Original Article

Minimizing Macrophage Extracellular Trap Formation in the Development of Pulmonary Tuberculosis

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Abstract

Background: Due to the rising frequency of antimicrobial treatment resistance and issues in vaccine development. To eradicate the microbiological infection with *Mycobacterium tuberculosis* can cause macrophages to release proinflammatory cytokines such as IL-1β, IFN-γ, and TNF-α. **Objectives:** The aim of the current study was to investigate the role of the TLR2 receptor in positively regulating IFN-γ and TNF-α production during *M. bovis* infected macrophages *in vivo* using human monocytic cell lines THP-1 and developed a new treatment to eliminate TB using TLR2 short interfering ribonucleic acid knockdown. **Materials and Methods:** The human monocytic cell line THP-1 cells were grown in the culture media that were prepared according to the manufacturer's instructions. Short interfering ribonucleic acid (siRNA) was transfected into cells using (lipofectamine) DNA-fectin TM Plus transfection reagent. Total RNA from the macrophages was extracted, and the total RNA was measured using a NanoDrop spectrophotometer. The mRNA in the total RNA was reverse transcribed to complementary DNA using a cDNA synthesis Kit. **Results:** Showed that the relative expression of both TLR2 and IFN-γ was scaled down to 0.001% in TLR2 siRNA knockdown (TLR2-KD) samples compared to (TLR2-C) control without BCG vaccine, due to the TLR2 siRNA knockdown, whereas the TNFα relative expression was stabilized in both (TLR2-KD) and (TLR2-C) samples without BCG vaccine. **Conclusion:** TLR2 expression in THP-1 cells was consistent with our goal of reducing TLR2 expression. Our results showed a decrease in gene expression of TNF-α and IFN-γ in the TLR2 siRNA knockdown samples pluse BCG challenged after only 4h, which, in turn, reduced the synergistic effect of these genes (TNF-α and IFN-γ) in granuloma formation during pulmonary tuberculosis.

Keywords: BCG, IFN-γ, THP-1 cells, TLR2 siRNA knockdown, TNF-α

Introduction

Mycobacterium tuberculosis (M.T) is well-evolved intracellular pathogens that can thrive indefinitely in their hosts. The recruitment of host macrophages to the bacteria, their phagocytosis, and the transit of infected macrophages into deeper tissues all occur as a result of infection. Infected macrophages recruit further macrophages and other immune cells to form granulomas, pathological characteristics of tuberculosis.[1] A granuloma may hold bacteria for years or decades before permitting the creation of viable bacteria (reactivation) for unknown reasons.[2] The toll-like receptors (TLRs) are proteins that play an vital role in battling invading pathogens and generating the innate immune response. TLRs may recognize pathogen-associated microbial patterns of microbes as well as damage-associated molecular patterns, which are precursors of cell debris.[3]

The recognition of M.T is mediated by different groups of pattern recognition receptors including TLRs, Nod-like receptors and C-type lectin receptors, TLRs family members such as TLR2, 4, and TLR9 with the adaptor molecule MyD88 which have molecules of importance in the immediate immune response to tuberculosis infection. The re-sensitization of macrophages to infections by increasing the expression of TLR2 and mCD14 genes obviously. Due to TLR2 forms heterophilic dimers with other receptors such as TLR1 and 6, which are functionally linked to TLR2 on macrophage surfaces and identify *M. tuberculosis* triacyl and diacyl lipopeptides, respectively.[4]

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Polymorphisms in the TLR2 gene may increase the risk of *M. tuberculosis* vulnerability. Impairing the TLR2 functions in murine phagocytes by gene knockout or blocking antibodies abolishes the TNF-α and IL-6, when those cells are challenged with Gram-positive bacteria.[5] Furthermore, *in vivo* research that followed has led to the dominant viewpoint that TNF-α is important for granuloma development and maintenance, based on the results of disordered granulomas following infections of TNF-deficient mice.[6] TNF-α treatment of macrophages (THP-1) resulted in enhanced maturation of phagosomes carrying *M. bovis* or *M. tuberculosis.* When used specific monoclonal antibodies (pentoxifylline) of TNF-α and neutralization its or targeting of gene in mice chronically infected with *M. tuberculosis* disrupts the integrity of granulomas, augments infection, and leads to death.[7] IFN-γ was the first macrophage-activating factor to be discovered,[8] and it was crucial for stimulating proinflammatory cytokine generation, phagocytosis, antigen-presenting capacity, and nonspecific cytocidal activity against *M. tuberculosis* infections and malignancies in these cells.[9] Individuals with mutations in IFN-γ signaling are vulnerable to mycobacterial infection and infection spread after Bacillus Calmette–Guérin (BCG) vaccination, thus IFN-γ action is lowest in individuals with the most acute manifestations of tuberculosis.[10]

Macrophages have recently been found to release extracellular traps bacteria, fungi, and parasites were trapped and removed by macrophage extracellular traps.[11] *M. tuberculosis* produces pore-forming toxins known as 6 kDa-early secretory antigenic target (ESAT-6). Importance of ESAT-6 in virulence was further supported when it was discovered that removing it from *M. bovis* lowered pathogenicity in guinea pigs, ESX-1 was required for extracellular trap formation by human macrophages and triggered a caspase-1–independent cell death pathway.[12] IFN-γ may worsen tuberculosis–triggered necrosis in a multiplicity of infection and a more virulent *M. tuberculosis* strain-dependent manner.[13] Immunopathological responses in tuberculosis, aforementioned also as the head mediator of the destruction of pulmonary tissue.[10] Elevated levels of TNF-α are related to excessive inflammation with necrosis and cachexy.[10,11] *M. tuberculosis* evolved and has developed mechanisms which interact and modulate the host immune response. Mycobacterium expresses surface antigens that can induce the production of IL-10 and IL-4, which typically have anti-inflammatory effects.[8,9] The high expression of IL-4 has been implicated as a virulence factor, both for its anti-inflammatory ability and for its apparent capacity to promote tissue damage in association with TNF-α.[12] These studies suggest that IL-4 (alone or jointly with TNF-α) may play a role in tissue destruction and/or cell death during infection by *M. tuberculosis*. TNF-α is one of the most powerful controlling factors for the recruitment of monocytes and is a potent inducer of cell death by apoptosis.[13] Necrosis, on the other hand, is associated with the lysis of the infected cell, the release of feasible *M. tuberculosis*, and damage to the surrounding tissues.[13] TNF-α is also a key cytokine involved in this event.

Materials and Methods

Preparation of cell culture media

The following components were combined to create complete cell culture medium: 8.2 g of RPMI 1640, 10 g of $NaCO_3$, 500 mL of T.D.W., 50 mL of FBS (10%), and 0.2 mM L-glutamine. The ingredients were combined, stirred, and sterilized using a 0.22 m syringe filter. Following sterilization, the prepared antibiotics 100 U/mL penicillin and 100 g/mL streptomycin were added at 37°C in a humid environment with 5% CO_2. Following the manufacturer's instructions, THP-1 cells were grown to 80% confluence in culture media, seeded in 12-well plates, and transfected with the constructs using lipofectamine DNA-fectin TM Plus.

Short interfering ribonucleic acid (siRNA) and transfection

TLR2 siRNAs targeting: Three were designed by (abm. Cat. No. G487, CANADA), siRNA was transfected into cells using (lipofectamine) DNA-fectin TM Plus transfection reagent according to the manufacturer's instructions. Each siRNA oligo was dissolved in 62.5 μL of DEPC water to prepare 20 μM. Each siRNA was mixed with 1.2 μL of lipofectamine and left for 20 min before adding the mix to the cells. In parallel, 62.5 nmole of the negative control were mixed with 1.2 μL of lipofectamine.

The TLR2 siRNA was transfected into macrophages in accordance with the manufacturer's instructions to inhibit the expression of TLR2. In brief, THP-1cells were incubated in the siRNA transfection medium (abm.Cat. No. G487, CANADA), at a density of 2×10^6 cells/well in 12-well cell culture plates, Hemocytometer results showed that the TLR2 siRNA or negative control siRNA was added next and that after 4 days of incubation at 37°, the transfection efficiency was >95%. (Superior. Germany), each well contained 1 mL medium, 0.8 μg DNA(μg), 2 μL DNAfectinTM Plus, 100 μL transfection medium, and then were added of BCG vaccine at a concentration of 8×10^6 cells/mL to six wells opposite six wells without BCG vaccine. The 12-well plate was incubated at 37°C for 4 h and subsequently harvested.

Quantitative reverse transcription PCR

Following the manufacturer's instructions, total RNA from the macrophages was extracted using an RNA extraction kit (Promega, USA). Using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), the extracted total RNA was measured by absorbance at 280 nm. The mRNA in the total RNA was reverse transcribed to complementary DNA using a cDNA

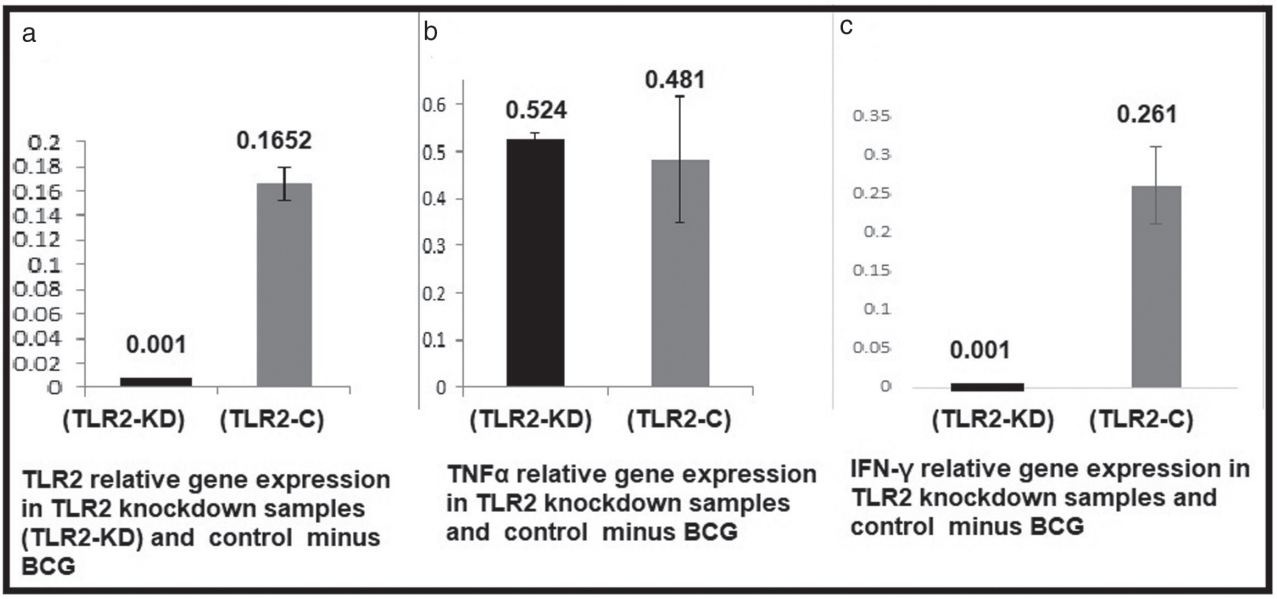


Figure 1: TLR2, TNF, and IFN-γ relative gene expression in TLR2 knockdown samples (TLR2-KD) and non-TLR2 knockdown control (TLR2-C) samples without BCG. All RNA was extracted after 4 h, reverse transcribed and the synthesized cDNA was used as a template for qPCR relative expression assay using SYBR Green master mix. The averages and standard deviation were considered for three replicates. (a) TLR2 relative expression was raised about 0.001% in (TLR2-KD) samples compared with its control. (b) TNFα expression was stabilized in both of (TLR2-KD) and (TLR2-C) samples. (c) IFN-γ expression was about 0.0014% in (TLR2-KD) samples. The TLR2 and IFN-γ were reduced expression due to the siRNA knockdown (*t* test $P = 0.0031$ significant)

synthesis Kit (Promega, USA). qPCR was performed in the I Cycler iQ5 (Bio-Rad) using SYBR RT permix (England) with the following conditions: 10 s at 95°C, 45 cycles of 15 s at 95°C, and 30 s at 59°C. The mRNA expression levels, which were normalized against β-actin, were calculated and expressed as ΔΔCT. The primers used for qPCR were as follows: β-actin; F-5′-GATTACTGCTCTG GCTCCTAGC-3′ and R-5′-GACTCATCGTACTCCTGCTTGC-3′ and for TRL2: F-5′-AAG AGGAAGCCCAAGAAAGC-3′ and R-5′-CAATGG GAATCCTGCT CACT-3′ and for TNFα; F-ATGAGCACTGAAAGCATGATCC and R-GAGGGCTGATTAGAGAGAGGTC and for IFN-γ F-GATCCA-GCACAAAGCTGTCA and R-GACTCCTTT-TCCGCTTCCTT.

Statistical analysis

Differences between groups were compared using the Wilcoxon test (TIMER2.0) or Student *t* test (Oncomine and UALCAN). Results are shown as mean ± SD, and the difference was statistically significant when $P < 0.05$.

Results

Relative gene expression of TLR2, TNFα, and IFN-γ in TLR2-siRNA knockdown and normalized to 1 by fold change analysis without BCG vaccine after 4 h

To measure the expression of the TLR2 gene, which is important for recognizing both endogenous and foreign ligands, and to check both TNFα and IFN-γ which are linked with TLR2 gene expression, the templates of cDNA were used in qPCR relative expression assay with a master mix of SYBR green and specific primers for TLR2, TNFα, and IFN-γ genes. The averages and standard deviation were considered for three replicates. Our findings showed that the relative expression of both TLR2 and IFN-γ was scale down to 0.001% in TLR2 siRNA knockdown (TLR2-KD) samples compared to (TLR2-C) control without BCG vaccine, due to the TLR2 siRNA knockdown, whereas the TNFα relative expression which was stabilized expression in both (TLR2-KD) and (TLR2-C) samples without BCG vaccine [Figure 1a–c].

The outcomes were based on ΔΔCTs analysis after deduction the house-keeping gene (β-actin). Furthermore, to accurately verify the fold change, the values of all samples were normalized to one by fold change analysis, and their values in TLR2 knockdown (TLR2-KD) samples and (TLR2-C) control were compared to that 1. Our findings showed that the relative expression of TLR2 was reduced to 99% in (TLR2-KD) and IFN-γ was decreased to 96% in (TLR2-KD), due to the TLR2 siRNA knockdown, whereas the TNFα relative expression which was equalized to 1 in both (TLR2-KD) and (TLR2-C) samples without BCG vaccine [Figure 2a–c].

Relative gene expression of TLR2, TNFα ,and IFN-γ in TLR2-siRNA knockdown and normalized to one by fold change analysis plus BCG vaccine, after 4 h

When the TLR2, TNFα, and IFN-γ gene expressions have been shown by qPCR machine through the reaction

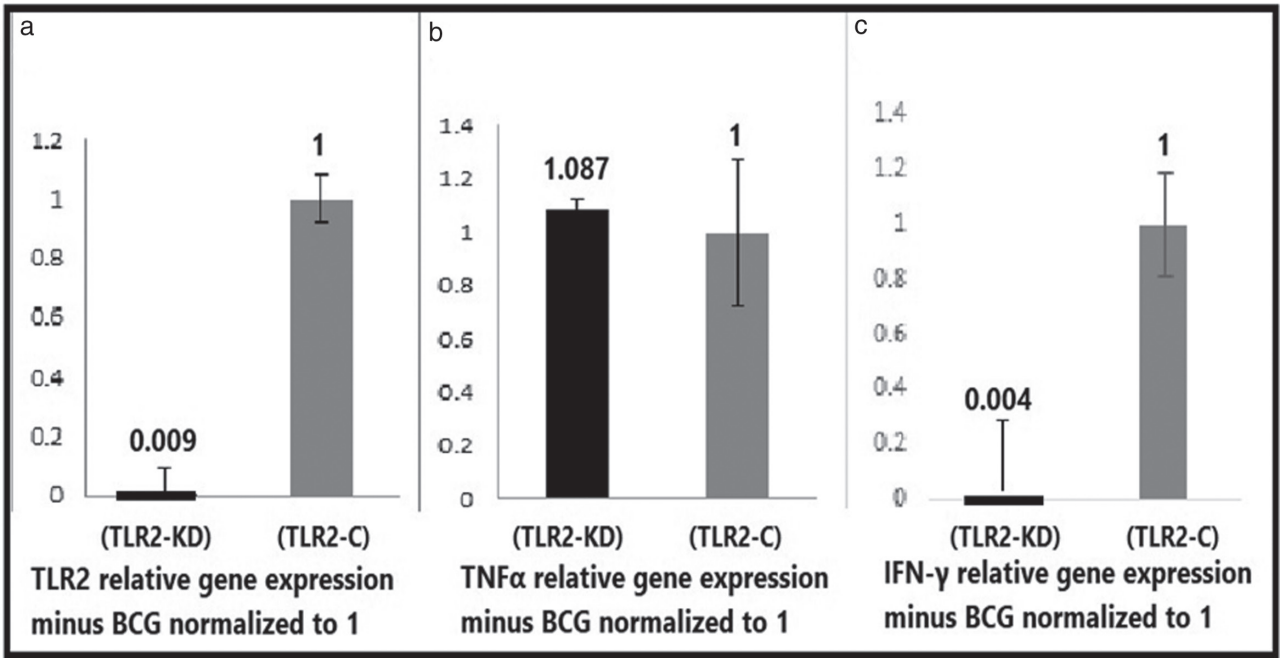


Figure 2: The relative gene expression of TLR2, TNFα, and IFN-γ in TLR2 knockdown (TLR2-KD) and control (TLR2-C) samples without BCG was analyzed by ΔΔCTs and normalized to (β actin) housekeeping gene which was equalized to 1. (a) TLR2 expression was reduced to 99% one-fold in (TLR2-KD) samples minus BCG compared with its control, **(b)** TNFα relative expression was equalized to 1 in both (TLR2-KD) and (TLR2-C) samples, (C) IFN-γ was reduced to 96% one-fold in (TLR2-KD) samples minus BCG compared with its control (*t* test $P = 0.00032$ significant)

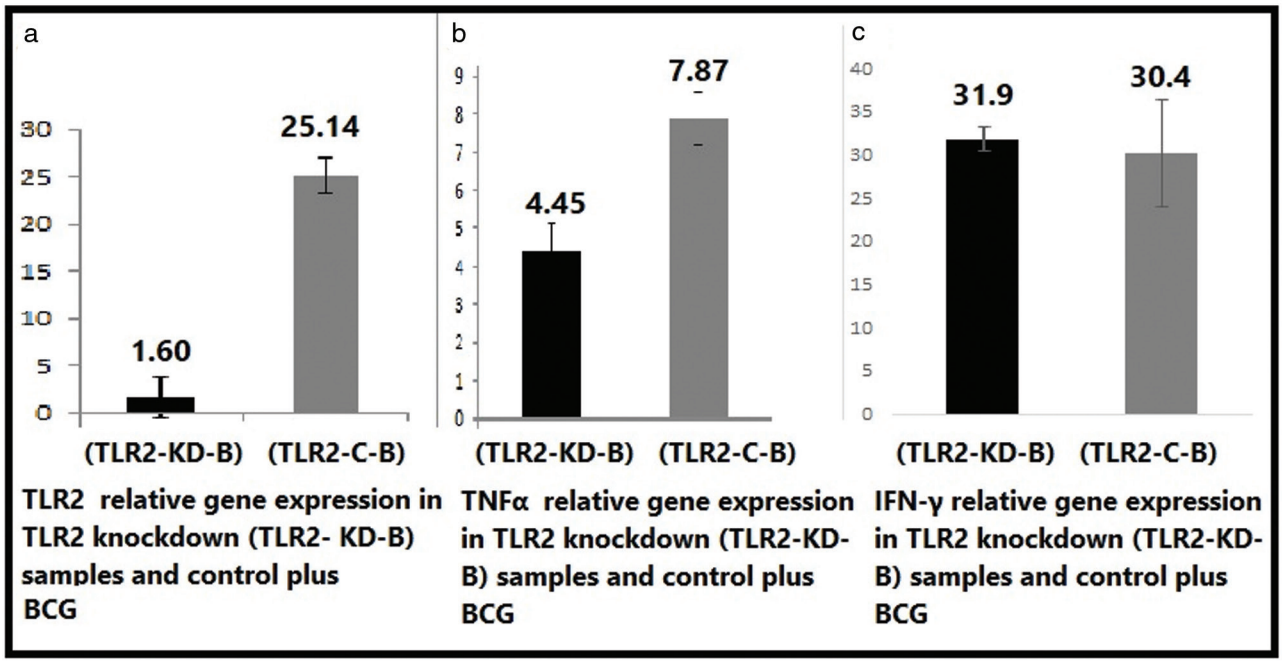
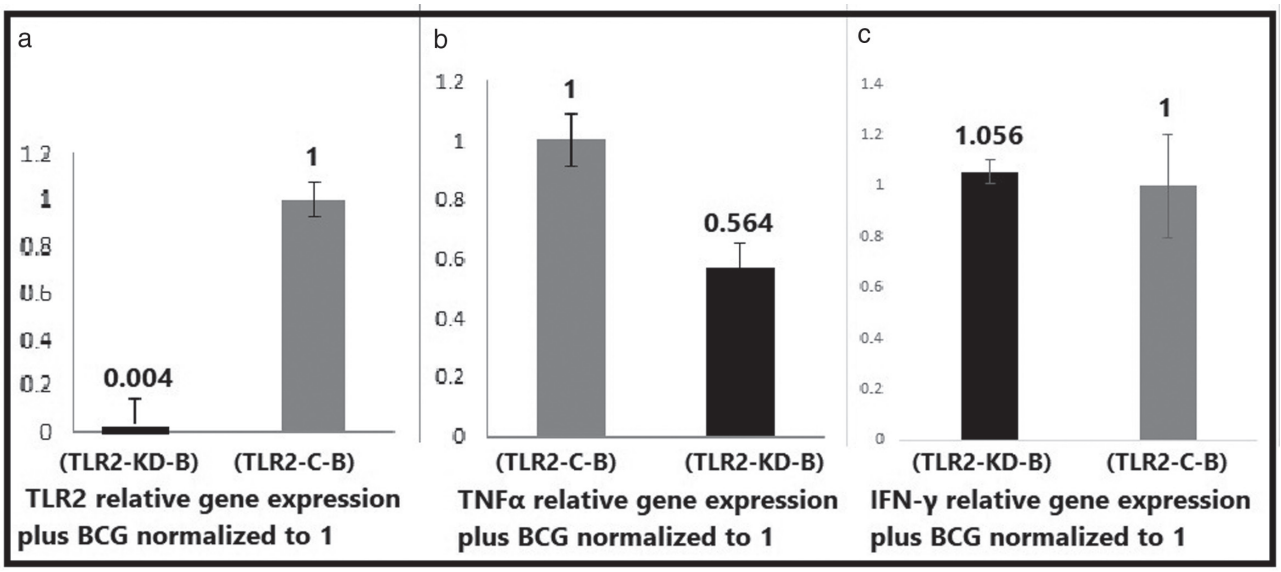


Figure 3: TLR2, TNFα, and IFN-γ relative gene expression in TLR2 knockdown samples (TLR2-KD-B) and control (TLR2-C-B) samples plus BCG. All RNA was extracted after 4 h, reverse transcribed and the synthesized cDNA was used as a template for qPCR relative expression assay using SYBR green master mix, the averages, and standard deviation were considered for three replicates. (a) TLR2 relative expression was few expression about 1.6% in (TLR2-KD-B) samples compared with high in control plus BCG vaccine. (b) TNFα expression was decreased to 4.45% in (TLR2-KD-B) plus BCG vaccine samples compared with control. (c) IFN-γ equal expression in control and (TLR2-KD-B) samples plus BCG vaccine was approaching 31.5% (*t* test $P = 0.00131$significant)

consisting of mixing SYBR Green master mix and specialized primers with cDNA templates from samples having BCG vaccine. The averages and standard deviation were considered for three replicates for each sample.



Figur 4: The relative gene expression of TLR2, TNFα, and IFN-γ in TLR2 knockdown (TLR2-KD-B) and control (TLR2-C-B) samples plus BCG after 4 h was analyzed by ΔΔCTs and normalized to (β actin) housekeeping gene, which was equalized to 1. (a) TLR2 relative expression was reduced to 56% only in knockdown samples (TLR2-B-KD) compared to 1 in the control. (b) TNF-α gene expression was reduced to 50% in (TLR2-KD-B) compared to 1 in the control (TLR2-C-B). (c) IFN-γ equal expression in control and knockdown samples plus BCG vaccine (*t* test $P = 0.0030$ significant)

Visibly, the results showed that TLR2 gene expression was low at 1.6% in the TLR2 knockdown plus BCG vaccine samples (TLR2-KD-B) and continued high in the control plus BCG vaccine (TLR2-C-B), but the TNFα expression decreased to half expression in TLR2 knockdown plus BCG vaccine samples compared with its control 4.45%, whereas the IFN-γ gene expression increased to become equal in both (TLR2- KD-B) and (TLR2-C-B) 31.4%, due to its important role in the inflammatory response of the immune system [Figure 3a–c]. The results were analyzed by ΔΔCT analysis and normalized to β-actin, the values of all samples were normalized to 1 by fold change analysis and their values in the TLR2 knockdown with its control were compared to normalized one. Our results demonstrated that the TLR2 relative expression was reduced to 56% in knockdown samples (TLR2-B-KD) compared to 1, due to the siRNA knockdown plus the BCG, After BCG challenge, the TNF-*α* gene was reduced to 50% in knockdown samples (TLR2-KD-B) compared to 1 in the control (TLR2-C-B), the IFN-γ equal expression in (TLR2-C-B) and (TLR2-KD-B) samples plus the BCG vaccine [Figure 4a–c].

Discussion

The best members of the TLR family are TLR2, 4, and TLR9 with their adaptor molecule MyD88, these receptors are an important part of the initiation of immunity against *M. tuberculosis*, TLR2 receptor recognizes a serious of *M. tuberculosis* components.[14] The current findings showed that siRNA knockdown lowered TLR2 gene expression to 99% one-fold in TLR2 knockdown samples without BCG vaccine-challenged after 4 h [Figure 2a], whereas the TNFα expression was equalized to 1 in both of TLR2 knockdown samples and (TLR2-C) control minus BCG vaccine [Figure 2b] while the IFN-γ was reduced to 96% one-fold in (TLR2-KD) samples minus BCG compare with its control [Figure 2c].

To analyses this result, the decreasing in TLR2 expression, due to successful TLR2 knockdown process without BCG, but decreasing in IFN-γ gene in (TLR2-KD) samples, it is well known that the IFN-γ response is an immunological correlate of M. *tuberculosis* infection, the decreased in both TLR2 and IFN-γ expression is not include TNFα expression under the same conditions without BCG vaccine-challenged, due to there is evidence that TNFα expression is necessary to cause TLR2 expression in primary microglia,[15] although recent research has shown that the nuclear factor-kappa B (NF-B), SP-1, and mitogen-activated protein kinase signaling pathways are involved in controlling TLR2 expression in monocytes and macrophages.[16] In other side, the results revealed the TLR2 gene expression was increased to 44% one-fold at siRNA knockdown samples challenged with BCG vaccine [Figure 4a], which indicates that up regulation level of TLR2 gene expression in siRNA knockdown samples with BCG challenged was due to the mimic of *M. tuberculosis* infection.

TLR2 expression is controlled through different mechanisms. LPS, synthetic lipid A, and cell-wall fractions – lipoarabinomannan–peptidoglycan complex (PGN) led to upregulate TLR2 gene expression and NF-κB activation into macrophages response *in vitro* and *in vivo* through a dose- and time-dependent

manner.[17] This result was consistent with report by Xiong *et al*. [18] who found that the TLR2 levels increased clearly after longer exposure with LPS-stimulated, not with shorty time. A new study finds that in adipocytes, a new expression of TLR2 is transmitted inside the cell, requiring a half-time of (3–3.5 h) and then rising on the surface. Therefore, our results were consistent with these data, as the TLR2 knockdown samples spent only 4 h with BCG challenged [Figure 4a]. The findings also demonstrated that the TNF-α gene was reduced to 50% of one-fold in (TLR2-KD-B) compared to 1 in control (TLR2-C-B) [Figure 4b] and equal expression of IFN-γ in the control and knockdown samples plus BCG vaccine, which was 1.05% one-fold when normalized to one [Figure 4c].

Due to *M. tuberculosis* is an obligate aerobic mycobacterium that can survive inside an immune-competent host and has a distinctive cell-wall structure, so various components of mycobacterium induce the expression of a wide variety of genes by macrophages in a TLR2-dependent manner, ESAT-6 protein of mycobacterium increases macrophages apoptosis through TLR2/NF-κB activation. Lipoprotein and mycolic acid components of mycobacterium which enables it to avoid host immunity in down regulation of major histocompatibility complex class II molecules and reduction of proinflammatory responses, together are conducted by TLR2-dependent manner.[15]

In addition, phagosome maturation of *M. tuberculosis* is conducted through TLR2, good survival strategies for mycobacterium, TLRs signals regulate the expression of proinflammatory cytokines such as TNFα, IFN-γ, IL-1, and IL-8, in turn, TNFα and IFN-γ could augments TLR expression in macrophages.[19] Our result revealed that the TNF-α gene was reduced to 50% in (TLR2-KD-B) compared to 1 in control (TLR2-C-B) with BCG after 4 h [Figure 4b], with IFN-γ equal expression in control and knockdown samples plus BCG vaccine [Figure 4c], which mean good results for us due to the need for new therapeutics for controlling tuberculosis and multidrug- and extensively drug-resistant *Mycobacterium* threaten human health globally,[20] because of, at the time of tuberculosis diagnosis, TNFα response to M. tuberculosis antigens may be increased while the IFN-γ response may be suppressed; a later reduction in TNFα and an increase in IFN-γ response are connected with successful disease resolution, Furthermore, individuals with previously treated mycobacterium disease have been seen to have higher TNFα levels at the time of relapse.[21]

On the other hand, TLRs upregulate anti-inflammatory cytokines such as IL-10 and IL-6 which creates kind of balance between pro- and anti-inflammatory responses, that is, triggered through TLR response and activity,[22] TNFα appears to have a fundamental function, this cytokine acts in synergy with IFN-γ, exciting the production of reactive nitrogen intermediates, so facilitates the tuberculostatic function of macrophages, and also exciting the migration of immune cells to the infection, therefor, any reduced in gene expression of TNFα and TLR2 it will effect on IFN-γ expression during tuberculosis infection.[23] Despite those cells producing inflammatory substances are unable to reduce the pathogenicity of *M. tuberculosis* with the support of IFN-γ and *Mycobacterium* infected macrophages more susceptible for necrosis when IFN-γ is present.[24] Accordingly, our findings suggest the TLR2 siRNA knockdown process will decrease TLR2 expression on macrophages in turn minimize interactions between macrophages and *M. tuberculosis* which is in turn reduces IFN-γ expression, also have seen to have TNF-α blockers decreased IFN-γ induced phagosome-lysosome fusion and acidification, which mean the TLR2 siRNA knockdown process led to decreased TNF-α expression in our study in turn led to reduced IFN-γ expression in THP-1 cells and prevent IFN-γ from producing non-protective necrosis in macrophages humanity through prevent synergistic effects of TNF-α with IFN-γ and ESX-1of M. tuberculosis and necrosis macrophages, due to the enhanced effect of IFN-γ dependent on ESX-1.[25] Given the importance of TNF-α in granuloma development and maintenance, it is perhaps not surprising that utilized TLR2 siRNA technique to diminish rather than block TNF-α expression and thus minimize granuloma formation during *M. tuberculosis* infection due to anti-TNF-α therapy has been associated with increase susceptibility to infection with *M. tuberculosis* comparable,[17] There is evidence that TNF- is participate in granuloma formation indicate that the granuloma is important to bacterial virulence, consistently TNF-α expression was affected withTLR2 knockdown, due to their expression decreased to 50% one-fold [Figure 4], in turn, led to reduce the IFN-γ expression bout 99% one-fold, due to the TNF-α synergized with IFN-γ induced phagosome-lysosome fusion and acidification and both of them are responsible in granuloma formation and maintenance.[20]

Given the importance of TNF-α in granuloma development and maintenance, it is perhaps not surprising that utilized TLR2 siRNA technique to diminish rather than block TNF-α expression and thus minimize granuloma formation during M. tuberculosis infection due to anti-TNF-α therapy has been associated with increased susceptibility to infection with *M. tuberculosis*.[19-21] There is a need for new therapeutics for tuberculosis (TB), the leading cause of death by a single infectious agent worldwide. Although much effort has been focused on controlling TB, multidrug- and extensively drug-resistant TB threaten human health globally.[26-28]

Conclusion

For *M. tuberculosis* survival, the TLR2 signaling pathway is advantageous. Since, this prolonged immune response

creates favorable conditions for *M. tuberculosis* survival in macrophages by producing inflammatory cytokines. Granulomas are produced as a result of the disease drawing immune cells. TLR2 thereby encourages *M. tuberculosis* survival and long-term endurance in macrophages. As a result, TLR2 expression in THP-1 cells was consistent with our goal of reducing TLR2 expression. Our results showed a decrease in genes expression of TNF-α and IFN-γ in the TLR2 siRNA knockdown samples pluse BCG challenged after only 4 h, which, in turn, reduced a synergistic effect of these genes (TNF-α and IFN-γ) in granulomas formation during pulmonary tuberculosis.

Ethical approval
Not applicable.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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