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# Comparison study between typhoid fever and tuberculosis patients to induce and produce of Th-1 cytokines (IL-18 and IFN-γ)

Hussein Hafedh Abbas<sup>1</sup>, Karrar Hussein Ali<sup>1</sup>, Dhargam Muhammed Ibrahem<sup>1</sup>, Omar Farhan Ammar<sup>2</sup>, and Mariam Tariq Abd-alwahed<sup>3</sup>

<sup>1</sup>Ministry of Higher Education and Scientific Research, Al-Yarmouk University College / Dept. of Medical Laboratories Techniques, Baghdad, Iraq <sup>2</sup>Ministry of Higher Education and Scientific Research, Usoul Al-deen University College / Dept. of Medical Laboratories Techniques, Baghdad, Iraq <sup>3</sup>Ministry of Health, Al-kadhraa Hospital, Baghdad, Iraq

## ABSTRACT

Typhoid fever (TF) is one of the most common infectious diseases in developing countries. Early and definitive diagnosis of the disease is not only important in relieving patients' suffering, but also critical in avoiding fatal complications such as perforation of the intestines. It also makes possible specific treatment at an early stage, which leads to the rapid elimination of the pathogen from the chronic patient's excreta, especially stool, become a constant source of spread of the disease. Tuberculosis (TB) is one of the oldest known human infectious diseases, primarily affects lungs and causes Pulmonary TB (PTB), also can affect intestine, meninges, bones and joints, lymph glands, skin and other tissues of the body. It's one of the three primary diseases of poverty along with AIDS and malaria, it may cause to death ranging between 10-15 people every year in the developing world. The results of present study after measuring the concentration levels of IFN-y and IL-18 in serum of TF and TB patients were showed higher levels (422.624±33.824, 252.467±129.773pg/ml respectively) more than levels in serum of typhoid patients group were highly levels ( $137.833\pm23.424$ ,  $377.357\pm106.585pg/ml$  respectively) than those in healthy control group. The serum IFN-y level was elevated in TF patients compared to control group with clearly significance ( $P \le 0.01$ ), and higher elevated in the TB patients compared to control group with higher significant ( $P \le 0.01$ ), and serum IFN- $\gamma$ level was highly elevated in TB patients compared to TF patients with highly significant ( $P \le 0.01$ ). In addition, IL-18 level was shown higher elevated in serum of TF patients compared to control group with higher significant  $(P \leq 0.01)$ , the serum IL-18 level in TB patients higher elevation compared to control with higher significance ( $P \leq 0.01$ ), and serum IL-18 level was shown elevation in TF patients compared to TB patients with highly significance ( $P \leq 0.01$ ). The present study showed significance of correlation coefficients between the maximum levels of cytokines in each patients groups including both of TB and TF compared to control group when the IFN-y level correlated significantly with IL-18 level (P=0.014 with statistical significance P<0.05). As conclusion, Cytokines are indicators and communicates signals to ability of immune cells in human response to resist many microbial infection especially Typhoid and tuberculosis diseases.

Key words: Th1-cytokine, Typhoid fever, and Tuberculosis.

## INTRODUCTION

Typhoid fever (TF) is an enteric disease and one of the major health problems in the developing countries, fostered by many interrelated factors, including increased urbanization, inadequate supplies of clean water, antibiotic resistance, and the variable efficacies of vaccine preparation (1).

*Salmonella* is a member of the family Enterobacteriaceae consist of more than 2500 serovars, and infections caused by *Salmonella* constitute a major public health problem worldwide (2).

These pathogens can affect both human and animals, causing food-borne disease ranging from mild gastroenteritis to life threating systemic infections, such as those caused by *Salmonella enterica* serovar *typhi* known as (*S. typhi*)(3).

Clinical studies demonstrated that *S. typhi* infection stimulates both an intestinal mucosal and systemic humoral and cellular immune response, which are play roles in controlling and clearing *S. typhi* infection (4; 5), circulating proinflammatory and anti-inflammatory cytokines in patients with typhoid is increasing levels compared to patients with other severe disease (6).

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M.tuberculosis*), Robert Koch identified *M.tuberculosis* as a causative agent of TB at 1882 (7). Since that TB has been one of the most important infectious diseases for human being. According to the World Health Organization (WHO) report regarding the global burden of TB in 2009, there were 9.4 million incident cases of TB with approximately one third of the world total population being infected (8). *M.tuberculosis* is a facultative intracellular bacterium that survives in alveolar macrophages.

Generally, the effective of immune responses against intracellular pathogens are based on the cellular arm (T cells), not on the humoral arm (antibodies) of immune responses (9; 10). The effective cell subsets have been important for protection against *M.tuberculosis* infection.

Intracellular bacteria in phagocytes cells including *M.tuberculosis* are processed via Major Histocompatibility Complex (MHC) class II-mediated processing and presenting of antigen pathway to  $CD4^+$  T cells (10; 11; 12). Therefore, Th1 has been considered to play a pivotal role for protection against *M.tuberculosis* infection. Both  $CD4^+$  and  $CD8^+$  T cells provide protection against *M.tuberculosis* (13).

Immune response to infection with *Mycobacterium or Salmonella* which activated macrophages and CD4<sup>+</sup> T cells produce and secrete proinflammatory cytokines, and Type-1 cytokines such as Interferon- $\gamma$  (IFN- $\gamma$ ), and Interleukins 12 and 18 (IL-12 and 18) (14).

This study objective is to estimate Th1 cytokine (INT-18 & INF-  $\gamma$ ) levels in the serum of typhoid fever and tuberculosis patients groups compare to healthy group; determination which patients of TB and/or TF diseases are inducer more levels of IL-18 & INF- $\gamma$ ; and investigate their role in the pathophysiology of TF and/or TB in humans infect and investigate their role on diagnosis of patients.

#### MATERIALS AND METHODS

#### Selection the clinical cases of TF and TB:

Based on the clinical status of patients that investigated by consultant clinic of Al-Kadhimiya Teaching hospital and bacteriological /serological results of blood culture, Widal test and Rose Bengal test ( $\geq 1/160$  of widal test with negative result of rose Bengal to check patients which suspected infect by typhoid fever. In additionally, according to the clinical status of TB patients that investigated by consultant clinic of Al-Thuwaiba solitary hospital and microbiology laboratory result for positive acid fast staining.

A total number of patients were 200 included (49 males and 51 females) aging from 10-60 years for each group of patients. All patients did not received antibiotics for any reason for at least 2 weeks ago; twenty apparently healthy individuals, age and sex matched were enrolled as a control group.

Blood samples of both TF and TB patient groups with control group were taken in the period of time from October–December 2012.

#### **Collection of clinical specimen:**

A total volume of blood samples collected from each patient was (10ml) by vein puncture using disposable syringes. Collected blood was allowed to clot at room temperature for 30min., and then centrifuged for 10min. at 4000 rpm, the serum separated in tubes and stored at  $-20^{\circ}$  C, and thawed immediately prior to estimate of IL-18 and IFN- $\gamma$  levels.

## Laboratory investigations:

#### For diagnosis of typhoid fever:

- *Bacteriological examination*: blood cultures were performed and cultured organisms were identified by customary methods (15). Briefly 5ml blood was inoculated in blood culture bottles (Hi-media, India) incubated overnight at 37°C and then sub cultured on *Salmonella Shigella* (SS) agar (Amersham, England) and Bismuth sulphate agar (Hi-Media, India) plates. All plates were incubated at 37°C for 24 hours. A maximum of 10 suspected colonies from positive agar plates were selected and examined by Gram staining for complete identification. Bacterial colonies were identified with API 20E (Biomereax, Marcy Etoile France). *Salmonella* serovars were determined by agglutination with anti-O and anti-H antisera (Sanafi, Diagnostic Pasteur) by following the Kauffmann-White scheme (16).

#### -Serological/Hematological tests:

**\*Widal test:** the Widal test was performed with standardized S.Typhi O and H-antigens (Sanofi Diagnostics Pasteur, Marnes la coquette, France). Serial dilutions of sera were made with 0.9% saline. Tubes containing O and H antigens and sera were incubated at 37°C for 2h centrifuged at 1400 g for 5 min and examined for visible agglutination. Appropriate positive and negative control sera were included.

\*\* **Rose Bengal test:** the rose Bengal test was performed with standardized Brucella antigen (Linear, spinach). One drop of *Brucella* antigen was put onto dry, clean slide, then one drop of patient serum was added on bacterial antigen drop and compared to one drop of positive and negative control, then they were mixed them at room temperature. The results were observed at the end of 1 min. under high intensity light.

\*\*\* **Hematological investigations**: Total leucocytes count and differential leucocytes count were performed and interpreted according to routine laboratory procedures.

#### For diagnosis of TB:

- **Sputum examinations:** which included smear and sputumculture, and tuberculin skin were performed and interpreted according to advance laboratory procedures.

- Chest radiographic: were finding with knowledge of clinical information of each patient.

**Cytokine measurement:** Quantitative determination of Th1 cytokines (IL-18 and IFN- $\gamma$ ) in sera samples were done by using the commercially available of enzyme linked immunosorbent assay (EL1SA) kits.

#### - IL-18 Assay:

IL-18 was measured in sera samples to both of patient groups with control by using IL-18 EIA kit (Cell,USA).IL-18 assay was performed according to kit protocol described method:

**\*Preparation of standard solution:** Reconstitute the lyophilized recombinant protein by added 1ml sample diluent buffer to a tube of lyophilized protein to make a 10000pg/ml of II-18 solution at room temperature for 10min. and mixed thoroughly. 0.9ml of the sample diluent buffer was aliquoted into tube labeled as 10000pg/ml protein standard, 0.1ml of the mixed 10000pg/ml IL-18 solution was added to tube containing 0.9ml diluent buffer and mixed to make a 1000pg/ml IL-18 solution, Labels 6 tubes with the protein concentration to be prepared by serial dilution: 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml and 15.6pg/ml, 0.3ml was aliquoted of the sample diluent buffer to the labeled tubes, 0.3ml was transferred from the 1000pg/ml IL-18 solution to the 500pg/ml tube and mixed thoroughly, Then 0.3ml transferred from each tube to another to prepare series dilute of protein standards into their respectively and store at 4°C until uses.

**\*\*** Loading the samples into the micro-plate: 0.1ml of the sample diluent buffer was aliquoted into a control well to serve as the blank, 0.1ml of the serial standard protein solutions were aliquoted into empty wells of the pre-coated well plate. The serum samples was diluted by taking 50µl of sample and 50µl of diluent buffer and mixed thoroughly to prepared 1:2 from sample working dilution, 0.1ml of each diluted test samples was aliquoted to empty wells, the wells plate were covered and incubated at 37°C for 90min., During incubating, a stock biotinylated antibody working solution were prepared according to protocol kit, and the working solution used within 2hr., the cover of the well plate was removed and plate well contents were discarded and the plate blotted onto paper towel, 0.1ml of the biotinylated 1:100 antibody working solution was added to each well and the plate was incubated at 37°C for 60min. During incubation period, a stock of Avidin-Biotin-Peroxidase Complex (ABC) working solution was prepared according to kit protocol, pre-warm the ABC working solution at 37°C for 30min. before use with in1hr., the plate was washed 3 times with 0.3ml of PBS and the washing buffer discarded and the plate blotted onto a filter paper No.1, 0.1ml of ABC working solution was added to each well and the plate was incubated at 37°C for

30min., During the incubation period, pre-warm TMB color at  $37^{\circ}$ C for 30min. before used, the plate was washed 5 times with 0.3ml of PBS and the washing buffer was discarded and the plate was blotted onto a paper towel,  $90\mu$ l of TMB color developing agent was added into each well and incubated at  $37^{\circ}$ C for 11min.

0.1ml of TMB stop solution was added to each well to convert the color in well from blue to yellow. The absorbance was read at 450nm.

#### - IFN-γ Assay:

IFN-γ was measured according to ELISA kit protocol (Ray Bio, USA) described method:

\* **Preparation of standard solution:** 400µl assay diluent A was added to vial item C to prepare a 50ng/ml standard and mixed thoroughly, 180µl of IFN- $\gamma$  standard from containers was added into tube containing 420µl from assay diluent A to prepare a 15000pg/ml stock standard solution, 7tubes were labeled with the IFN- $\gamma$  concentration to prepare serial diluent: 5000pg/ml, 1666.7pg/ml, 555.6pg/ml, 185.2pg/ml, 61.7pg/ml, 20.6pg/ml, and 0pg/ml, 400µl was transferred from assay diluent A, Then transfer 0.2µl of 15000pg/ml stock standard solution to first tube of group to prepare 5000pg/ml and 200µl was transferred from 5000pg/ml tube to second tube to prepare1666.7pg/ml and transfer 200µl from each tube to another tubes to prepare serial dilute standards into their respectively and mixed thoroughly, and then store at 4oC until uses.

\*\* **Loading samples in micro-plate:**  $100\mu$ l of each of the serial standard solutions was added into empty wells of the pre-coated well plate,  $100\mu$ l from each samples test was added into appropriate wells, well was covered, mixed gently and incubated at 4°C for overnight, Prepared wash buffer(1x) by dilute 20ml of wash buffer concentrate into 400ml D.D.W., and the solution was discarded and washed 4 times with  $300\mu$ l of (1x) wash buffer and the plate was blotted onto a filter paper No.1, 100µl of prepared biotin antibody according to kit protocol was added to each well and incubate for 1hr. at room temperature with gentle mixing, the solution was discarded and the washing process repeated, 100µl of prepared streptavidin solution according to kit protocol was added to each well and incubate for 45min. at room temperature with gentle mixing, the solution was discarded and the washing process repeated, 100µl of TMB reagent was added to each well and incubated for 30min. at room temperature in the dark with gentle mixing, 50µl of stop solution was added to each well, the absorbance was read at 450nm immediately.

#### **Statistical Analysis:**

Statistical calculator software was used to statistical analysis of significance value (in 0.01 value) of difference mean between two groups was assessed by Independent group's t-test between means, and Statistical package of social science (T- test) was used for statistical analysis of the results were shown as mean± standard deviation (17).

Person correlation coefficient was used to determine the correlation between serum cytokines levels in typhoid fever and tuberculosis patients.

#### RESULTS

#### Estimate of cytokines concentration levels in serum of typhoid and tuberculosis patients:

In the present study, the concentrated levels of IFN- $\gamma$  and IL-18 were determined in typhoid patients compared to healthy control group were higher levels(377.357±106.585, 137.833±23.424pg/ml respectively) with highly significant ( $P \le 0.01$ ) than those in healthy control group that showed in table (1).

Table (1) the mean levels of Cytokines (IFN-γ and IL-18) in healthy and typhoid Patients groups							
Cytokines		T dant					
	Control group	Typhoid Patients groups	1 - test				
IFN-γ pg./ml	75.816±0.209	137.187±0.703	77.51*				
IL-18 pg./ml	10.333±1.958	377.357±106.585	34.978*				
Where: $*P \leq 0.01$ , SE: Standard error.							

Table (2) the mean levels of Cytokines (IFN- $\gamma$ and IL-18) in healthy and TB Patients groups							
Critalring	М	T tost					
Cytokines	Control group	Control group TB Patients groups					
IFN-γ pg./ml	75.816±0.209	422.624±33.824	12.49**				
IL-18 pg./ml	9.556±1.0333	252.467±29.773	9.247*				
Where: $P \leq 0.01$ , $P \leq 0.05$ , SE: Standard error.							

In additionally, Serum IFN- $\gamma$  and IL-18 levels in TB patients group were higher levels(422.624±33.824, 252.467±129.773pg/ml respectively) with highly significant ( $P \le 0.05$  and  $P \le 0.01$ ) than those in healthy control group that showed in table (2).

#### Comparison between TB and TF patients in inducing and producing of IFN-γ and IL-18:

After measuring the concentration levels of IFN- $\gamma$  and IL-18 in serum of TF and TB patients were showed higher levels in serum of TB patients group (422.624±33.824, 252.467±129.773pg/ml respectively) more than levels in serum of typhoid patients group were highly levels (137.833±23.424, 377.357±106.585,pg/ml respectively) than those in healthy control group.

The serum IFN- $\gamma$  level was elevated in TF patients compared to control group with clearly significance ( $P \le 0.01$ ), and higher elevated in the TB patients compared to control group with higher significant ( $P \le 0.01$ ), and serum IFN- $\gamma$  level was highly elevated in TB patients compared to TF patients with highly significant ( $P \le 0.01$ ).

In addition, IL-18 level was shown higher elevated in serum of TF patients compared to control group with higher significant ( $P \le 0.01$ ), the serum IL-18 level in TB patients higher elevation compared to control with higher significance ( $P \le 0.01$ ), and serum IL-18 level was shown elevation in TF patients compared to TB patients with highly significance ( $P \le 0.01$ ), that showed in table (3).

Table (3) Comparison between TB and TF patients in inducing and producing of IFN-γ and IL-18.										
Patients and control groups	IFN-γ pg/ml			IL-18 pg/ml						
	T-test	P value	S value	C value	T-test	P value	S value	C value		
TF vs. control	5.152	0.01*	0.106	0.18	3.096	0.01***	0.889	0.06		
TB vs. control	3.379	0.01***	0.812	0.614	7.108	0.01***	0.036	0.74		
TF vs. TB	83.95	0.01**	0.988	0.12	1.196	0.01**	0.21	0.278		
Where: P: is probability, S: significant value, C: correlation coefficient value, $*$ : is clear significant (P $\leq 0.01$ ), $**$ : is highly significant (P $\leq 0.01$ ),										
*** is higher significant ( $P < 0.01$ )										

## DISCUSSION

Typhoid fever is caused by the facultative intracellular gram negative bacillus *S. typhi*, the clinical features of typhoid fever confused with other febrile disease (18). Cytokines have been shown to play principal roles in the defense against *Salmonella* infection; IFN- $\gamma$  is one of the representatives of cytokines involved in the clearance of intracellular pathogens. IL-18 is a Th1-inducing cytokine; IL-18 shares in many of the biological properties and plays a critical role in the host defense against intracellular pathogens through T cell activation. In this study, IFN- $\gamma$  and IL-18 are significantly increased in concentration levels in serum of typhoid patients compared the control, this result supports that IFN- $\gamma$  and IL-18 are implicated in the pathogenesis of typhoid fever and agrees with the result of (19) who reported that the levels of IFN- $\gamma$  and IL-18 are elevated in typhoid patients compared to control.

TB, a granulomatous disorder, is an infection caused by *Mycobacterium tuberculosis* in which the cellular immune response plays an important role in TB by produced different cytokines have been shown to play principal roles in the defense against *mycobacteria* infection; IFN- $\gamma$  is one of the representatives of cytokines involved in the clearance of intracellular pathogens. IL-18 is a Th1-inducing cytokine.In this study, IFN- $\gamma$  and IL-18 are significantly increased in concentration levels in serum of TB patients compared the control, this result supported by hypothesis which has believed: The amount of mycobacterial replication is controlled by many host immune factors: Effector T cells (including CD4<sup>+</sup> and CD8<sup>+</sup> T cells) and macrophages participate in controlling of mycobacterial infection. IFN- $\gamma$  and TNF- $\alpha$  produced by T cells are important macrophage activators. Macrophage activation promotes phagosomal maturation, vacuole acidification and the production of antimicrobial molecules, such as reactive nitrogen intermediates (RNIs) by nitric oxide synthase 2 (NOS2), reactive oxygen intermediates (ROIs), and antimicrobial peptides, which can block bacterial replication. The production of the proinflammatory cytokines such as TNF- $\alpha$  and IL-18 by activated macrophages also contributes to controlling the intracellular replication of mycobacteria. The ability of mycobacteria to inhibit the secretion of IL-12 by infected macrophages might contribute to bacterial survival, as this cytokine normally functions to induce the production of IFN- $\gamma$  (20).

The present study showed significance of correlation coefficients between the maximum levels of cytokines in each patients groups including both of TB and TF compared to control group when the IFN- $\gamma$  level correlated significantly with IL-18 level (P=0.014 with statistical significance p<0.05).

The results indicated a stronger level of IFN- $\gamma$  in TB patients more than TF patients compared to control; and agrees with the result of (19) who reported that the level of IFN- $\gamma$  are elevated in systemic disease such as pulmonary tuberculosis more than gastroenteric diseases such as typhoid fever compared to control.

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The inflammatory processes trigger various types of cells, macrophages and monocytes, to release many cytokines. The released cytokines trigger other cells and initiate the cascade of cytokine release which can contribute to activating of appropriate host defenses (18). The key association between IFN- $\gamma$  and cell mediated immune response that IFN- $\gamma$  activating macrophages; and because of higher macrophages has revolved strategies to manipulate that macrophages interactions with numbers of immune cells and lead to granuloma formation (aggregation of macrophages) (21). The infected macrophage and DCs secret cytokines (which includes IL-12, IL-18 and IFN- $\gamma$ ), these cytokines contribute in the protective immunity against TB especially produced IFN- $\gamma$  and that interpretive the results of this study.

In addition, the result of present study showed a stronger level of IL-18 in TF patients more than TB patients compared to control; Because of IL-18 works by binding together with IL-12 to induced cell mediated immunity following infection with microbial product like (LPS and flagella) which is considered as virulence factor of *S. typhi* (22). The decrease level of IL-18 in TB patients may be due to insufficient binding of macrophages to virulence factor of *M. tuberculosis* (e.g. LPS). IL-18 is a central for successful host defense against *Salmonella* infection; because neutralization of IL-18 leads to increased bacterial numbers in spleen and liver and decreased host survival, while IL-18 treatment decreases bacterial counts in spleen and liver and increases host survival (23). This shows that IL-18 plays an important role in host defense against *Salmonella*. This role is effective to be mediated of IFN- $\gamma$  production (24).

#### CONCLUSION

Increasing significantly of IFN- $\gamma$  and IL-18 levels in serum of typhoid and tuberculosis patients with correlate significantly between IFN- $\gamma$  and IL-18 levels which will indicate and supports that IFN- $\gamma$  and IL-18 were implicated in the pathogenesis of these diseases.

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