Using Flow cytometry technique to characterize Toll- Like Receptors (TLR2 and TLR4) among patient with tuberculosis in Basrah-IRAQ

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Abstract

A case control study has been carried among patients with Tuberculosis, who attended to the Consultation Center of Chest and Respiratory diseases of Basrah province during 1st September 2020 to 1st June 2021. From total number of (176) patients with Tuberculosis (TB) were taken from Basrah province that included in the present study. In flow cytometry results, the present study found that TLR2 present on monocytes surface in percentage 32.08% and in control group was 14.8%, and TLR4 appeared in 3.4% among TB patients and in control group was 0.8%.

Key words: tuberculosis, Basrah, flow cytometry, Toll- Like Receptors

Introduction

Tuberculosing bacteria are disseminated by little droplets discharged into the air by coughing and sneezing, singing or simply talk from one person to the next, and from one person to the next. The neighboring people can respire and become infected with these bacteria. An infectious disease, known as Tuberculosis (TB), remains one of the world's largest bacterial infections. TB-related problems have been identified a recognized in the past, but their severity has been more recently
emphasized because of emerging antibiotic resistance in TB and the risk of re-infection. Innate immunity shown a major role in protecting the host from early infection with TB, as indicated by the majority of TB-exposed individuals being able naturally control the infection although a conspicuous delay of acquired immunity. The immune system, including adaptive and innate immunological mechanisms, modulate host response to tuberculosis infection (both active and latent). To stop the successful incorporation of TB infection in the lungs, host immune cells, and various nonclassical immune cells in the airway are fortified with a clusters of cell-surface and intracellular Pattern Recognition Receptors (PRRs) to recognize the occupying of mycobacteria, such as Toll-like receptors. At the meeting of host mucosal immunity and TB pathogenes, these innate immune sensors play a vital role. The early warning part of the recognizing bacteria was the natural immune system through its own receptors such as Toll-like receptors (TLRs), these were a group of distinct single membrane-spanning receptors consist of (1 to 10) types have been founded in humans, in both immune and non-immune cells and the (11 to 13) types in non-humans. The Toll-like receptors that expressed on cell surface were TLRs (1, 2, 4, 5, and 6), while TLRs (3, 7, 8, and 9) founded absolutely inside endosomes and these who known to be involved in recognition of Mycobacterium tuberculosis (MTB) were TLR2, TLR4, TLR9 and probably TLR8. Normally TLRs play an important role in both innate immune responses and the induction of adaptive immunity to TB. Really, polymorphisms of TLRs have been related with mutated susceptibility to tuberculosis among different populations. The TLRs are transmembrane proteins that illustrated as a key in the innate immune system considered pattern recognition receptors (PRRs), binding to Pathogen-Associated Molecular Patterns (PAMPs). Their function is Recognition of pathogens; and stimulation of immune responses directed against those pathogens. The primary innate immune cells participating in TB infection are macrophages, neutrophils, dendritic cells, and natural killer cells. PRRs expressed on innate immune cells recognize PAMPs present in MTB and have an important function in the initiation
responses of innate immunity. 

*Mycobacterium tuberculosis* can escape immune responses. And interrupt the crosstalk between acquired and innate immunities. Host defense systems initiate various strategies for eliminating TB such as activating proinflammatory responses. Producing reactive intermediates such as Reactive Oxygen Species (ROS) and reactive nitrogen species. And inducing cell death to inhibit the spread of TB infection.

TB also has several strategies to disturb these defenses, such as

- interference with phagosomal maturation and acidification,
- resistance to oxidative stresses,
- escape to the cytosol,
- formation of granulomas,
- modulation of host cell death.

And, TB can inhibit host innate immune systems by producing cellular envelope glycolipids and tetra-acylated sulfolipids, which are antagonists of TLR2, thereby inhibiting its role in pathogen recognition. Tuberculosis begins with ingestion of *Mycobacterium tuberculosis* through inhaled into the pulmonary alveoli. TB is identified by phagocytic cells of the innate immune system such as macrophages and denderitic cells (DCs), natural killer cells, and neutrophils, interact with various mycobacterial components, which represents the first line of host defense. These cells express many Pattern Recognition receptors (PRRs), including Toll-like receptors (TLRs), C-lectin type receptors (CLRs), complement receptor 1 (CR1), complement receptoer 3 (CR3), dendritic cellspecific intracellular adhesion molecule-3–grabbing nonintegrin, mannose receptors, surfactant protein A receptors, class A scavenger receptors, mannose-binding lectin and NOD like receptors (NLRs), which are recognize antigenic molecules expressed by *Mycobacterium tuberculosis* called pathogen associated with the molecular pattern (PAMPs). The Toll-like receptors (TLRs) have an vital role in
Mycobacterium infection, these receptors are associated with particular ligands exist on the bacteria to facilitate the absorption of \textit{MTB} in to the cells, which leads to the inducement phagocytic cells to produce cytokines, chemokines which serve as a sign of infection and crucial to stimulate the adaptive immune defenses and to stop growing of bacteria. As a result, TLRs serve as a connection between innate and adaptive immune defenses against Mycobacterium infection \textsuperscript{27,28,29}. The alveolar macrophages and dendritic cells with engulfed bacilli migrate to the regional lymph node and prime T cells (both CD4+ and CD8+) against mycobacterial antigens \textsuperscript{30}. The specific immune response produces primed T cells which migrate back to the focus of infection, guided by the chemokines produced by the infected cells \textsuperscript{31}. The accumulation of macrophages, T cells, and other host cells (dendritic cells, fibroblasts and endothelial cells) leads to the formation of granuloma at the site of infection \textsuperscript{32,33,34,35,36}. The formation of granulomas is barriers away from the other lung tissue tuberculosis and limits the body bacterial spread, as well as the interaction of macrophages and other immune cells and cytokines that these cells produced \textsuperscript{37}. The CD4+ T lymphocytes which produce IFN-\(\gamma\) detect and destroy infected macrophages presented with \textit{MTB} antigens \textsuperscript{38-40}. The infection progression is halted; however, some resistant bacilli capable of surviving under the stressful conditions generated by the host escape killing and enter a state of dormancy and persist by avoiding elimination by the immune system \textsuperscript{41-46}.

**Materials and methods**

**Sampling and source**

This case control study was carried out in the province of Basrah between 1\textsuperscript{st} September 2020 and 1\textsuperscript{st} June 2021. During the process of collecting data, the patients' names, age, gender, marital status, medical family history, personal information and clinical disease findings were reported on a single questionnaire for each patient. Samples of blood have been gathered from the symptomatic patients of the Chest and Respiratory Diseases consultation center of the province of Basrah. Every samples of
patients and control group were investigated in this study with age ranged from 14 years to equal or less than 78 years. Most of patients suffer symptoms like (Fever, chills, night sweats, loss of appetite, weight loss, fatigue). The blood samples were collected from patients after examination by the Pulmonologist and confirm as Tuberculosis according to clinical criteria.

**Control Group**

A total of 88 individuals without pulmonry problem, infectious diseases and allergies they were regarded as control group.

The number of patients group are calculated according to minimum size equation based on the ratio of disease which about 11 %.

**Exclusion criteria**

1. All patients how have atopic diseases.
2. All patients how have autoimmune diseases.
3. Patients how have an infectious diseases

**Blood samples:**

Five ml of venous blood was drawn by vein puncture using disposable syringes from each participant; 2 ml which will keep in EDTA tube and the other 3ml in disposable, non-pyrogenic, and non-endotoxin plastic tube which placed as a whole blood sample at room temperature for 2 hours and centrifugation for 20 minutes at approximately 1000 revolution per minute (rpm), blood collection tubes should be undergone centrifugation where the serum will obtained and preserved at (-20) °C till be used.

**Flow cytometry instruments and kits :**

Table (1) figures (1 and 2) show the instrument and Kits of flow cytometry.
Table (1) Flow cytometry instrument and kits

<table>
<thead>
<tr>
<th>Item</th>
<th>Model</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry</td>
<td>Bricyte E6</td>
<td>Mindray</td>
<td>China</td>
</tr>
<tr>
<td>Anti-h TLR2</td>
<td>ABCW021805</td>
<td>Minneapolis</td>
<td>USA</td>
</tr>
<tr>
<td>Anti-h TLR4</td>
<td>ABOG0217081</td>
<td>Minneapolis</td>
<td>USA</td>
</tr>
</tbody>
</table>

Figure(1) Flow cytometry kits for TLR2 and TLR4.

Figure(2) Flow cytometry analyzer. Bricyte E6, Mindray, China
The results

A case control study was carried on an overall cases of tuberculosis patients were (88) that taken from the Consultation Ceter of Chest and Respiratory diseases of Basrah province through period of 1st September 2020 to 1st June 2021, their age were ranged (14 - 78) years. Cutch up with (88) individuals regarded as control group were checked and confirmed to be free from any respiratory diseases or any other health problems that also studied, the number of cases are obtained according to minimum size equation that depend on the ratio of disease.

TB patients and controls distribution within age groups:

Table (2) documented that the highest age group of patients with tuberculosis was the third decades (20-29) years were 23 (26.1 %) from total study patients 88 (100.0%), fallowed by the fourth decades (30-39) years were 16 (18.2 %) from total study patients, at the fifth decades (40-49) years they were 14 (15.9%), closely with the second decades (10-19) years TB patients were 13 (14.8%), while less cases of TB patients appeared at the age (>60) years were 10 (11.4%) from total study cases 88 (100.0%). Statistically this differences were non- significant (P-value=0.45)

Table (2): Distribution of studied group according to age group (Years)

<table>
<thead>
<tr>
<th>Categorial age group (Yrs.)</th>
<th>Studied Groups</th>
<th>Total</th>
<th>Chi-square</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient</td>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10-19)</td>
<td>13</td>
<td>11</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>14.8%</td>
<td>12.5%</td>
<td>13.6%</td>
<td></td>
</tr>
<tr>
<td>(20-29)</td>
<td>23</td>
<td>17</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>26.1%</td>
<td>19.3%</td>
<td>22.7%</td>
<td></td>
</tr>
<tr>
<td>(30-39)</td>
<td>16</td>
<td>22</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>18.2%</td>
<td>25.0%</td>
<td>21.6%</td>
<td></td>
</tr>
<tr>
<td>(40-49)</td>
<td>14</td>
<td>22</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>15.9%</td>
<td>25.0%</td>
<td>20.5%</td>
<td></td>
</tr>
<tr>
<td>(50-59)</td>
<td>12</td>
<td>9</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>13.6%</td>
<td>10.2%</td>
<td>11.9%</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>10</td>
<td>7</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>11.4%</td>
<td>8.0%</td>
<td>9.7%</td>
<td></td>
</tr>
</tbody>
</table>
TB patients and controls distribution within Gender

Figure (3) documented that the most cases of tuberculosis recorded among male groups 50 (56.8 %) versus 38 (43.2 %) for female group from total study patients 88 (100.0%). Statistically this differences was non-significant (P-value=0.6) as shown below the following figure.

<table>
<thead>
<tr>
<th></th>
<th>Count</th>
<th>%</th>
<th></th>
<th>Count</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>88</td>
<td>100%</td>
<td>88</td>
<td>100%</td>
<td>176</td>
</tr>
</tbody>
</table>

Figure (3): Distribution of studied groups according to gender, $X^2=0.21$, $P=0.6$

Flow Cytometry

Flow cytometry plots for TB patients and control group

Figure (4) shows the getting area of flow cytometry after lysis process of blood cells for control sample, flow cytometry divide the cells according to size and shape, the shape include the cytoplasmic contents of cell and their granules and also shape of nucleus therefore, because the sample was a blood samples after passing the lysing steps by breakdown of RBCs and Platelets and get only WBCs.
Figure (4): Shown Getting area of flow cytometry after lysis process

Flow cytometry plots for TLR2 in TB patients and control group

Figure (5) shows histogram of flow cytometry that explains the availability of TLR2-CD marker for various samples with percentage of TLR2 for patients with tuberculosis and control group.
Flow cytometry plots for TLR4 in TB patients and control group

Figure (6) shows histogram of flow cytometry that explains the availability of TLR4-CD marker for various samples with percentage of TLR4 for patients with tuberculosis and control group.
Figure (6): Show the availability of cells for certain CD marker for various samples with percentage of TLR4 for tuberculosis patients and controls.

Number of TLR2 and TLR4 on Monocytes

Table (3) show the number and percentage of TLR2 and TLR4 among patients with tuberculosis and control group that found TLR2 in tuberculosis patients was 3.41% and in control group was 0.8%, and found TLR4 was 32.08% with tuberculosis patients and in control group was 14.8% by total monocytes from total white blood cells.
Table(3): show the number and percentage of TLR2 and TLR4 on Monocytes in blood component of tuberculosis patients in comparison with control.

<table>
<thead>
<tr>
<th>TLRs on Monocytes</th>
<th>TB Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2 (%)</td>
<td>32.08</td>
<td>14.8</td>
</tr>
<tr>
<td>No. of monocytes from total monocytes× 10⁹/L</td>
<td>0.133</td>
<td>0.048</td>
</tr>
<tr>
<td>TLR4 (%)</td>
<td>3.41</td>
<td>0.8</td>
</tr>
<tr>
<td>No. of monocytes from total monocytes× 10⁹/L</td>
<td>0.014</td>
<td>0.002</td>
</tr>
<tr>
<td>Total Monocyte From total WBCs (%)</td>
<td>3.9</td>
<td>6.1</td>
</tr>
<tr>
<td>No. of monocytes × 10⁹/L</td>
<td>0.414</td>
<td>0.324</td>
</tr>
</tbody>
</table>

W.B.Cs counts among tuberculosis patients and control group:

Table (4) show number of W.B.Cs among patients with tuberculosis and control group. That found the number of W.B.Cs in tuberculosis patients was 10.61×10⁹/L with Monocytes ratio 3.9% , Lymphocytes ratio 30.0% and Granulocytes ratio 66.1%.

Table (4): illustrate the number and percentage of white blood cells.

<table>
<thead>
<tr>
<th>WBCs</th>
<th>Normal Values</th>
<th>TB Patient</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBCs count×10⁹/L</td>
<td>4.0 – 10.0</td>
<td>10.61</td>
<td>5.32</td>
</tr>
<tr>
<td>Monocyte %</td>
<td>3.0 – 8.0</td>
<td>3.9</td>
<td>6.1</td>
</tr>
<tr>
<td>count×10⁹/L</td>
<td>1.0 – 1.5</td>
<td>0.412</td>
<td>0.324</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>20.0 – 40.0</td>
<td>30.0</td>
<td>46.5</td>
</tr>
<tr>
<td>count×10⁹/L</td>
<td>0.5 – 1.0</td>
<td>3.183</td>
<td>2.473</td>
</tr>
<tr>
<td>Granulocytes %</td>
<td>50.0 – 70.0</td>
<td>66.1</td>
<td>47.4</td>
</tr>
<tr>
<td>count×10⁹/L</td>
<td>1.2 – 8.0</td>
<td>7.013</td>
<td>2.521</td>
</tr>
<tr>
<td>Total %</td>
<td>100.0 %</td>
<td>100.0 %</td>
<td>100.0 %</td>
</tr>
</tbody>
</table>
The Discussion

Tuberculosis is a contagious disease that is remains one of the major bacterial infections worldwide. Tuberculosis-related problems were identified in the past but their severity was impressive as TB antibiotic resistance was emerging and re-infection risk. A case control study was carried on an overall cases of tuberculosis patients were (88), thier age were arounded (14 - 78) years. In addition to (88) persons observed as control group, in this investigation the highest age group of patients with tuberculosis was (20-29) years were 23 (26.1%) and fallowed by the fourth decades (30-39) were 16 (18.2%) from total study patients, at the fifth decades (40-49) were 14 (15.9%), closely with it the second decades (10-19) were 13 (14.8%), while less cases of tuberculosis appeared at the age (>60) were 10 (11.4%) from total study cases. This results similler with that observed during adolescence (age 15–19 years), there is a rapid increase in risk with a second peak between the ages of (20–30) years, this supported by the study of that found TB primerly affect adolescent and adults, and other studies evidences our results and give more explianation about distribution of tuberculosis within age group like that conclude the age distribution of tuberculosis case s mirrors global patterns, with a low number of cases in childhood and a high number in young adulthood. Other study disease of poverty affecting mostly young adults in their most productive years. The vast majority of TB deaths are in the developing world. And other study saying that the incidence of TB varies with age, while the study of that found TB is mainly a disease of older people, or of the immune compromised.

Toll Like Receptors (TLRs), a family of single membrane-spanning receptors, the nature involved thirteen types of TLRs, those 1 to 10 have been designated in humans, were expressed on cells of immune and non-immune system and others 11 to 13 in non-human beings these innate immune sensors play critical roles at the interface of host mucosal immunity and TB. In the current study that selected of (TLR2, TLR4 and TLR9) to be study thier asociasion with TB in Basrha province. Supported by other studies, TLRs had been recorded mainly in the
recognition of tuberculosis were TLRs (2, 4, and 9) there was a possibility TLR8. In addition study of which selected these TLR genes (TLR2, TLR4 and TLR9), due to the strong biological evidence that supports their role in TB. In this results that the mean concentration of TLRs (ng/ml) was higher among tuberculosis patients (100.0), of TLR2 were (0.65 ±0.27), while in TLR4 (3.19 ± 1.78) (ng\ml), and TLR9 were (1.92 ± 1.06), statistically the differences were highly-significant.

TLR2 a member of pattern recognition receptors (PRRs) plays critical role in host immune response against TB infection. TLR2, which is a well-known receptor forming with TLR1 or TLR6, heterodimers, involves the recognition and response of innate immune cells the dendritic and macrophagous cells. TLR2 is the central receptor for mycobacterial detection in particular. TLR2 is used to recognize the presence of fungi, parasites and virus in a broad range of bacteria. In the current study documented that the mean concentrations of TLR-2 (ng/ml) among male and female of tuberculosis patients (0.63±0.26) (0.67±0.26) respectively, was higher than male and female of control group (0.22±0.10) (0.20±0.13) respectively, statistically the differences was highly significant. In these results that the concentration of TLR2 in TB female patients slightly more than male, suggested may according to thier hormonical activity differences. In other studies observed that TLRs can prompt T-lymphocyte activation, adjust and ruler the aquired immunity, and keep the body's immune system balanced. In other way shown that TLR2 and TLR4 participate in recognizing and promoting inflammatory reactions to tuberculosis and associated metabolites. In study of was carried out to TLR2, TLR4, TNF-α, IFN-α, IL-2, IL-6, and IL-10 expressions were investigated in HIV patients infected with TB. These findings suggested that concentration of TLR2 associated with the activity of TB infection and the patient immunity responses after clear comparison with TLR2 concentration of control group. In addition study TLR2 is thought to be important to initiate innate host protection through its stimulatory effect on TNFα macrophage production. An important role for the stimulation of IL-1β production was found of TLR2 and TLR6 as well as important for macrophage release of IL-12. A few
studies did not find a correlation between TLR2 polymorphism and TB susceptibility.

TLR4, a member of pattern recognition receptors (PRRs), plays a critical role in the host immune response against TB infection. In the current investigations, observed that the mean concentrations of TLR4 (ng/ml) among male and female tuberculosis patients were (3.35 ± 2.03) (2.99 ± 1.41) respectively, was higher than male and female of control group (1.09 ± 0.45) (1.00 ± 0.58) respectively, statistically, the differences were highly significant. In these results, the concentration of TLR4 in TB male patients slightly more than females. That matched with the study of which shown that in *Mycobacterium tuberculosis*, TLR4 recognizes the cell wall lipids, glycoproteins, and antigens. The surface of TLR4 expression on lymphocytes in TB patients was also reported as much as that in healthy control persons in the apparent expression of both TLR4 and TLR2. In addition, studies from West Africa TLR4 is necessary to detect Gram-negative bacteria's endotoxins and has been associated with pulmonary TB.

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