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Original Article

Evaluating the Effects of Imiquimod on Paths of TLRs and Inflammatory Cytokines Signaling in Infected Macrophages with *Leishmania major* in Vitro and in Vivo

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Abstract

Background: *Leishmania major* is an obligate and intracellular pathogen and the macrophages are the cell hosts for *L. major*. Imiquimod stimulates macrophages to secrete different cytokines via the expression of TLRs.

Methods: This study was carried out in the Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, in 2018. The effect of imiquimod was investigated on non-infected and infected macrophages with *L. major* on the expression of Toll-like receptors (TLRs) and inflammatory cytokines. TLRs play an important role in enhancing the proceeding of phagocytosis and killing parasites. Moreover, the cytokines such as TNFα, IL6, and IL1, are often identified in inflammatory conditions as interfering targets in treatment. Healthy macrophages and macrophages infected with *Leishmania major* parasites were affected by different concentrations of imiquimod, after that the expression of TLR genes (TLR1, TLR2, TLR3, TLR4, TLR7 and TLR9) and cytokines were evaluated by real time RT-PCR. For experiments in laboratory animals, infected BALB/c mice were exposed to imiquimod and then isolated peritoneal macrophages.

Results: The expression of TLR2 decreased in non-infected macrophages were affected by the imiquimod. The expression level of TLR7 in healthy macrophages, decreased and the difference with control group was significant. Imiquimod increases the expression of inflammatory cytokines and IL12 in mouse macrophages and also decrease the expression of IL10.

Conclusion: This suggests that imiquimod may improve the therapeutic effects in infected mice with *Leishmania major*. Imiquimod causes that TLR2 decreased expression but TLR7 and TLR9 increased expression. Imiquimod as TLR7 agonist, enhance the recovery of leishmaniasis.

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Introduction

Leishmaniasis is one of the neglected tropical diseases that cause a wide range of human infections, from spontaneous healing skin lesions to diffuse cutaneous-visceral forms (1). At present, the common treatment, pentavalent antimony compounds, has side effects, drug resistance and recurrence of the disease, especially in visceral leishmaniasis so replacing it with drugs that have less side effects and more efficiency seems necessary (2-6).

Imiquimod is a TLR7 agonist that stimulates macrophages, monocytes and Th1 cells to secrete IFN-α, IFN-γ, IL-1β, TNF-α ,IL-6, IL-12 and IL-8. It has been shown to stimulate the synthesis of nitric oxide (NO) in macrophages in mice infected with Leishmania major and thus has a lethal effect on *Leishmania*. In a leishmaniasis study, the improvement in the group receiving 5% imiquimod cream with meglumine antimonate (20 mg/kg/d for 20 d) was greater than in the group receiving meglumine antimonate alone (7). Imiquimod has activity in treatment dermal lesions caused by viral infections such as genital warts, genital herpes and molluscum contagiosum (8). Imiquimod has therapeutically and prophylactically effects against acute toxoplasmosis (9).

Imiquimod can modulate the immune response and this is the reason for using against a lot of infectious diseases and cancers. Imiquimod can activate the immune cells system such as macrophages, monocytes and dendritic cells to stimulate and produce cytokines (IFNα, IL-6, IL-12, IL-1α and tumor necrosis factor (TNF)) (10-13). Imiquimod can be used to treat mice infected by stimulating the synthesis of nitric oxide (NO) by macrophages (14).

TLRs are related to the both innate and adaptive immunity with an important role in control the infection specialy intracellular organisms (15-17). Receptors in different cell types, when stimulated by microbial compounds, inducing intracellular responses that ultimately lead to inflammatory cytokines and chemotactic factors production. There is evidence that cytokines are activated by various transcription factors such as interferon regulatory factor (IRF), activating protein-1 (AP-1), nuclear factor-κB (NF-κB) and then two different messaging paths (MyD88 protein-dependent pathway and TRIF protein-dependent pathway) are generated. Stimulation of TLRs by homogenous ligands (AP-1 and NF-κB) induces the production of anti-inflammatory cytokines and chemotactic factors, while IRF factors induce interferon production (18,19). These pathways, in turn, cause the production of cytokines and the activation of other cells. However, activation of individual TLRs elicits a strong innate immune response (20,21).

A brief outline of TLRs and their ligands in MyD88-related paths in TLRs signaling and their effects on inflammatory cytokines is shown in Fig. 1. In this study, the effect of imiquimod was evaluated on macrophages infected with Leishmania major as an immunomodulator agent and TLR-7 agonist, and evaluating its effects on paths of TLRs and inflammatory cytokines signaling in macrophages.

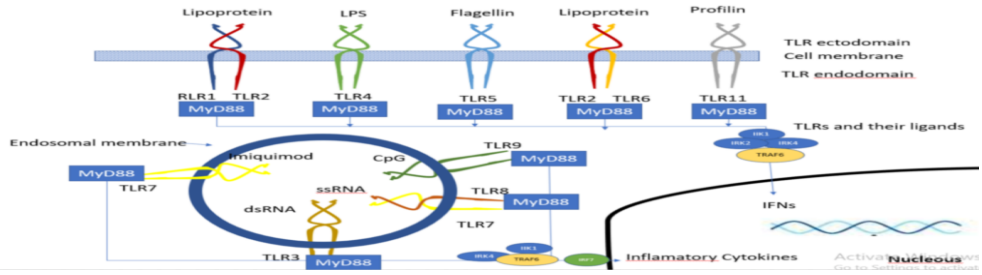


Fig. 1: A brief TLRs and their ligands in MyD88-related paths in TLRs signaling and their effects on inflammatory cytokines

Materials and Methods

The approval number of this study that approved by Ethical Committee is: 52/D/8207.

Parasite, macrophage and drug preparation

We used *L. major* (MRHO/IR/75/ER) kept in the Tarbiat Modares University. Imiquimod was purchased from Invitro Gen-San Diego, USA (dry powder). One mg of imiquimod was dissolved in 1 ml of its specific solvent (commercially available). In this study cell line macrophages J774 A.1 was used (a gift from Professor Marcel Hommel with many thanks).

In vitro Assay

In this study promastigotes, healthy and infected macrophages with *L. major* exposed to imiquimod 0.01 µg/ml and also with 50 µg/ml of glucantime as control group. After 24 h the macrophages were washed with cold PBS and collected by centrifugation. Then RNA extraction and cDNA were prepared from collected macrophages. By real time PCR, cDNA was used for assessment of TLRs and cytokines expression (22,23).

Real Time PCR

TLRs cytokines and GAPDH and β_2 microglobulin genes primers were used from Panday et al. and Tauer et al. and Ebrahimisadr. Qiagen Mastercycler was used for quantitative real-time PCR assays. The expression was analyzed by relative fold change of gene by comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. Data of expression for target gene were normalized by both GAPDH and β_2 microglobulin genes (24,25).

In vivo Assay

Female BALB/c mice (6-8 wk old) were provided by the Pasteur Institute of Iran and kept under standard conditions. Mice were subcutaneously infected with 2×10^6 stationary phase promastigotes. Forty mice were divided into four groups:

1. Infected, untreated group (control).
2. Uninfected, untreated group (control).
3. Infected, treated topically with 5% imiquimod cream (Aldara) three times a week for four wk.
4. Infected, treated with glucantime (20 mg/kg/day for 28 d). Half of the mice in each group were sacrificed four weeks post-treatment. Spleen lymphocytes were isolated for cytokine assays (IL-4 and IFN-γ) using U-CyTech kits (Netherlands). Spleen samples were also used to evaluate parasite load. RNA was extracted from macrophages to prepare cDNA and assess TLR expression via real-time PCR. The other half of the mice were monitored for survival and lesion size (22,23)."

Parasite burden assay by quantitative Real Time PCR

In this study we evaluated the parasite burden in spleen tissue. The DNA extraction from spleen tissue carried out by Sinagen company kit. For standard curve we used six samples contain 10^1-10^6 parasite copies. The parasite burden evaluated for all treated and control groups (22).

Measurement of lesion size and survival rate

After treatment the lesion size measured weekly for 22 wk. In this time the survival rate weekly recorded too.

Evaluating the expression of TLRs and cytokines of macrophages

For in vitro assay the J774A.1 macrophages in treated and control groups and for in vivo assay the peritoneal mice of all groups of mice collected and after the extract the RNA, the cDNA was made. The expression of TLRs (TLR1, 2, 3, 4, 7, 9), cytokines (IL-1, IL-6, IL-10, TNFα, IL-12 p35 and IL-12 p40) was carried out by using Real time PCR. The primers are showed in Table 1. The test was per-

formed as following conditions for 40 cycles: 95 °C for 15 min (95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec). The genes expression was normalized by β_2 microglobulin and GAPDH genes, also measured using fold changes with $2^{-\Delta\Delta Ct}$ method (22).

Table 1: The primers sequences of the TLRs, cytokines and housekeeping genes (GAPDH and β_2 microglubolin)

TLRs	**TLR1**	**F, 5'-CAATGTGGAAACAACGTGGA-3' R, 5'-TGTAACTTTGGGGGAAGCTG-3'**
	TLR2	F, 5'-AAGAGGAAGCCCAAGAAAGC-3' R, 5'-CGATGGAATCGATGATGTTG-3'
	TLR3	F, 5'-TCCTTGCGTTGCGAAGTGAA-3' R, 5'-TTGGGCGTTGTTCAAGAGGA-3'
	TLR4	F, 5'-ACCTGGCTGGTTTACACGTC-3' R, 5'-CTGCCAGAGACATTGCAGAA-3'
	TLR7	F, 5'-TGCAACTGTGATGCTGTGTGGT-3' R, 5'-TTTGACCTTTGTGTGCTCCTGG-3'
	TLR9	F, 5'-ACTGAGCACCCCTGCTTCTA-3' R, 5'-AGATTAGTCAGCGGCAGGAA-3'
Cytokines	IL1	F, 5'-CAAGGGGACATTAGGCAGCA-3' R, 5'-TGAAAGACCTCAGTGCAGGC-3'
	IL6	F, 5'- AAAGAAATGATGGATGCTACCAAAC -3' R, 5'- CTTGTTATCTTTTAAGTTGTTCTTCATGTACTC -3'
	IL10	F, 5'- GGCGCTGTCATCGATTTCTC-3' R, 5'- GACACCTTGGTCTTGGAGCTTATTAA-3'
	TNFα	F, 5'- CATCTTCTCAAAATTCGAGTGACAA -3' R, 5'- TGGGAGTAGACAAGGTACAACCC -3'
	IL12 P35	F, 5'- TACTAGAGAGACTTCTTCCACAACAAGAG -3' R, 5'- GATTCTGAAGTGCTGCGTTGAT -3'
	IL12 P40	F, 5'- GGAAGCACGGCAGCAGAATA -3' R, 5'- AACTTGAGGGAGAAGTAGGAATGG -3'
Housekeeping genes	GAPDH	F, 5'-ATGGACTGTGGTCATGAGCC-3' R,5'-ATTGTCAGCAATGCATCCTG-3'
	β_2 microglubolin	F, 5'-TTCAGTCGCGGTCGCTTCAGTC-3' R , 5'-CAATGTGAGGCGGGTGGAACTG-3'

Statistical analysis

For statistical analysis we used SPSS software, Graph Pad Prism and online site RT^2-Profiler at (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Kolmogrov-Spirnov test, One-Way ANOVA and LSD and Student's t-test was performed to check the difference between means of the representative treatments. Differences were considered significant at *P*-value<0.05.

Results

Parasite burden and Lesion size

The results of parasite burden are shown in Table 2. The less parasite burden is related to group treated with imiquimod. The reduction of lesion sizes for treated groups are shown in Table 3. The reduction percent in lesion after 12th week relative to control was 70.83%.

Table 2: Cycle of Threshold (CT) and parasite load test copy/reaction according to Real Time PCR method for all treated and control groups

Sample Type	Cycle of Threshold for Test	Parasite load Test Copy/Reaction
Control	20.89	195177
Imiquimod	28.39	987
Glucantime	26.63	3575

Table 3: The results of reduction percent in lesion diameter at 7 and 12 wk after treatment, compared to the control group

Sample Type	Reduction percent in lesion after 7th week relative to control	Reduction percent in lesion after 12th week relative to control
Imiquimod	35%	70.83%
Glucantime	32.5%	62.5%

Cytokine Assay

The amount of IFNγ and IL4 and also the ratio of IFNγ/IL4 are coming in Table 4. The highest ratio was seen in the imiquimod group.

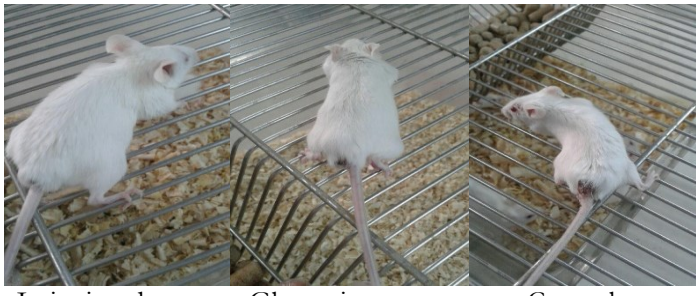
Table 4: Mean and standard deviation of interleukin 4 (IL-4) and interferon gamma (IFNγ) concentrations (pg/ml) and IFNγ/IL4 ratio in treated and control groups

Group Name	IL-4 (pg/ml)	IFNγ (pg/ml)	IFNγ/IL4 Ratio
Control	168±8.5	526±43.2	3.14
Imiquimod	173.25±9.45	645.75±16.4	3.87
Glucantime	238±14.15	620±13	2.60
Healthy group	20.46±1.3	52.4±2.7	2.62

*Statistical differences with control group ($P \leq 0.05$)

Lesion size and survival rate

The results of reduction percent in lesion diameter at 7 and 12 wk after treatment, compared to the control group (Table 3). The comparison between lesions in treated mice with imiquimod, glucantime and control is shown in Fig. 2.



Imiquimod Glucantime Control

Fig. 2: The figures of lesions in treated mice with imiquimod, glucantime and control

The percentage of survival rate of mice ten weeks after the treatment for mice treated with imiquimod, glucantime and control groups was 80%, 60% and 20% respectively.

Expression of TLRs in uninfected J774 A.1 macrophages and J774 A.1 macrophages infected with L. major

In uninfected macrophages that treated with imiquimod in comparison to untreated macrophages (control group), the expression of all TLRs was significantly decreased (Fig. 3).

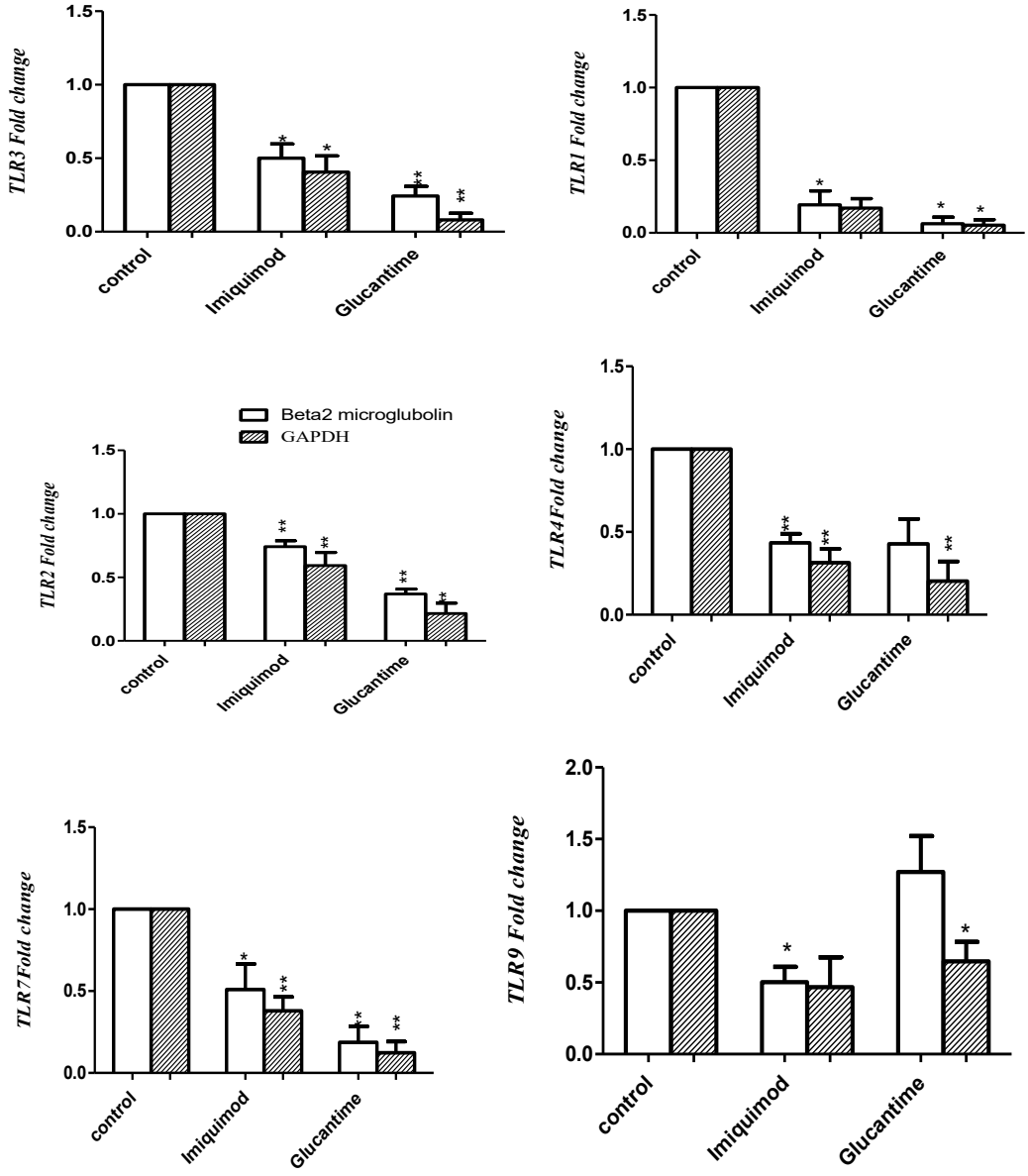


Fig. 3: Mean & SD of expression of TLRs in uninfected J774 A.1 macrophages normalized with β2 microglubolin, GAPDH (I 0.01µg/ml, G 50µg/ml) *(P<0.05), **(P<0.01)

In group of infected macrophages with *L. major* treated with imiquimod, TLR1 and TLR9 expression was significantly increased and TLR4 and TLR7 genes expression was decreased significantly (Fig. 4).

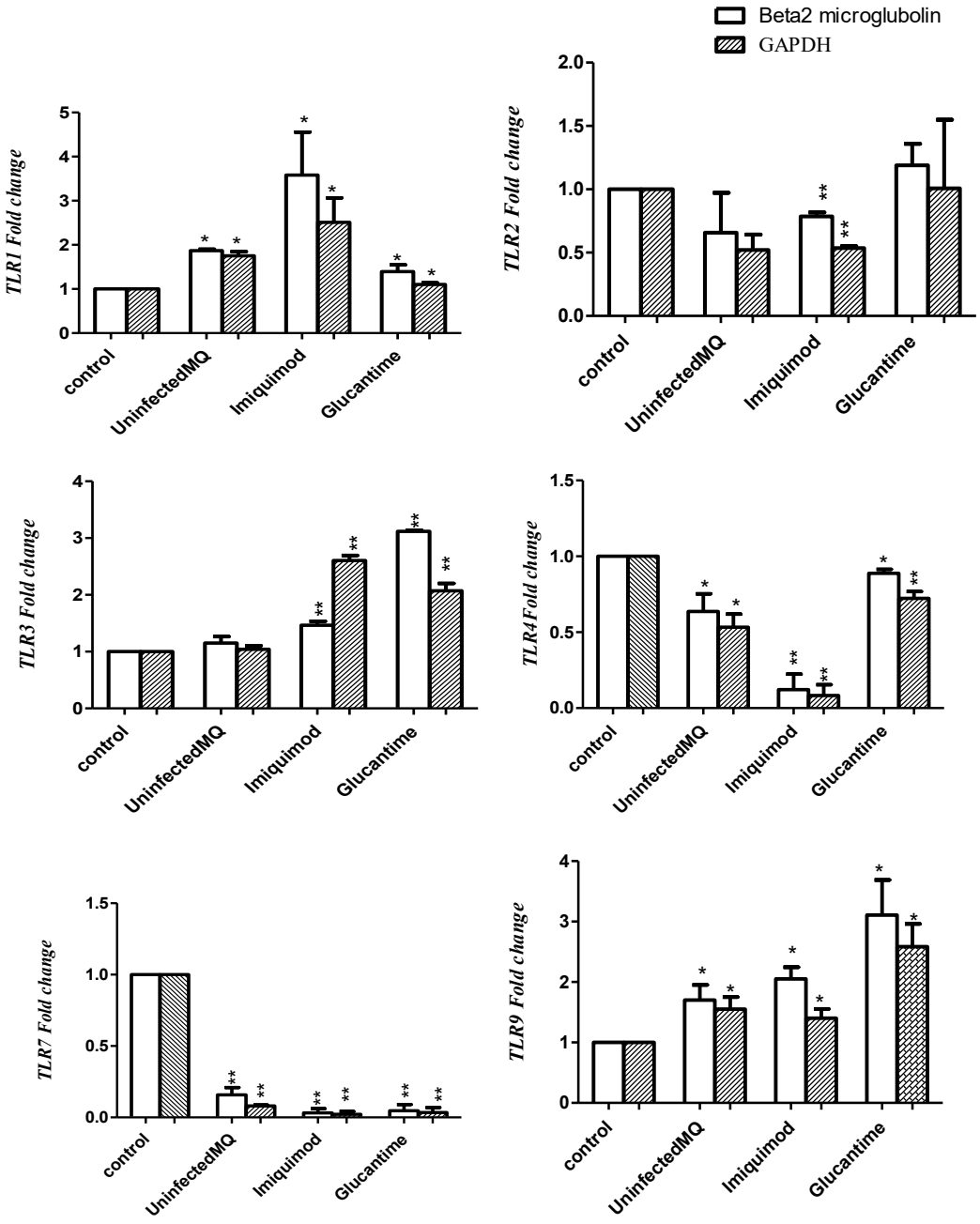


Fig. 4: Mean & SD of expression of TLRs in infected J774 A.1 macrophages normalized with β2 microglubolin, GAPDH. (I 0.01µg/ml, G 50µg /ml), **(*P*<0.01),*(*P*<0.05)

Expression of TLRs in mouse macrophages

In mouse macrophages, an increased expression of TLR1 was observed in both the imiquimod and glucantime-treated groups. All treated groups of mouse macrophages showed an increase in TLR2, TLR3, TLR4, TLR7, and TLR9 expression. However, in healthy mice, the expression of TLR4 and TLR9 was decreased, as illustrated in Fig. 5. These findings suggest that imiquimod and glucantime have distinct effects on TLR

expression in healthy versus infected macrophages, potentially contributing to their therapeutic effects against Leishmania major.

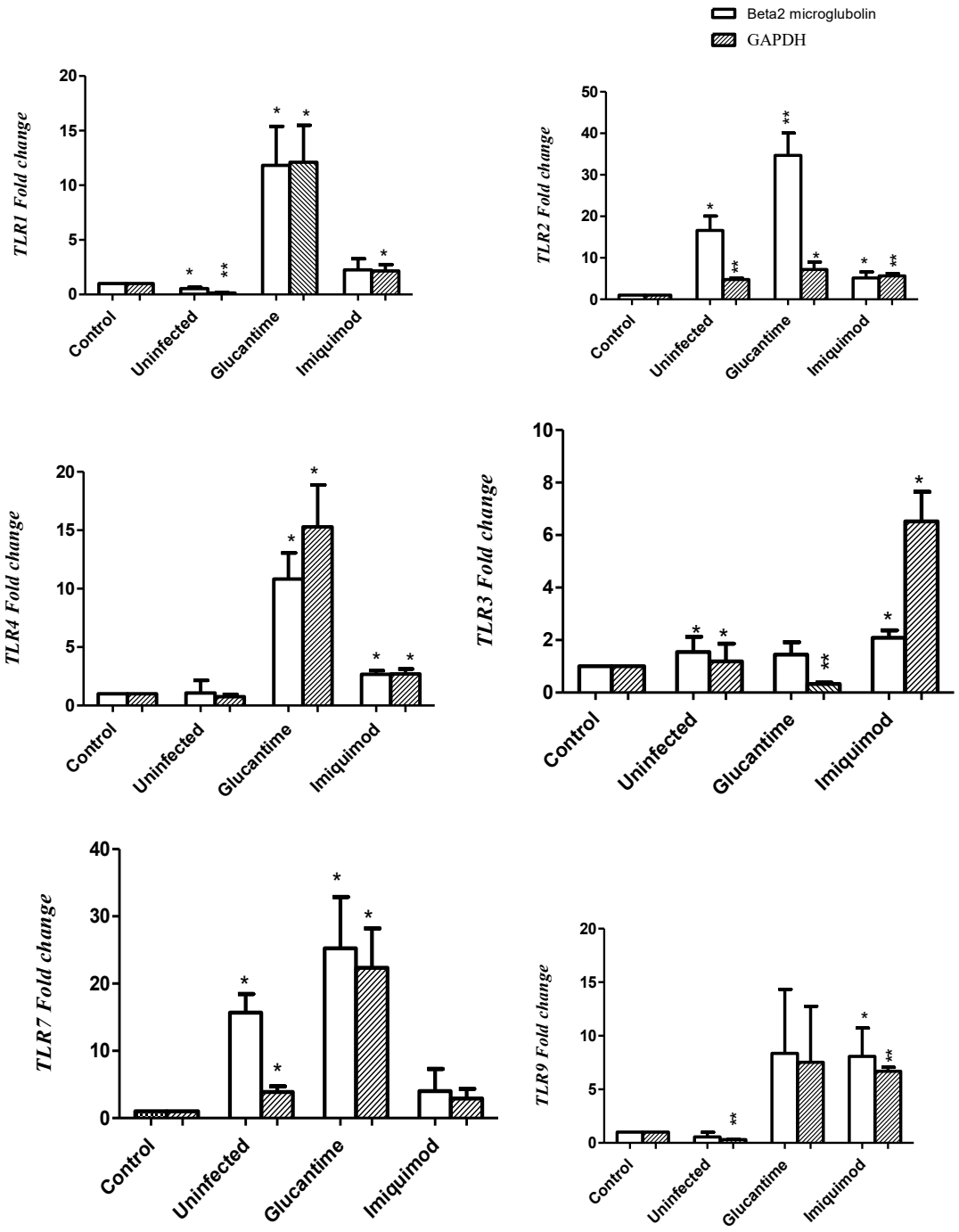


Fig. 5: Mean & SD of expression of TLRs in infected mice macrophages normalized with β2 microglubolin, GAPDH Control (infected mice), Uninfected (healthy mice), G (20mg/kg Glucantime), I(cream 5%imiquimod)

Expression of inflammatory cytokines in J774 and mouse macrophages

The results for expression of inflammatory cytokines in uninfected J774 (Fig.6) and infected J774 (Fig.7) and mouse macrophages (Fig. 8) showed in Figures 6-8. The most expression cytokine in mice macrophages treated with imiquimod was for IL12p35.

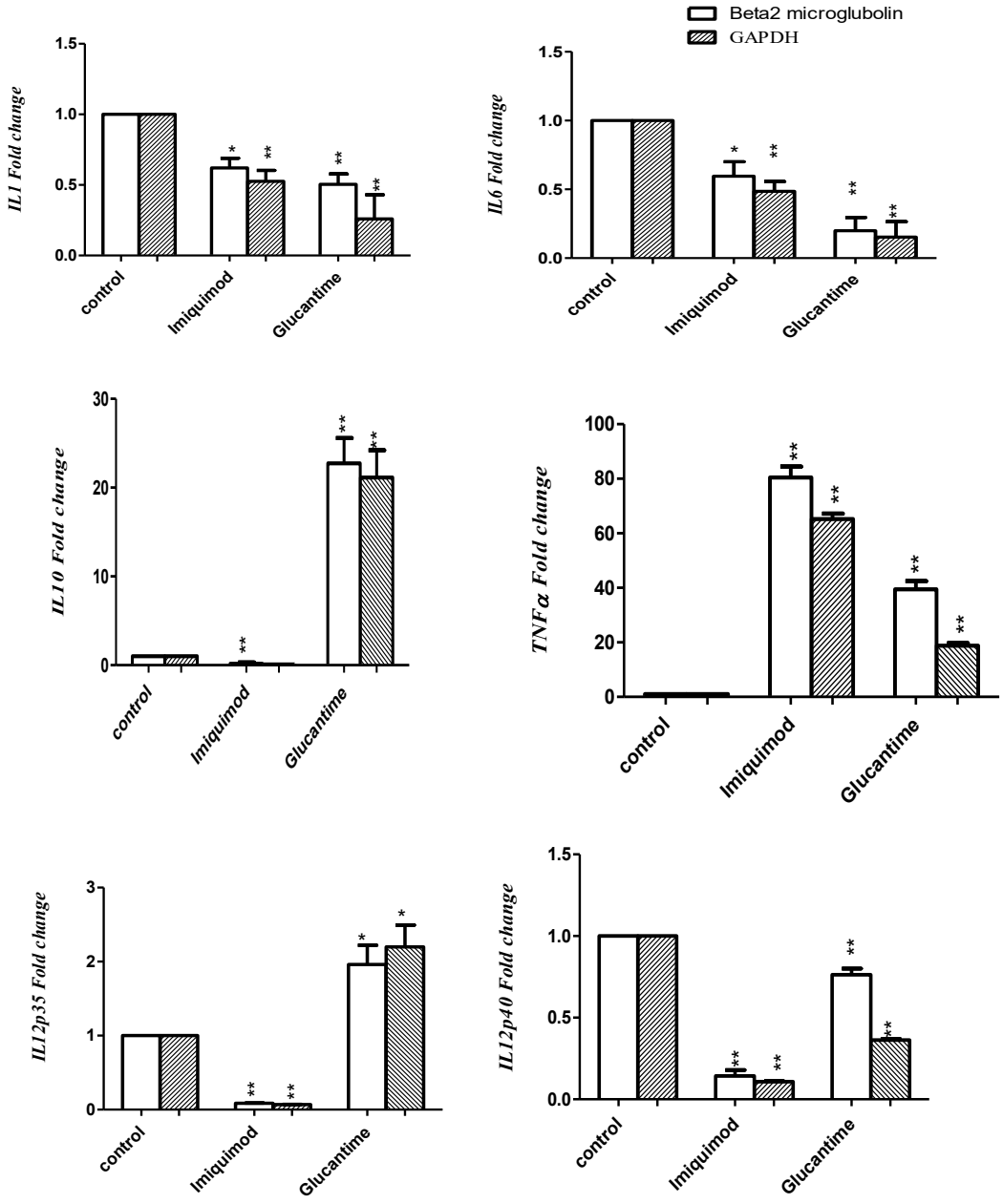


Fig. 6: Mean & SD of expression of cytokines as a relative fold change by real time PCR in uninfected J774 macrophages compared to untreated control by using ΔΔCt method normalized with β2 microglubolin, GAPDH .Imiquimod (0.01 µg/ml), glucantime (50 µg/ml) were applied. Control group (uninfected J774 macrophages). *(P<0.05),**(P<0.01)

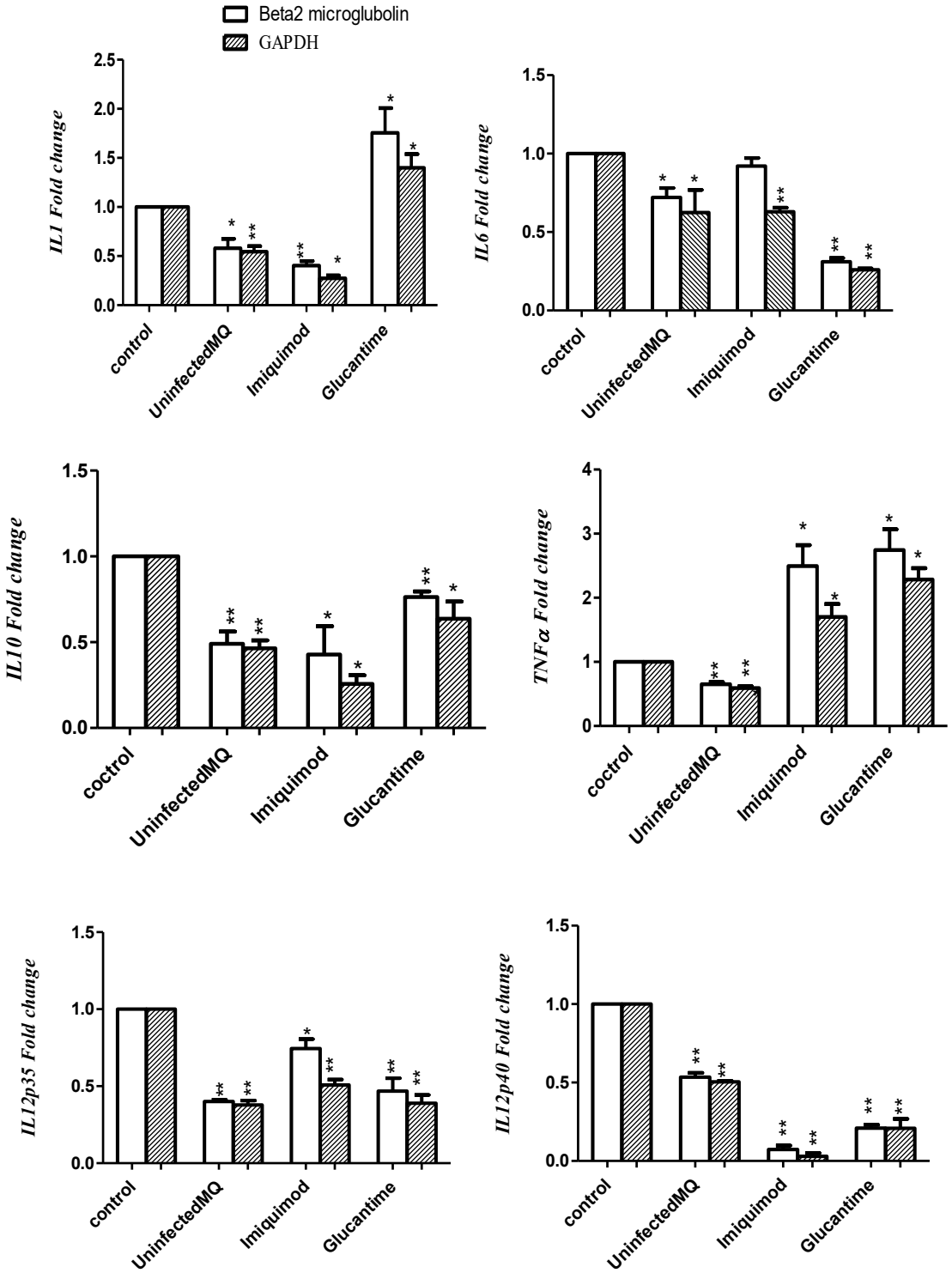


Fig. 7: Mean & SD of expression of cytokines as a relative fold change by real time PCR in infected J774 macrophages with amastigotes of *Leishmania major* compared to untreated control by using ΔΔCt method normalized with β2 microglubolin, GAPDH. Imiquimod (0.01 µg/ml) ,glucantime (50 µg/ml) were applied. Control group (J774 macrophages infected by *Leishmania major*)).*(P<0.05),**(P<0.01)

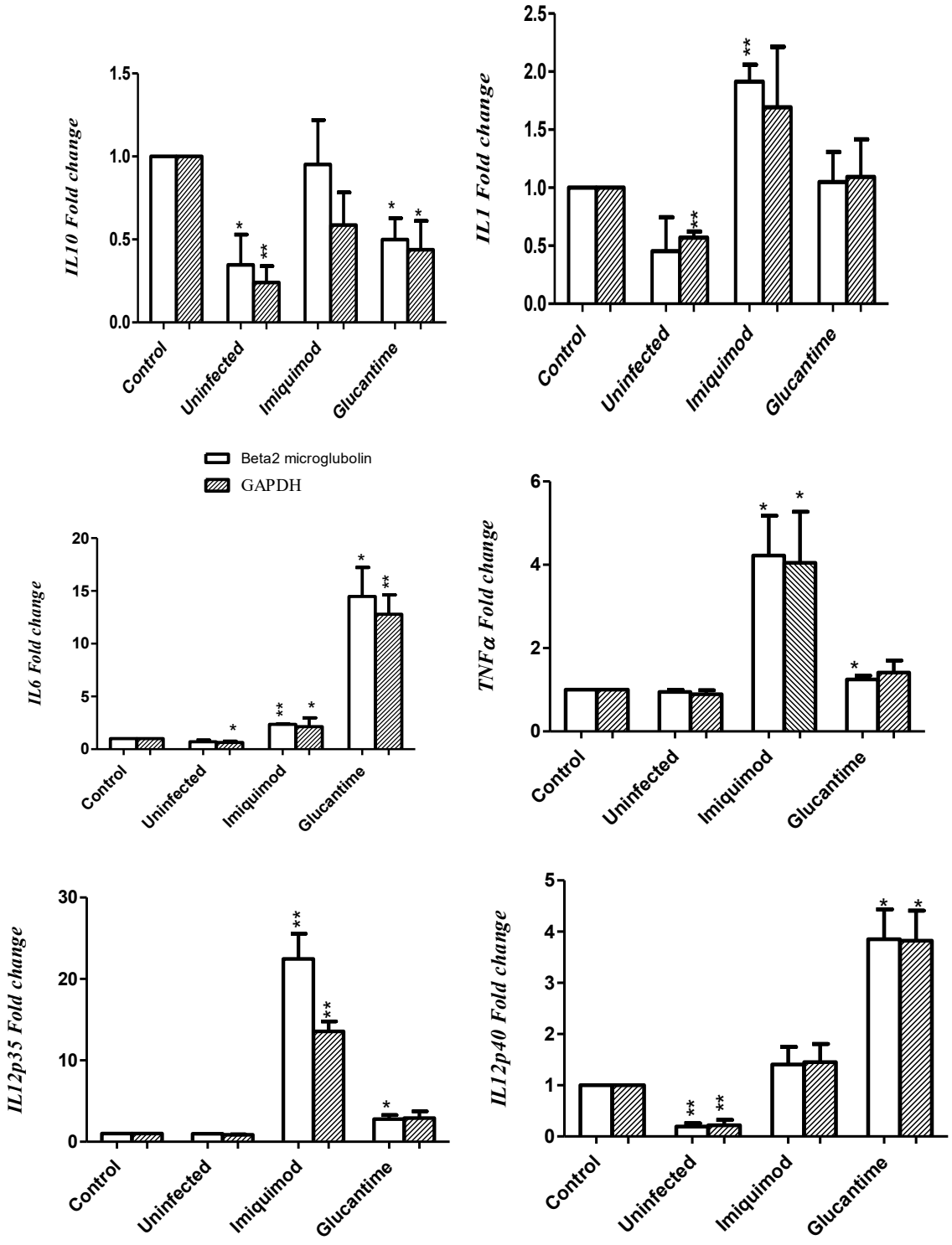


Fig. 8: Mean & SD of expression of cytokines as a relative fold change by real time PCR in infected mice macrophages normalized with β2 microglubolin ,GAPDH .Control(infected mice), Uninfected (healthy mice), Glucantime (20mg/kg), Imiquimod (cream 5%) *(P<0.05),**(P<0.01)

Discussion

As Leishmania is an obligate intracellular pathogen, macrophages have conditions that are necessary for the continued survival of the parasite (26, 27). Macrophages phagocytize both amastigotes and other amastigote-infected cells that have undergone apoptosis. (28).

Imiquimod is the most prominent, widely used and is very effective in the treatment of a lot of viral and cancer diseases (29). In addition, imiquimod has been effective in treating many tumors such as melanoma, lung sarcoma, breast cancer, and toxoplasma. The mechanism of action of imiquimod is still unknown (9, 30-33). In this study the effect of imiquimod on macrophage as the most suitable cellular host for Leishmania parasite was investigated and the effect of this drug on Toll-like receptors in macrophages and also the expression of inflammatory, proinflammatory and regulatory cytokines by macrophages were investigated.

TLR3 is rarely detectable in naive macrophages but is expressed in interferon-gamma-treated macrophages. In our study we found that imiquimod decreased the expression of TLR3 in intact macrophages but increase the expression in infected mice with amastigotes. TLR2 and TLR3 are involved in the secretion of NO and TNF-α by macrophages infected with *L. donovani* (34). In non-infected macrophages normalized by two housekeeping, in most groups affected by imiquimod TLR3 expression decreased, while in infected macrophages normalized with 2β microglobulin in all groups, TLR3 expression increased in macrophages extracted from infected mice. The two groups normalized with GAPDH and β2 microglobulin had the highest increase in TLR3 expression related to the glucantime group.

Research by Kropf et al. showed that TLR4 has an effective roll in control of *L. major* in mice (35). Mice lacking with TLR4 gene cannot effectively control the disease and skin wounds do not heal (36). The effect of TLR4 in control of *L. major* is likely through of nitric oxide synthesis induction in mice (35). Ribeiro et al. in two study showed *L. major* control via TLR4 (26, 37).

However, no direct reaction between *L. major* and TLR4-derived molecules has been reported. TLR4 has been shown to be essential role for the both innate and acquired immune response against *L. major* (38, 39). Macrophages infected with the parasite decreased expression and, in all cases, decreased expression was observed while in macrophages extracted from infected mice increased expression was observed.

Imiquimod has been shown to be an agonist for (TLR7) in macrophages and dendritic cells (40). TLR7 can develop Th1 type immune response. Such immune responses are essential for the treatment of leishmaniasis (41). The expression of TLR7 in healthy macrophages decreased in this study and in macrophages extracted from infected mice, the results obtained from the group treated with normalized imiquimod with both housekeeping decreased expression and their differences with the control group were significant. The highest increase in expression was observed in the glucantime group (25.23, P=0.0317)= Fold change) 2 β microglobulin and 25.36, P=0.0240)=Fold change).

Imiquimod has an effective and incremental role in TNFα, which increases TNFα increases Th1 responses, which has an effective and positive role in leishmaniasis. Imiquimod activates macrophages and produces cytokines such as TNFα and IL12, thereby enhancing the immune response. In mouse macrophages, the highest expression of TNFα is related to imiquimod, which confirms the role of imiquimod in enhancing TNFα expression. In this study, we report that IL12p35 expression significantly increased in the mice group treated with imiquimod in comparison to control group. Potentiating effect of imiquimod on

IL12 expression, which is the most important cytokine in the control of leishmaniasis is very important finding in this research. In the healthy macrophage group, the highest increase in IL12 is related to the groups treated with imiquimod, which indicates the adjuvant role of imiquimod.

In infected macrophages of BALB/c mice, an increase in TNFα expression in the groups treated with imiquimod.

IL6 in vivo decreased expression in the imiquimod-treated group. In healthy macrophages, a decrease in IL6 expression was observed under the influence of imiquimod, so imiquimod can play a preventive and rejuvenating role.

IL10 regulates the innate immune system and suppresses macrophage activity and dendritic cell maturation. IL10 produced by Th1 cells inhibits the immune response against intracellular parasites such as *Leishmania major* and *Toxoplasma gondii*. This cytokine is associated with susceptibility to leishmaniasis and parasite resistance to infections (42,43).

Decreased IL10 expression and increased TNFα expression were observed in infected and healthy macrophages in BALB / c mice under the influence of imiquimod.

Conclusion

Imiquimod increases the expression of the IL12 and also inflammatory cytokines in mouse macrophages and decreases the expression of IL10. This suggests that imiquimod may improve the therapeutic effects against leishmaniasis in infected macrophages. Imiquimod causes decreasing the expression of TLR2 but increasing the expression of TLR7 and TLR9. CpG Oligodeoxynucleotides as TLR9 Agonist and imiquimod as TLR7 agonist, the both enhance the recovery of leishmaniasis.

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Conflict of interest

The authors declare that there is no conflict of interests.

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