Research Article

Role of Toll Like Receptor 2 and 4 (TLR2 and TLR4) in Susceptibility and Resistance to *Mycobacterium Tuberculosis* Infection among Sudanese Patients

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Abstract

Background: Tuberculosis (TB), an infectious disease caused by *Mycobacterium Tuberculosis* (MTB). Toll-like receptors (TLRs) are the best-studied class of PRRs, and they recognize specific pathogen-associated molecular patterns (PAMPs) from various microorganisms.

Objectives: This study aimed to determine and measure cellular TLR2 and TLR4 in active TB, latent TB and healthy individuals. Also aimed to define correlation between gene expression and present of TLR4 and TLR2 in study volunteers.

Materials & Methods: This Multi-site, prospective unmatched age and sex case-control, hospital and community-based study. 244 Consented volunteersor patients diagnosed as active tuberculosis, diagnosed latent TBI and a community controls volunteers with negative both TST and INF γ release assay were enrolled. Three-color Flow cytometry using Heparinized blood to study surface expression of TLR4 (CD284) and TLR2 (CD282), by Mean channel fluorescence intensity (MFI).

EDTA blood was used for mRNA expression of both receptors using Quantitative RT-PCR.

Results: TLR2, TLR4 cell surface expression by Flow cytometry was decreased in all study volunteers with no significance differences (*p.value* 0.24).mRNA expression of TLR2 were increased in active TB compared to Latent TBI and healthy individuals,while mRNA TLR4 decreased in active TB and showed increased pattern in latent and low level of expression in healthy volunteers, associated with no significant difference in the expression of TLR-2 mRNA between patients and close contacts with LTBI (p. *value* 0.045), compared with significance correlation between cellular TLR2 and expression and BCG status (p. *value* 0.00) respectively. There was highly significance correlation between TST indurations and level of both TLR2 and TLR4 (p. *value* 0.00). Also there was significant correlation between mRNA expression of TLR2 and disease types (*p value* 0.01). Single Nucleotide Polymorphisms (SNPs) in TLR2and TLR4,in active Tb Patients Showed three alleles,group one were heterozygous allele, homozygous wild type in group two, while homozygous mutant present in group three with statistical significant with disease stages, (*P.value* 0.02), while LTBI volunteers had only homozygous mutant present and group two had undetermined alleles, and four of them develop active TB infection, while healthy controls had similar alleles.

Conclusion: Up-regulation of TLR-2 and TLR-4 due to SNPs may be involved in the process of tuberculosis infection in Sudanese individuals.

Keywords: Toll like receptor; Latent TB; Active TB

Introduction:

Tuberculosis (TB) is chronic and infectious disease that is caused by *Mycobacterium Tuberculosis* and remains an important public health problem worldwide[1], in 2017, it was one of the 10 major causes of death and about1.5 million diagnosed cases globally[2]. *M. kansasii* and *M. avium-intracellulare* (MAI) shown similar pathological and clinical appearance to *M. Tubercluosis*[3].

Both innate and acquired immunity play a crucial roles in tuberculosis infection.Pro-inflammatory and anti-inflammatory cytokines is mediate the body immune response, which they are secreted by dendritic cells and macrophages[4]. This rapid immune response prevents bacterial reproduction and aids in suppress of the infection. an important role played by phagocytic cells in antigen presentation and T-cell-mediated immunity[5]. The bacilli protect themselves by generating immune response-avoiding mechanisms that antagonize the body immunity.

Mycobacteria have evolved to escape from macrophage by using elaborate evasion mechanisms[6]. Although, the healthy immune system is control the acute infection, a subclinical latent or chronic infection may persist[7]. Immunological pressure results from Inhibition of iNOS, or T cell depletion due to neutralized immune mediators such as IFN γ or TNF, with continuous activation of phagocytes most likely in associated activated T cells. Toll-like receptors (TLRs) are a class of pattern recognition receptors (PRRs) that initiate the innate immune response by sensing conserved molecular patterns for early immune recognition of a pathogen[8]. Several Functions of TLRs have been shown, such as the recognition of self and non-self-antigens; detection of invading pathogens; bridging the innate and adaptive immunity; and regulation of cytokine production, proliferation, and survival[9-12].

A key receptors that recognize *mycobacterial* antigens and activating macrophages and dendritic cells are believed to be Toll-Like Receptors (TLRs). Recognition of the whole *mycobacterial* and

purified *mycobacterial* lipoarabinomannans, lipomannans, phosphatidyl-*myo*-inositol mannoside, and the 19-kDa lipoprotein by TLR2, and to some extent, TLR4 participate[13]. Also, most researches postulated that a proinflammatory response generated due to this interaction[13].

TLR2 is a principal receptor implicated in recognizing of Gram-positive bacteria. Peptidoglycan is an essential and major component of Gram-positive bacteria cell walls[14]. TLR4 is known to recognize a broad spectrum of microbial products associated with bacteria, fungi, protozoa, and viruses[15]. TLRs can be expressed extracellularly and/or intracellularly depending on the receptor examined. TLR2 and TLR4 are localized on the cell surface for recognition of extracellular, mostly bacterial, pathogens[16]. During infection TLR2 and TLR4 are expressed in higher magnitude. TLRs and the Toll-interleukin 1 (IL-1) receptor (TIR) domains of myeloid differentiation protein 88 (MyD88) pathway are incorporate with pathogen-associated molecules. Indeed, muted susceptibility to tuberculosis among different populations associated with polymorphisms of TLRs[17]. The most risk factor for tuberculosis infection is TLR2 polymorphism[18].

The objective of present study is to determine the role of Toll-Like Receptor 2 (TLR2) and Toll-Like Receptor 4 (TLR4) in susceptibility and resistance to mycobacterial infections among Sudanese individuals.

Materials and methods:

A Multi-site, prospective, age and sex unmatched, hospital and community-based case-control study took place at Abuanga, Teaching Hospital, Khartoum State and Greater Shendi, River Nile State Sudan, in the period from January 2010 – January 2012.

Consenting adult volunteers or patients diagnosed as active tuberculosis with smear-positive for acid fast bacilli were collected from Abuanga Tuberculosis Treatment Centre and Latent TBI diagnosed by positive or negative Tuberculin skin test and positive INF- γ release assay (TST > 5mm diameter, IFN-γ > 0.35 IU/ml) and community controls volunteers with non-reactive (induration 00 mm) Tuberculin skin test and INF γ release assay from River Nile state were enrolled in this study. While Immunocompromised subjects were excluded, including individuals with HIV infection, those with diabetes mellitus requiring medication, and those taking Immunomodulation medications as well as pregnant and lactating women.

One hundred twenty two TB patients were participate in this study, 61 active TB cases, 61 Latent TB in addition to 122 controls with Fleiss CC (Continuity Correction factors). 100µl of anticoagulated blood lithium heparin blood was transferred to 12×75mm polystyrene test tube (106 cells), 20 µl of (TLR2) CD282 FITC and (TLR4) CD284 was added and mixed gently with a vortex mixer. The mixture was incubated in dark at room temperature at 4°C for 30 minutes or at room temperature (20-25°C) for 15 minutes. 2ml of RBCs lysis buffer was added and mixed gently with a vortex mixer and incubated in dark at room temperature for 10 minutes or at room temperature (20-25°C) for 15 minutes. Centrifuged at 1000×g (1850) round per minute for 5 minutes. Gently the supernatant was aspirated and discarded left 50 µl of fluid. 2ml of phosphate buffer saline (0.01mol/l) was added and cells were re suspended using vortex. The mixture was incubated in dark at room temperature at 4°C for 30 minutes or at room temperature (20-25°C) for 15 minutes. Centrifuged at 1000×g (1850) round per minute for 5 minutes. Gently the supernatant was aspirated and discarded left 50 µl of fluid. 300µl of paraformaldehyde added and stored until work.

1ml EDTA blood was collected for RNA extraction to unstimulated leukocytes 0.75 ml of Trizol reagent was added to 0.2 ml of blood and 20 µl 5 N Acetic Acid. Vortexed and the tube standed at Room Temperature for 10 min. Chloroform 0.2 ml, was added, shaked, the tube stand at R.T. for 2-5 min. Centrifuged 1200 rpm for 15 min at 2-8ºC. The aqueous phase to fresh tube was taken and 500µl Isopropanol was added and mixed. Allow to stand for 5-10 min in R.T. centrifugation at 1200 rpm for 10 min at 2-8ºC. The RNA participate was form pellet on the side and bottom of the tube. The supernatant was removed and washed the pellet by adding 1 ml of ethanol 75%. Vortexed, centrifuged at 7500 rpm for 5 min at 2-8ºC. Ethanol was removed and dissolved in 50µl RNase free water.

The mRNA was transcribed into cDNA, using the Omni script reverse transcription kit (Quiagen, Dusseldorf, Germany) with oligodT primers, (according to the manufacturer's instructions), the concentration calculated from the optical density using a Gene Quant spectrophotometer (Amersham Biosciences, Amersham, UK) and was stored at −20°C.

PCR was carried out in a total volume of 50 µl with 1 µg of cDNA using the HotStarTaq Master Mix kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Standard curves of quantifies and dilute PCR product, and at least eight negative controls per 96-well plate, were included in each PCR run. Primers were designed for the following targets: TLR2 sense: 5'-TTTCA CTGCTTTCAACTGGTA-3', TLR**2** anti-sense: 5'-TGGAGAGG CTGATGATGAC-3', TLR**2**TaqMan probe:5'-FAM/CAAGAC CCACACCATCCACAA-BHQ-1/-3'; TLR4 sense: 5'-CGATTC CATTGCTTCTTG-3', TLR4 anti-sense: 5'-GCTCAGGTCCAG GTTCTT-3',

TLR4 TaqMan probe: 5'-FAM/CAATGCATGG AGCTGAATTTCT-BHQ-1/-3Cycling conditions of an initial activation step of 15 min at 95°C follows by 45 cycles of 30s at 94°C, 30s at 60°C and 1 min at 72°C were used for each primer pair.

Statistical analysis

All statistical analyses were carried out using Epidemiological Information (Epi Info) soft- ware version 3.4.3.0. Which includes: make view, enter data, analyze data, visualize data and word processor was used for data entry, data cleaning and analysis. Mean ± STD used to compared surface cell expression of receptors and mRNA levels. Pearson correlation test was used to compare TST and disease phenotypes,MFI and SNP and *P values* of <0.05 were considered significant.

Results:

Two hundred and forty four consented volunteers were enrolled in the study with a mean age of 31.5 ±12.1 years. The majority (234/244; 95.9% were adults (> 15 years) and a male: female ratio of 1:1.6.

According to phenotypes of Tuberculosis they were grouped into: pulmonary TB patients (n=61); latent TB infection volunteers (n=61) diagnosed by reactive TST (in duration ≥10 mm) and Interferon-γ release assays (TST (induration< 5mm) and Interferon- γ release assays (IGRA <0.35 IU/ml).IGRAs; ≥0.35 IU/ml) and apparently healthy volunteers (n=122) with non-reactivity for both.

The mean ESR for the study groups was 53.2 ± 39.1 mm/hour, while sixty one percent showed an ESR of ≥ 50mm/hour. There was significant correlation between disease phenotypes and mean of TST (*p value* 0.008) Table (1).

Table 1: Demographic data, TST, MFI and mRNA levels of active TB and healthy volunteers.

Disease phenotypes	**Active TB** ***n= 61***	**Healthy volunteers** ***n= 122***	**Comments**
Sex			
M: F	**47**[77% : **14**[23%] **3:1**	**27**[22%]:**95**[78%] **1:3**	
Age			
Age group > 15 years **Age Group ≤ 15 years**	**61**[100%] **0**[0 %] **36.6** ± 12.8	**116**[93.4%] **6**[6.6%] **29.6** ± 12.3	*p*= 0.001* OR= 2.48
TST Mean ± STD **< 5 mm**	**2.0** ± 1.6 100%	**3.5** ± 5.2 > mm[74.5%]	*p*= 0.75 OR=1.33
ESR Mean ± STD	**108.3** ± 23.2	**36.3** ± 23.4	*p*= 0.00* OR=6.00
TLR2			
MFI ≤ 1 **MFI > 1**	**47**[77%] **14**[23 %]	**92**[75%] **30**[25%]	*p*= 0.92 OR=23.04
mRNA level (copies/ml)	**2972**.4 ± 1112.4	**258**± 93.4	*p*= 0.00* OR= 20
Single nucleotide polymorphism			
Homo. wild type **Homo. Mutant** **Heterozygous**	**43**[70.5 %] **10**[16.4 %] **08**[13.1%]	**121**[99.2%] **01**[0.8%] **0**0[00 %]	***p*= 0.00* OR=0.14** ***p*= 0.12 OR=5**

Continuous variable are expressed as means ±SD.**Significant difference (p<0.05)*

The mean of cell surface expression of TLR2 in all volunteers was 0.71 ± 0.37, while the mean of cell surface expression of TLR4 was 0.72 ± 0.38. The meanTLR2 mRNA levels in study population was 1164.9 ± 1447.6 copies/ml. While the mean of TLR4 mRNA TLR4 levels was 1077.9 ±1330.3 copies/ml, with no statistical differences between mean cell surface expression and mRNA levels of for receptors (*p value* 0.9).

Sixty one active TB patients (61/244, 25%) from Abu Anga Teaching Hospital, Omdurman were enrolled in the study. All were adults (age >15yrs), with a mean age of 36.6 ±12.8 years, and a male: female ratio of 3:1. The ESR mean was 108.3 ±23.2 mm, while the TST indurations mean 2.0 ± 1.6mm, with no statistical differences compared to other disease phenotypes (*p*=0.06).

Mean TLRs receptors cell surface expression was low for both TLR2 and TLR4 receptors in active TB patients with MFI 0.74 ± 0.34 and0.77 ± 0.32 respectively. Mean channel fluorescence intensity (MFI) for TLR2 (CD282) was decreased (<1) in the majority of patients (47/61, 77 %). Similarly, TLR4 (CD284) MFI was < 1 in most patients (50/61, 82%) Table1.

mRNA levels TLR2 was low compared to TB patients with a mean of 258 ± 93.38 copies/ml. Mean TLR4 mRNA (90.16 ± 85.49 copies/ml) was low compared to TLR2. Mean TLR2 mRNA level was lower than that seen in TB patients put was significantly lower than that seen in LTBI volunteers (*p value 0.00*). Similarly, mean mRNA of TLR4 which lower than that seen in TB patients and LTBI volunteers (= p=0.00) respectively Table 2,3.

Table 2: Demographic data, TST, MFI and mRNA levels of LTBI and health

Disease phenotypes	**LTBI** ***n= 61***	**Healthy volunteers** ***n= 122***	**Comments**
Sex			
M: F	**20**[32.8] % :**41**[67.2%] **1:2**	**27**[22%] : **95**[78%] **1:3**	
Age			
Age group > 15 years **Age Group ≤ 15 years** **Mean ± STD**	**57**[93.4%] **4**[6.6%] **30.7**±10.7	**116**[95%] **6**[5%] **29.6** ± 12.3	*p*= 0.005OR= 2.07
TST Mean ± STD **< 5 mm**	**8.0** ± 5.3 [74.5%]	**3.5** ± 5.2 [82.6 %]	*p*= 0.008*OR= 5.33
ESR Mean ± STD	**31.9** ± 20.5	**36.3** ± 23.4	*p*=0.06 OR= 1.72
TLR2			
MFI ≤ 1 **MFI > 1**	**43**[70.5%] **18**[29.5%]	**92**[75%] **30**[25%]	*p* = 0.78 OR= 0.93
mRNA level (Copies / ml)	**227.6**± 118.6	**258**± 93.4	*p*= 0.00*OR= 1.67
Single nucleotide polymorphism			
Homo. wild type **Homo. Mutant** **Heterozygous**	**48**[78.7%] **05**[8.2%] **08**[13.1%]	**121**[99.2%] **01**[0.8%] **0**0[00 %]	*p*= 0.31OR=0.79 *p*=0.01* OR= 10 *p*=0.00*OR= 14

Continuous variable are expressed as means ±SD.**Significant difference (p<0.05)*

Table 3: Demographic data, TST, MFI and mRNA levels of LTBI and active TB population

Disease phenotypes	**LTBI** ***n= 61***	**Active TB** ***n= 61***	**Comments**
Sex			
M: F	**20**[32.8] :**41**[67.2%] **1:2**	**47**[77% : **14**[23%] **3:1**	
Age			
Age group > 15years **Age Group ≤ 15 years** **Mean ± STD**	**57**[93.4%] **4**[6.6%] **30.7**±10.7	**61**[100%] **0**[0 %] **36.6** ± 12.8	*p*=0.55 OR= 0.83
TSTMean ± STD **< 5 mm**	**8.0** ± 5.3 [74.5]	**2.0** ± 1.6 100%	*p*= 0.06 OR=4
ESRMean ± STD	**31.9** ± 20.5	**108.3** ± 23.2	*p*= 0.00* OR=0.54
TLR2			
MFI ≤ 1 **MFI > 1**	**43**[70.5%] **18**[29.5%]	**47**[77%] **14**[23 %]	*p*= 0.74 OR=0.91
m RNA level (Copies / ml)	**227.6**± 118.6	**2972**.4 ± 1112.4	*p*= 0.00*OR=8
Single nucleotide polymorphism			
Homo. wild type **Homo. Mutant** **Heterozygous**	**48**[78.7%] **05**[8.2%] **08**[13.1%]	**43**[70.5 %] **10**[16.4 %] **08**[13.1%]	*p*= 0.69 OR= 1.12 *p*= 0.22 OR= 0.54 *p*= 1 OR= 1
TLR4			
MFI ≤ 1 **MFI > 1**	**47**[77 %] **14**[23 %]	**50**[82 %] **11** [18 %]	*p*= 0.82 OR=6.94
m RNA level (Copies / ml)	**2719.7**± 981.5	**294.0**± 146.9	*p*= 0.00* OR= 9.25
Single nucleotide polymorphism			
Homo. wild type **Homo. Mutant**	**38**[62.2%] **03** [05 %]	**40**[65.5%] **03** [05 %]	*p*= 0.85OR= 0.95 *p*= 1.00OR1

Heterozygous	**20**[32.8%]	**18**[29.5%]	*p*=0.77OR = 1.11

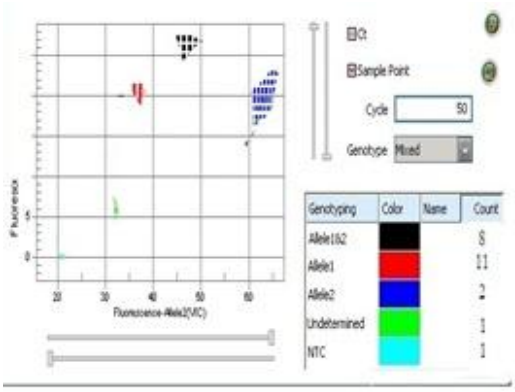
Continuous variable are expressed as means ±SD.**Significant difference (p<0.05)*

Sixty one latent TB volunteers (61/244, 25%) from River Nile State were enrolled with a mean age 30.67 ± 10.65 years and a male: female ratio of 1:2. Most were adults (57/61; 93.4%). The mean of ESR was 31.88 ± 20.45 mm/hour and with 88% having ESR <50mm/hour. TST induration of >5mm (mean 8.02 ± 5.3mm) was see in the majority of volunteers (74.5%). There was significance correlation with LTBI disease phenotype compared to healthy volunteers [TST mean 3.5 ± 5.2mm] (*p*= 0.008) Table 3.

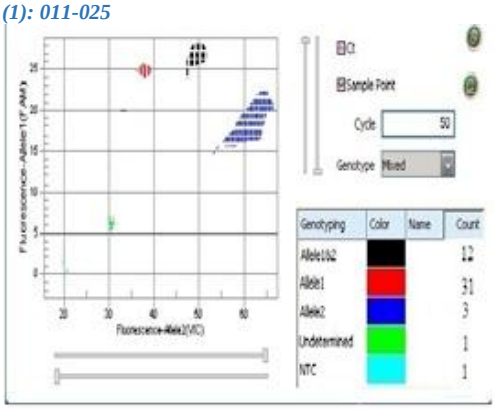
Cellular expression (MFI) of both TLR2 and TLR4 in these volunteers group was similar to active TB patients with low TLR2/TLR4 expression (*p value* 0.2). The mean TLR2 and TLR4 were 0.78 ±0.38 and 0.78 ±0.38 respectively. Mean channel fluorescence intensity (MFI) of TLR2 (CD282) was low (<1) in the majority of volunteers (43/61; 70.5%), compared to 29.5% (18/61) who had high level (>1) of TLR2 (CD282) expression. TLR4 (CD284) showed low cellular expression (MFI<1) in most volunteers (47/61, 77%) compared to 23% (14/61), who had MFI of >10. There was no statistically significance difference between TLR2 and TLR4 cellular expression (MFII between active TB and LTBI volunteers (p=0.8; p=0.9) respectively Table 3.

Mean TLR2 mRNA level was low (227.6 ± 118.6 copies / ml) compared to mean TLR4 mRNA level (2719.7 ±981.5 copies / ml). Mean mRNA levels for TLR2 and TLR4 were significantly different from those seen in overt TB patients (*p=0.00*; *=0.00*) Table 3.

Single nucleotide polymorphisms (SNPs) in *TLR2* and *TLR4* genes among active TB patients showed that seventy percent (43/61, 70.5%) had homozygous wild type TLR2 alleles. Homozygous mutant TLR2 alleles were seen in 16.4% (10/61), while 13.1% (08/61) had heterozygous mutant alleles (Figures 1). For TLR4, sixty five per cent (40/61) showed homozygous wild type allele, while heterozygous mutant genotypes wereseen in 29.5% (18/61). Only 5% (3/61) had homozygous mutant genotypes (Figures 2).



Figures (1): qPCR printout of TLR 2 Single nucleotide polymorphisms among TB Patients



Figures (2): qPCR printout of TLR4 single nucleotide polymorphisms among TB Patients

Single nucleotide polymorphisms (SNPs) for TLR2 showed that most of volunteers (78.7%; 48/61) had homozygous wild type genotype. Homozygous mutant genotype was seen in 8.2% (5/61), while 13.1% (08/61) had heterozygous mutant genotype (Figures 3). For TLR4, 62.2% (38/61) had homozygous wild type genotype, minority 5% (3/61) had homozygous mutant genotype and 32.8% (20/61) had heterozygous mutations (Figures 4).

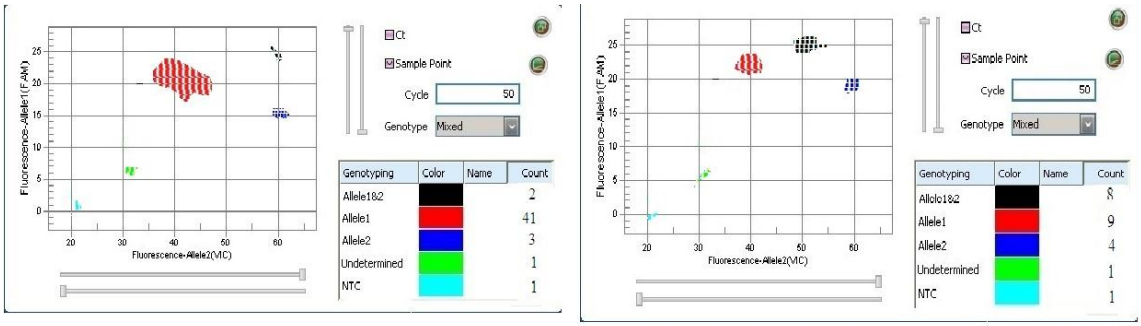


Figure (3): qPCR printout of TLR 2 Single nucleotide polymorphisms among LTBI volunteers

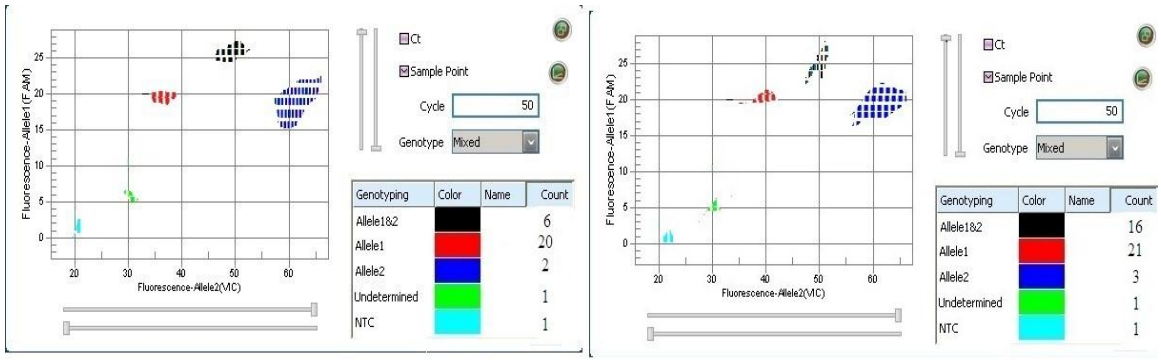


Figure (4): qPCR printout of TLR 4 Single nucleotide polymorphisms among LTBI volunteers

One hundred and twenty two (122/224, 50%) apparently healthy volunteers with non- reactive TST and IFN-γ release assay (>0.35 picograms/ml) were enrolled. The majority were adults (116/ 122, 95%) with a male: female ratio was 1:3 with a mean age of 29.6 ±12.3 years. And mean ESR of 36.3 ± 23.4 mm/first hour. More than eighty per cent (82.6%) had TST induration of < 5mm (mean 3.5 ± 5.2mm).

mRNA levels for TLR2 (2972.37 ± 1112.39 copies / ml) were increased in active TB compared to Latent TB and healthy individuals [p=0.00 for LTBI and p=0.00 for healthy volunteers. On the other hand, TLR4 mean mRNA level was relatively low in TB patients (294.01± 146.87 copies/ml), but was significantly lower than that seen in latent (2719.**7** ± 981.5 copies / ml**)** and healthy volunteers (90.2 ± 85.5 copies / ml**)** (*p value* 0.01, *p value 0.00*) (Table 4).

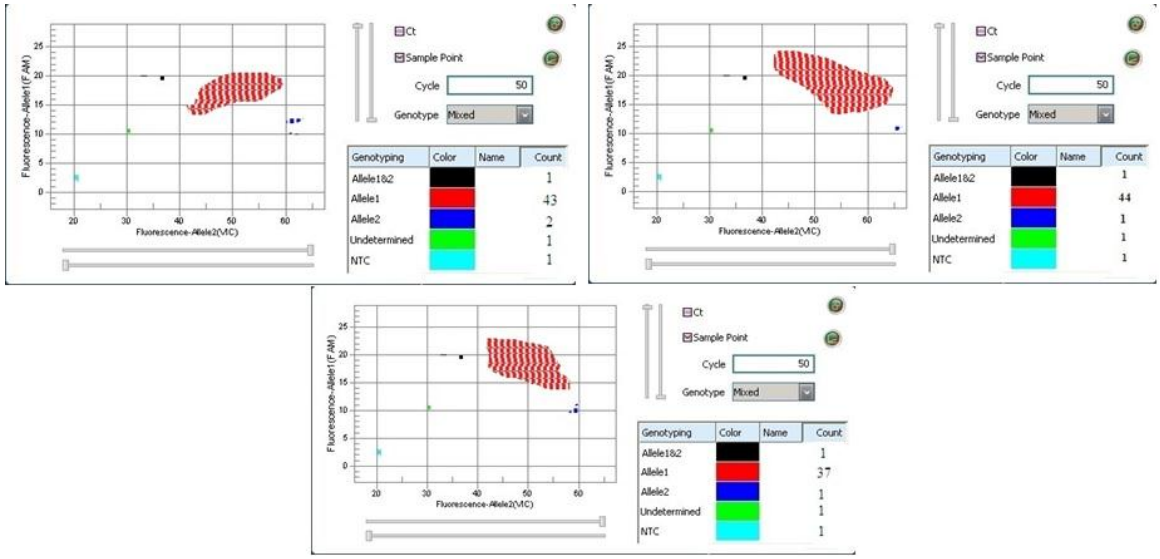
Cellular expression of both TLR2 and TLR4 receptors was similar to TB patients and LTBI volunteers. Mean MFI for TLR2 was 0.66 ±0.37, while mean for TLR4 was 0.67 ±0.40. Mean channel fluorescence intensity (MFI< 1) for TLR2 (CD282) was low in 75% of volunteers (92/122), with a quarter (30/122) having high levels of TLR2 (CD282) expression (MFI>1). Similarly, TLR4 (CD284) cellular expression (MFI<1) was seen in more than 83% of volunteers (102/122). Only 16% had high levels MFI>1 Table 4.

Table 4: Demographic data, TST, ESR LEVEL, MFI and mRNA levels of study population

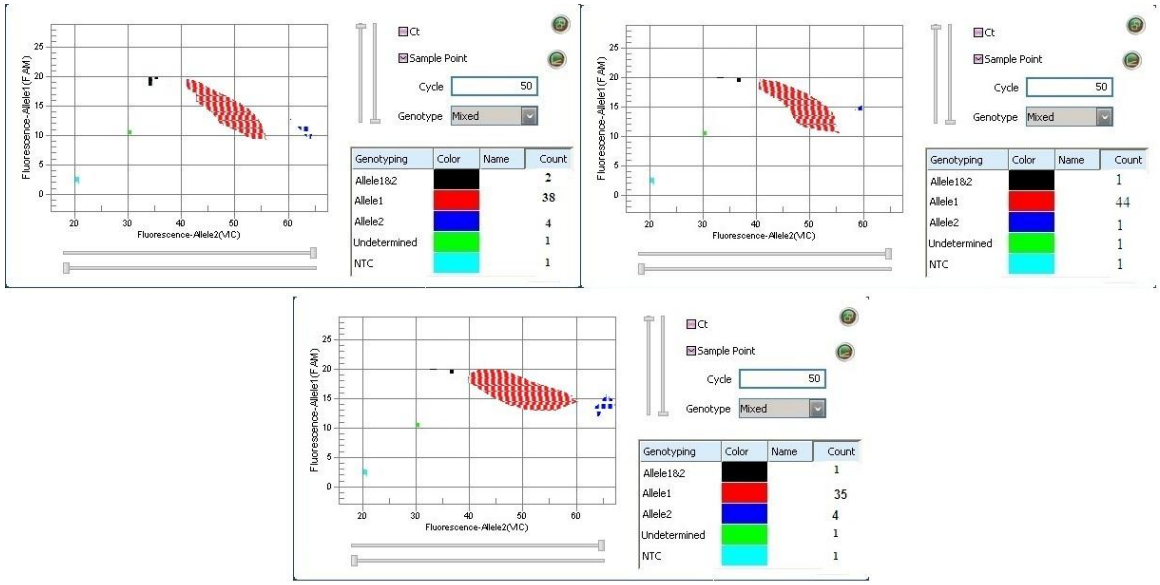
Disease phenotypes	**Active TB** ***n= 61***	**Latent TBI** ***n= 61***	**Healthy volunteers** ***n=122***
Sex			
M : F	**47**[77%] :**14**[23%]	**20**[32.8] % : **41**[67.2%]	**27[22%] : 95[78%]**
M : F ratio	**3:1**	**1:2**	**1:3**
Age			
Age group > 15 yrs	**61**[100%]	**57**[93.4%]	**116 [95%]**
< 15 yrs	0%	**4**[6.6%]	**6 [5%]**
Mean age ±**SD**	36.6 ± 12.8	30.7 ±10.7	**29.6 ± 12.3**
TST			
Mean TST ±**SD**	**2.0** ± 1.6 [100%]	**8.0** ± 5.3 [74.5%]	**3.5 ± 5.2 [82.6%]**
Mean ESR ±SD	f		
	108.3± 23.2	**31.9** ± 20.5	**36.3± 23.4**
TLR2			
MFI≤ 1			
MFI>1	**47** [77 %]	**43**[70.5%]	**92 [75%]**
	14[23 %]	**18**[29.5%]	**30 [25%]**
mRNA level (copies/ml)			
	2972.4 ± 1112.4	**227.6**± 118.6	**258± 93.4**
Single nucleotide polymorphism			
Homo. Wild type	**43**[70.5 %]	**48**[78.7%]	**121[99.2%]**
Homo. Mutant	**10**[16.4 %]	**05**[8.2%]	**01[0.8%]**
Heterozygous	**08**[13.1%]	**08**[13.1%]	**00[00 %]**

Continuous variable are expressed as means ±SD.**Significant difference (p<0.05)*

The majority (121/122, 99.2%) had homozygous wild type TLR2 alleles, while a minority (01/122, 0.8%) had homozygous TLR2 mutant alleles (Figures 5). TLR4 wide type allele was in the majority of healthy volunteers (115/122, 94.3%), while the mutant homozygous allele was seen in the minority (06/122, 04.9%). Heterozygous mutant genotype was seen in <1% (01/122, 0.8%) (Figures 6).



Figures (5): qPCR printout of TLR 2 Single nucleotide polymorphisms among healthy volunteers



Figures (6): qPCR printout of TLR 4 Single nucleotide polymorphisms among healthy volunteers

Discussion:

The innate immune system constitutes the first line of defense against pathogens such as mycobacteria. The recognition of pathogen associated molecular patterns by pathogen recognition receptors (PRR) is crucial for the initiation and coordination of cell-mediated responses[19]. This emphasizes further the current concepts that the adaptive immune response is partly under control of the innate immune system. Discovery of the central importance of TLR-mediated signaling to innate immune

mechanisms has transformed immunology and answered a fundamental question: how does the host sense the presence of foreign pathogens and how does that generate a signal to rapidly mount a vigorous defense. Mammalian Toll-like receptors (TLRs) play a key role in the innate immune response to infectious agents through their ability to discriminate conserved microbial structures, known as pathogen-associated molecular patterns (PAMPs) from self[20, 21]. TLRs recognition of PAMPs, such as lipopolysaccharide (LPS) initiates signal transduction through the NF-kB pathway. Nuclear translocation of NF-kB induces transcription of pro-inflammatory cytokine genes that are essential to mount a protective immune response and host defence.

TLR-2 in association with TLR-1, TLR-6 and TLR-4 has been implicated as receptors involved in the recognition of mycobacterial antigens and activation of macrophages and dendritic cells. Furthermore, it has been shown that TLR-2 is essential for the intracellular mycobacterial killing in macrophages, through the production of the antimicrobial peptide cathelicidin.

Although mice studies have shown that TLR -/- mice are more susceptible to mycobacterial infection, polymorphisms of human TLR2 or TLR4 can result in increased susceptibility to microbial infections[22]. Interestingly, mycobacterial infection and pro- inflammatory cytokines increase surface expression of TLR-2 and lower TLR4 in active TB patients. In the present study, that TLR2/TLR4 cell surface expression low in all study volunteers irrespective of the mycobacterial disease phenotype or healthiness. Lack of surface expression was not associated with presence or absence of TLR-2 or TLR-4 SNPs or the disease phenotype. This is in agreement with Druszczynska and colleages (2013) who showed no difference in the TLR2 expression on monocytes from TB or non-mycobacterial lung diseases (NMLD)[23].

The recent study showed significant increase in expression of TLR2 mRNA levelsin active TB compared to latent TBI and healthy individuals. These finding, probably indicate a rapid turn-over of surface TLR2 and possible indicate that TLR2 is involved in the active mycobacterial infection. This is in agreement with[22], who reported that the increase in expression of TLR2 in peripheral blood is surprising. This finding might indicate that expression of TLR2, TLR4 or TLR6 at the mRNA level is sensitive to increased circulating levels of pro-inflammatory cytokines or to circulating mycobacterial components. Alternatively, it may simply represent the re-circulating monocytes from the pulmonary tuberculosis lesions, which are expressing TLR-1, TLR-2 and TLR-4, as previous studies have shown[24]. But this does not explain the low surface expression of TLR2. Additional *in vitro* studies have shown that engagement of TLR2 with *MTB* ligands induces inhibition of macrophage MHC class-II antigen presentation and also blocks macrophage responsiveness to IFN-γ. A defensive mechanism that the organism use to disarm the macrophages[25].

That was a markedly increased TLR4 mRNA expression in Latent TB infected volunteers compared to active TB and non-infected groups. This was couple with low surface expression probably suggested a differential up-regulation of TLR4 in latently infected volunteers making it a possible diagnostic indicator for LTBI.

Presence of low *TLR2/TLR4* mutation across the study population probably indicates that TLR2 play a minor role in susceptibility to mycobacterial diseases. This is discordant with a Korean study that showed that polymorphism in *TLR2* (R 677W homozygosity TLR2) is significantly associated with susceptibility to TB infection[26]. Our findings are in partial agreement with Zhang*et al*., 2013 who showed in a Meta-analysis that *TLR2*

(G2258A) polymorphism is associated with increased TB risk, especially in Asians and Europeans[27]. Whereas, *TLR2* (T597C) and *TLR2* (T1350C) polymorphisms did not show significant association with TB. Another study showed variants of TLR 2 and 9 influence susceptibility to pulmonary tuberculosis in Caucasians, African-Americans and West Africans was found to be true to our Sudanese population. The strongest evidence for association came at an insertion (I)/deletion (D) polymorphism (-196 to -174) in TLR2 that associated with TB in both Caucasians and Africans[28]. Our findings are also agrees with Mohammed, 2013 who showed that TLR2 polymorphisms do not appear to be responsible for host susceptibility to *M. Tuberculosis* in a cohort of Sudanese population[29].

Similarly, our result showed that SNPs in TLR4 genes have no association with susceptibility to *M. Tuberculosis* infections. In disagreement with Branger and colleagues, who suggested that TLR4 plays a protective role against lung infection by *M. tuberculosis*[30]. Similar findings were reported in Gambian population[31]. A recent study from Sudan demonstrated that there are common polymorphisms in TLR4 gene associated with susceptibility to pulmonary tuberculosis in Sudanese. This study used a large sample size and sequencing technique[32].

Conclusion:

- TLR2/TLR4 cell surface expression in generally low and has no bearing to health status or mycobacterial disease phenotype. The low surface expression of TLR2/TLR4 couple with high mRNA of TLR2 or TLR4 probably indicates rapid TLR2/TLR4 molecules turn-over.
- Latent TBI volunteers despite having high TLR4 mRNA showed low cellular expression of both receptors.
- SNPs in TLR2 & TLR4 play little or no role in susceptibility to mycobacterial infections in this cohort Sudanese individual.

Ethical approval: Ethical clearance was obtained from Research Ethics Committee, Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan. Reference No: UKIENDERC 1/ 11 in 9 /5 /2011. Written and verbal inform consent was obtained from all patients, information taken from them used only for research.

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