophage and, by consequence, mycobacterial peptides cannot be presented in a MHC class I groove, through the classical pathway (5). Since mouse experiments proved a major role of CD8+ T cells in defence against tuberculosis it seems that alternative MHC class I pathways are used (8, 9).

Canaday *et al* 2001 reported similar, overlapping effector mechanisms for both CD4+ and CD8+ T cells, which are cytolytic and significantly inhibit growth of intracellular *M. tuberculosis* in monocytes. Cytokine-secreting T cells contribute indirectly to infection control by activating uninfected macrophages (10).

The robust, highly hydrophobic cell wall of the bacillus confers resistance against complement but the fatty acids and polysaccharides in the wall can be recognised by non-conventional T cells, either CD1 restricted or γδ (5).

All the above mentioned T cells participate in the acquired immune

response against *M.tuberculosis* by secreting interferon gamma (IFNγ) that activates macrophages (7). Another mechanism of action of T cells is cytotoxicity against the infected macrophages (11). The ensuing pro-inflamatory response, dominated by the cyokines, acts both in a paracrine and autocrine manner. IFNγ, TNFα and IL-12 implication in mycobacterial containment was documented in mice (12, 13).

MATERIALS AND METHODS

1. Study groups

The study group included 20 healthy medical employees, nurses and doctors, contacts of patients with sputum smear-positive and culture – positive TB. The employment duration in tuberculosis wards ranged between 2 and 38 years, with an average of 10.1 years.

The positive control group included 17 patients from the Clinical Hospital of Lung Diseases, Iasi Romania, aged between 24 and 50, recently diagnosed with active pulmonary tuberculosis. The patients were also investigated after 6 months of successful therapy. Active pulmonary tuberculosis was defined by clinical findings and thoracic radiograph images suggestive of tuberculosis and confirmed by the presence of at least two sputum smear positive for acid-fast bacilli or the growth of *M tuberculosis*.

The negative control group consisted of 12 healthy volunteers, aged between 27 and 58, with no exposure or history of active tuberculosis. All the subjects have been BCG vaccinated during childhood and were HIV-negative.

Peripheral blood obtained by venous puncture was heparin-treated and plasma was separated by blood centrifugation at 2500 x g.

2. Cellular immunity investigation

Flow-cytometric identification of lymphocyte subpopulations

The investigation of cellular immunity consisted in flowcytometric detection of the percentages of lymphocyte subpopulations in the peripheral blood.

White blood cells were counted in Burker-Turk counting chambers. Approximately 2 x 10^5 cells were aliquoted in tubes and incubated for 20 minutes in the dark, at room temperature, with mouse anti-human monoclonal antibodies specific for CD45, CD14, CD3, CD4, CD8 and CD19, conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridin chlorophyll protein (PerCP) (Becton Dickinson and Pharmingen). After labelling, red blood cells were lysed by incubating with BD FACS Lysing solution for 10 minutes, at room temperature. The cells were washed twice, resuspended in phosphate buffered saline and analysed the same day. A FACSCalibur flowcytometer and CellQuest software (Becton Dickinson) were used for the acquisition and analysis of data.

3. Humoral immunity investigation

Humoral immunity was explored by quantifying plasma immunoglobulins and Th1/Th2-type cytokines.

3.1. Plasma immunoglobulins quantification

Total plasma IgG, IgM and IgA levels were detected by simple immune diffusion Mancini assay, using immune plates ("Cantacuzino" Institute).

Measurement of total circulating IgE level was performed by chemi-

luminiscence, an immunometric assay that used Immulite Total IgE DPC (*Diagnostic Product Corporation*) reagents and an IMMULITE analyzer.

3.2. Cytokine determination

After separation by centrifugation of the venous blood at 2500 x g, plasma was stored at -80°C. The levels of IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ were quantitatively measured by flow-cytometry, using a FACSCalibur flow-cytometer and a Cytometric Bead Array Human Th1/Th2 Cytokine CBA Kit (*Becton Dickinson*). The amount of cytokine in each plasma sample was measured by using the BD CBA Software (*Pharmingen*).

4. Statistical analysis

Student's t Test was used to compare the mean values of the group results. A p value under 0.05 was considered indicative of statistically significant difference.

RESULTS

1. Cellular immunity investigation - identification of lymphocyte subpopulations

The peripheral blood lymphocyte subpopulations detected by flow-cytometry were T cells, defined as $CD45^{+hi}$ $CD14^{-}$ $CD3^{+}$ cells, with two subpopulations - $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ - and B lymphocytes identified as $CD45^{+hi}$ $CD14^{-}$ $CD19^{+}$ cells.

The normal range for T cells is 67 - 76% from the peripheral blood lymphocytes, with 48.7 - 59% $CD3^{+}CD4^{+}$ cells and 39.7 – 51.3 % $CD3^{+}CD8^{+}$ cells. B cells represent 11 – 16% of the circulating lymphocytes.

The percentages of T and B lymphocytes are represented in figure 1; B cells subpopulation was smaller in the exposed group and in patients when compared to controls ($p<0.001$).

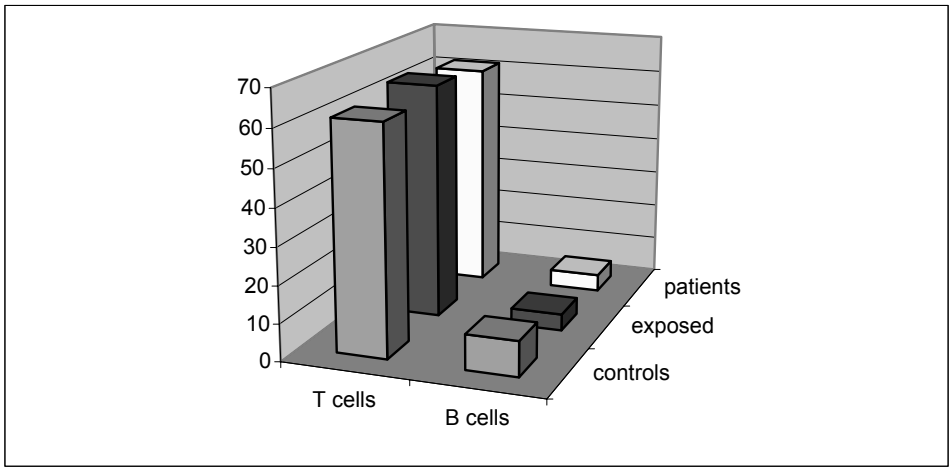


Fig. 1 Percentages of T and B lymphocytes in the peripheral blood of patients with active pulmonary tuberculosis

As figure 1 shows, *M. tuberculosis* occupationally exposed workers and controls reveal statistically significant differences between B lymphocytes in the exposed group or tuberculosis patients and controls.

CD4+ and CD8+ T cells subpopulations, represented in figure 2, were within the normal ranges for all the groups we investigated.

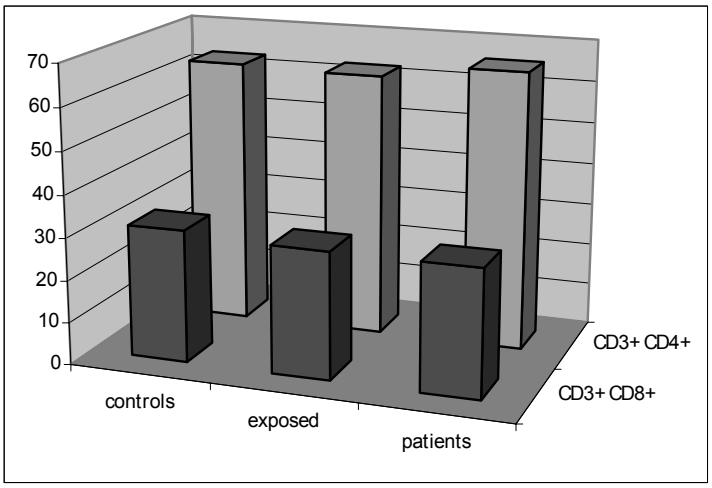


Fig. 2. Circulating T lymphocytes subpopulations had similar, normal percentages in the three groups

Exposed people had 63.73 ± 8.48 % T cells with 62.65 ± 14.79% $CD3^+CD4^+$ and 30.1 ± 13.15% $CD3^+CD8^+$ cells and B cells were 2.7 ± 2.32 % of the lymphocytes.

The mean percentages of T cells in controls were 61.33 ± 9.18 %, with 63.25 ± 8.43 % $CD3^+CD4^+$ and 31.33 ± 8.54 % $CD3^+CD8^+$ cells. B cells were 9.45 ± 2.48 % of the lymphocytes.

At diagnosis, tuberculosis patients had 61.38 ± 11.46 % T cells with 65.91 ± 7.03% $CD3^+CD4^+$ and 29.87 ± 7.18 % $CD3^+CD8^+$ subpopulations. B cells were 4.8 ± 2.92 % of the lymphocytes.

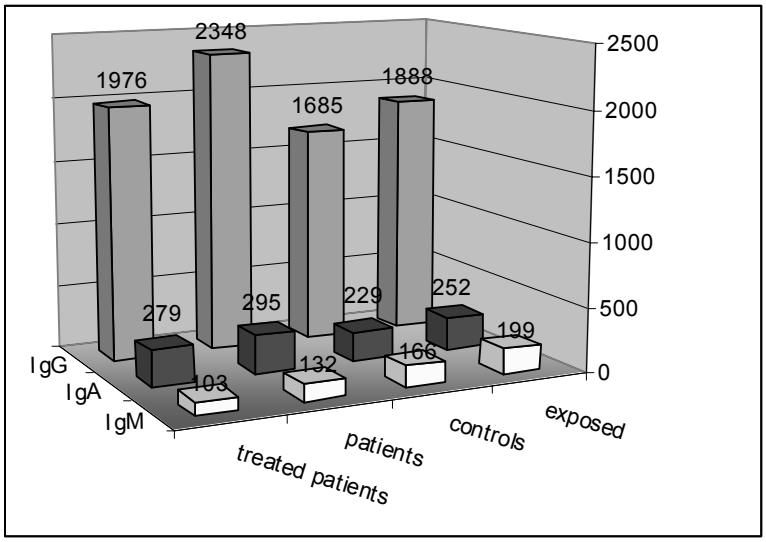
2. Humoral immunity investigation

2.1. Plasma immunoglobulins quantification

Total serum IgG, IgM and IgA plasma levels were detected by immune diffusion. The mean values are represented in figure 3.

Normal IgG values are within the 800 and 2000 mg/dl range. The mean value calculated for exposed people was 1888.27 mg/dl (SD 692.38), in healthy controls it was 1685.83 mg/dl (SD 449.85), in active tuberculosis patients at diagnosis it was 2348.8 mg/dl (SD 841.05) and after 6 months of therapy patients had 1976 mg/dl

(SD 479.19). Active tuberculosis patients had higher than normal IgG mean values before treatment; the values turned to normal after treatment. No statistically significant differences were observed between the four groups.



	Normal range (mg/dl)
IgG	800 – 2000
Ig A	90 - 450
Ig M	60 - 280

Fig. 3. Mean values of plasma total IgG, IgA and IgM (mg/dl)

Mean values of plasma total IgG, IgA and IgM (mg/dl) were in normal values range except for tuberculosis patients that had higher than normal IgG levels at diagnosis (fig 3).

Total IgA mean plasma levels were 252.53 mg/dl (SD 130.08) in exposed people, 229.85 mg/dl (SD 65.34) in healthy controls, 295.2 mg/dl (SD 119.64) in patients at diagnosis and 279.5 mg/dl (SD 53.35) in patients after treatment.

The mean plasma IgM concentration was 199.45 mg/dl (SD 168.19) in exposed people, 166 mg/dl (SD 44.38) in controls, 132 mg/dl (SD 38.36) in tuberculosis patients at diagnosis and 103 mg/dl (SD 30.65) after tuberculostatic treatment.

Plasma amounts of total IgE were quantified by chemiluminiscence. The mean values, represented in figure 4, were 71.65 kU/l (SD 102.84) for non-allergic tuberculosis - exposed workers, 19.44 kU/l (SD 14.16) for healthy controls, 667.75 kU/l (SD 551.75) for tuberculosis patients at diagnosis, and 861.51 kU/l (SD 884.25) for patients after treatment.

Statistically significant differences ($p < 0.05$) were found between total IgE mean levels in patients (both at diagnosis and after treatment) and tuberculosis exposed people or healthy controls.

M. tuberculosis occupationally exposed employees had higher levels than controls, and both groups had statistically significant lower IgE values than tuberculosis patients, even after 6 months of treatment. Normal values of serum total IgE are under 87 kU/l (fig 4.).

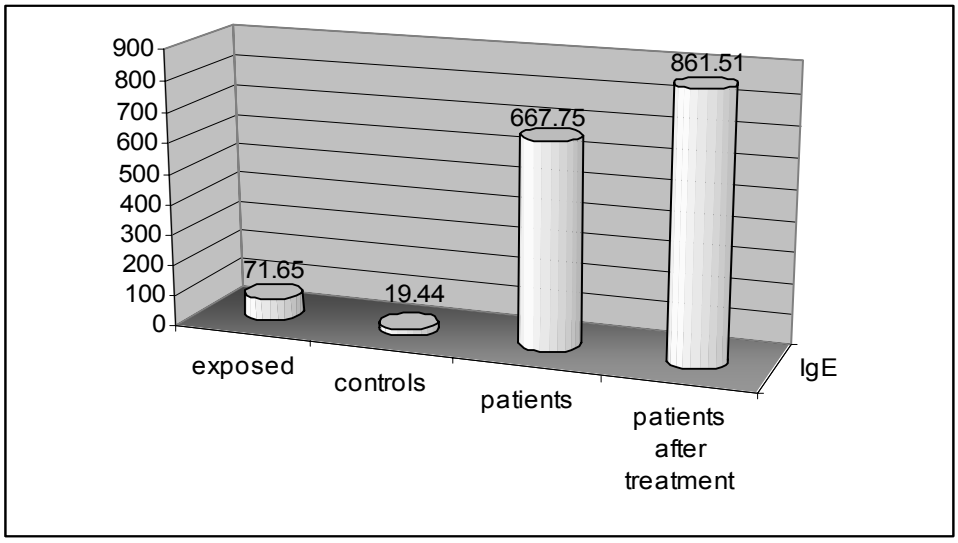


Fig. 4. Mean values of serum total IgE (in kU/l)

2.2. Cytokine determination

The levels of IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ were quantitatively measured by flow-cytometry and the mean values are presented in table 1.

Table 1. Mean values and standard deviations of the plasma levels of Th1/Th2 cytokines (pg/ml) in the four groups

		IFNγ	TNFα	IL-10	IL-5	IL-4	IL-2
Tuberculosis exposed employees	mean	2.94	1.84	3.27	2.10	3.42	4.85
	SD	5.21	1.14	1.84	0.73	2.21	10.09
Healthy controls	Mean	5.43	2.87	3.85	2.36	5.06	3.91
	SD	4.70	0.74	0.94	0.60	2.44	0.87
Tuberculosis patients before treatment	Mean	24.17	1.93	3.05	2.34	3.34	2.72
	SD	26.34	0.95	0.84	1.40	2.69	0.68
Patients after 6 months of therapy	Mean	21.65	2.4	3.41	2.82	3.02	2.86
	SD	65.80	1	1.15	2.61	2.48	1.36

For the exposed group statistically significant differences ($p < 0.05$) were calculated for IFN γ plasma level when compared to tuberculosis patients at diagnosis and for TNFα plasma level when compared to controls. TNFα had significantly lower levels in tuberculosis patients as well when compared to controls.

DISCUSSION

In tuberculosis control, a rapid and accurate identification of infected individuals is paramount. The simplest rapid method is to detect acid-fast bacilli by microscopy, but 40-60% of patients with pulmonary tuberculosis are smear negative and culture methods take several weeks to become positive (14).

Detection of lymphocyte subpopulation in the peripheral blood would provide a rapid monitoring way. In our study we found that peripheral blood lymphocytes of tuberculosis – exposed health care workers and those of patients contained fewer **B cells** ($p<0.001$) than controls. The same decrease of B cells in the peripheral blood of tuberculosis people was reported by Raju *et al*, 2001 (15).

There was no significant difference between the percentages of circulating T cells in the studied groups, an observation consistent with literature data. An important (fourfold) increase of CD4+ T cells was however detected in the tuberculosis involved lung lobe (15).

The kinetics of T cells immune response during *Mycobacterium bovis* bacille Calmette-Guerin (BCG) infection was studied by Lai *et al* (2003) in macaques - non-human primates (16). The primary BCG infection, after bronchial exposure, initially generates lymphocytopenia, followed by proliferation of CD4+, CD8+ and gammadelta T cells in the lungs that is non-detectable in the blood. Bronchial reinfection was followed by a rapid surge of specific T cells associated with a resolution of active BCG infection (16).

The higher than normal level of total plasma IgG in patients with active pulmonary tuberculosis might be attributed to the bacillar load, a great exposure to antigen resulting in a vigorous antibody response. After 6 months of treatment the values turned to normal, probably secondary to decreased mycobacterial load.

Normal values for seric IgE in adults without allergic or parasitic diseases were reported to be 46.65 kU/l (95% CI: 15.5-77.8) versus 204.29 kU/l (95% CI: 93.3-515) for allergic subjects (17). In our study **IgE** mean values were significantly higher ($p<0.05$) in tuberculosis patients at diagnosis (667.75 kU/l) and after treatment (861.51 kU/l) when compared to healthy controls and to tuberculosis exposed employees. Atopic persons were not included in our study and stool specimens were examined in order to detect parasite infestation that might interfere with IgE determinations.

High levels of IgE were also reported in tuberculosis patients from the Gambia and Guinee (18) and from South Africa 457 kU/l (19). The decrease of IgE level after tuberculosis treatment reported in the paper of

Adams *et al*, 1999 is suggestive for the implication of this class of immunoglobulins in host defence against *M. tuberculosis* (19).

A possible mechanism of action was suggested by Davidsson *et al* (2005) who detected significantly elevated serum levels of IgE in influenza, another respiratory infection. The significant increase of influenza-specific IgE after vaccination of non-allergic volunteers was interpreted as indicative for IgE participation in viral defence. After attaching to the virus, IgE would activate the mast cells, resulting in mediator release that would turn the immunological response towards a Th1 profile. Thus IgE might have a beneficial role, protecting against viral infection by inducing a rapid local inflammatory response (20).

Quantification of plasma levels (in pg/ml) of IFNγ, TNFα, IL-10, IL-5, IL-4, and IL-2 showed significantly higher levels of **IFNγ** in tuberculosis patients when compared to controls and exposed workers.

Gamma interferon, often used as a surrogate marker of the Th1 – type cellular response, acts primarily on infected macrophages, leading to their activation and driving the formation of granulomas and it is considered to be a key component of the anti- *M. tuberculosis* protection in humans (21, 22). Other literature data also reported increased levels of IFN γ in the peripheral blood of almost all patients with primary pulmonary tuberculosis (23, 24)

Tumor necrosis factor (TNFα) was significantly lower in exposed people and pulmonary tuberculosis patients when compared to controls.

In animal models TNFα was reported to play a central role in the host immune response to tuberculosis, including granuloma formation and containment of disease (25, 26)

In humans, an indirect role for TNFα protective role in tuberculosis was deduced after treating rheumatoid arthritis patients with Infliximab, a TNF-blocking agent that resulted in reactivation of latent tuberculosis. (27)

CONCLUSIONS

- The increased occupational risk in health care workers from pneumology clinics urges the need of close monitoring and finding reliable, early markers of transition to disease.
- There is a complex interplay between various cellular and humoral components of natural and adaptive immunity involved in the fight against *M. tuberculosis*. By consequence it is not possible to point to one component that would be more important than the others.
- Our study revealed statistically significant lower ($p<0.001$) percentages of B cells in the peripheral blood of tuberculosis patients and of exposed health care workers when compared to controls. Total IgE and IFNγ levels were significantly higher in patients compared to exposed health care employees or controls. TNFα was significantly lower in *M. tuberculosis* - occupationally exposed workers and

in pulmonary tuberculosis patients when compared to controls.

- A continuous monitoring of the clinical status, with periodical investigations of these immunological parameters in tuberculosis exposed health care employees will help us decide if these are reliable markers for seizing progression to disease.

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