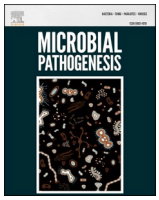
Microbial Pathogenesis 168 (2022) 105568

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath



Interferon inducible guanylate binding protein 1 restricts the growth of *Leishmania donovani* by modulating the level of cytokines/chemokines and MAP kinases

Ravindra Kumar, Pramod Kumar Kushawaha *

Department of Microbiology, School of Basic Sciences, Central University of Punjab, VPO Ghudda, Bathinda, Punjab, 151401, India

ARTICLE INFO

Keywords:
Leishmania donovani
Guanylate binding protein (GBP1)
Mitogen-activated protein kinase (MAPK)
Cytokines
and chemokines

ABSTRACT

Visceral Leishmaniasis (VL) is a zoonotic chronic endemic infectious disease caused by *Leishmania donovani* infection and a well-studied model for intracellular parasitism. Guanylate binding proteins (GBPs) are induced by interferons (IFNs), and play a crucial role in cell autonomous immunity and the regulation of inflammation. Guanylate-binding protein 1 (GBP1) has been shown vital for the host immune response against various pathogens. However, the role of GBP1 during VL is undefined. In the present study, we have investigated the role of GBP1 in *Leishmania donovani* infection using *in vitro* model. For that, knock down of the Gbp1 gene was carried out in both PMA differentiated human monocyte cell line THP-1 and mouse macrophages RAW264.7 cell line using siRNA based RNA interference. Infection of these cell lines revealed a high parasite load in knock down cells at 24 and 48h post infection as compared to control cells. A significant increase was observed in the level of different cytokines (IL-4, IL-10, IL-12b, IFN-γ, TNF-α) and chemokines (CXCL9, CXCL 10, and CXCL 11) in GBP1 knock down cell lines after post-infection. In GBP1 knock down cells the expression level of IFN effector molecules (iNOS and PKR) was found to be elevated in THP1 cells and remained almost unchanged in RAW264.7 cells after *Leishmania donovani* infection as compared to the control cells. Moreover, interestingly, the level of MAPK activated ERK1/2, and p38 MAPK were considerably induced by the parasite in knock down cells as compared to control after 24 h post-infection. This study, first time reported the involvement of GBP1 in *Leishmania donovani* infection by modulating the level of important cytokines, chemokines, IFN effector molecules, and MAP kinases.

1. Introduction

Leishmania is a well-studied model of protozoan parasitic infection because of its global incidence, affecting roughly 12 million people and over 90% of new cases were reported in 2020 in 10 countries from all five continents [1]. *Leishmania* spp. are transmitted by sand fly vectors in the promastigote form and in vertebrate hosts, mainly found inside macrophages, assume the round, oval and non-flagellated amastigote form. *Leishmania* promastigote is phagocytized by dendritic cells (DCs) and macrophages and forms a parasitophorous vacuole for its survival and propagation within the cell [2–4]. Further, they produced interleukin −12 (IL-12), which stimulates NK cells to produce interferon Interferon- γ (IFN-γ) which is responsible for skewing Th1 response [2,5] and stimulate the macrophage to produce reactive oxygen species (ROS) and nitric oxide (NO) for the oxidative killing of intracellular amastigotes thereby protects the host [6–9]. While the host's innate immune response against leishmaniasis is important, it is now clear that the T-cell mediated immunity and the cytokines produced from various immune cells play a crucial role in determining the disease outcome [10,11]. *Leishmania* parasites have evolved sophisticated mechanisms to subvert macrophage immune responses by altering the host cell signal transduction machinery, including inhibition of Janus kinase - signal transducer and activator of transcription (JAK-STAT) signaling and other transcription factors such as Activator protein 1 (AP-1), cAMP-response element-binding protein (CREB), nuclear factor-kappa B (NF-kB), and stimulation of mitogen-activated protein kinase (MAPK) activated extracellular signal-regulated kinase (ERK), and p38 MAPK [12–16].

In Visceral Leishmaniasis (VL) the balance between the microbicidal and suppressor functions exhibited by macrophages in association with

* Corresponding author.
E-mail address: kpramodk82@gmail.com (P.K. Kushawaha).

https://doi.org/10.1016/j.micpath.2022.105568
Received 21 March 2022; Received in revised form 1 May 2022; Accepted 4 May 2022
Available online 9 May 2022

parasitic adaptive strategies dictates disease progression. The production of NO by iNOS (or NOS2) is one of the main effector mechanisms for *Leishmania* elimination by macrophages. This enzyme is induced by IFN-γ and TNF-α, which are cytokines produced by $CD4^{+}$Th1 cells [17]. It has been also reported that the cross-talk between IFN- γ and CXCR3-cognate chemokines (C-X-C motif) ligand (CXCL): CXCL9/Mig (also known as monokine induced by interferon-gamma), CXCL10/IP-10 (also known as interferon-inducible protein-10), and CXCL11/I-TAC (also known as interferon-inducible T cell alpha-chemoattractant) play an important role against *Leishmania* infection [18]. However, the cytokines associated with Th2 responses, such as IL-4, IL-10, and TGF-β, not only inhibit the IFN-γ mediated killing of parasites but also directly favor the growth of *Leishmania* inside macrophages through the production of polyamines [19,20]. IFN-γ is an important T helper 1 (Th1) cell cytokine that strongly suppresses the growth and survival of intracellular pathogens and plays essential roles in the induction and regulation of innate and adaptive immune responses [21]. Robust gene expression of several effector molecules results after stimulation of innate immune cells such as macrophages and dendritic cells by IFN-γ. Immunity-related GTPases such as the Myxovirus resistance proteins (Mx), the small GTPases or immunity-related p47 GTPases (IRGs), and large GTPases or p65 guanylate-binding proteins (GBPs) [22,23] are well known among them. Furthermore, GBPs have recently been shown to induce antibacterial responses involving phagocytic oxidases, autophagic effectors, and inflammasome [24]. Thus, IFN-γ-inducible IRGs and GBPs play pivotal roles in antiviral and antibacterial immune systems. In humans, thirteen GBPs (hGBP1 to hGBP13), and in mice, eleven GBPs (mGBP1 to mGBP11) have been identified that have a high degree of sequence homology [25,26]. GBP1 is an interferon-inducible protein of the GTPase family [25,27] involved in the host immune response against microbial infections such as viral [28], bacteria [29,30], and protozoa [31,32]. However, the distinct role of GBP1 in *Leishmania* parasite infection is not clearly defined. Thus, we studied the role of GBP1 during *Leishmania donovani* infection in different *in vitro* models of intracellular parasitism.

2. Materials and methods

2.1. Parasite

Promastigotes of *L. donovani* strain Dd8 (MHOM/IN/80/Dd8) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma, USA), 100 U/ml penicillin (Sigma, USA), and 100 mg/ml streptomycin (Sigma, USA) at 26 °C [33]. The strain has also been maintained in hamsters through the serial passage, i.e. from amastigote to amastigote [34].

2.2. Cell lines, infection with the parasite, and stimulation with IFN-γ

The human monocyte THP-1 cell line was maintained in RPMI 1640 (Sigma, USA) and the RAW 264.7 murine macrophage cell line was cultured in DMEM (Sigma, USA). Both the cell lines were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin (Sigma, USA), and 100 mg/ml streptomycin (Sigma, USA) in a culture flask and placed at 37 °C with 5% CO_2. THP-1 monocyte cell line was further differentiated into macrophages using phorbol-12-myristate- 13-acetate (PMA) at 10 ng/ml [35]. For *in vitro* infection, both cells were infected with promastigote at a parasite: cell ratio of 20:1. These cell lines (1.5×10^6) were stimulated with 10 ng/ml of recombinant IFN-γ protein (PeproTech, USA).

2.3. Knockdown of GBP1 in cell lines and quantification of parasite load

Human/mice GBP1 siRNA (small interfering RNA) were purchased from Sigma-Aldrich, USA. For evaluation of gene silencing of GBP1, RAW 264.7 and PMA induced THP-1 cells (1×10^6 cells) were seeded in a 35 mm cell culture dish (Sigma, USA). The 75 mM of siRNA for THP-1 cells and 100 mM of siRNA for RAW 264.7 cells were used to prepare the transfection mixture in Opti-MEM™ I Reduced Serum Medium (Gibco, USA) containing 7.5 μl of X-tremeGENE™ 360 Transfection Reagent (Sigma, USA), followed as per instruction manual. Scrambled siRNA served as control. Both the cells were infected with promastigote at a parasite: cell ratio of 20:1. After 24 and 48h of incubation at 37 °C in a 5% CO_2 atmosphere, the cells were washed with phosphate-buffered saline (PBS) of pH 7.2, dried, and fixed with methanol. The cells were then stained with Giemsa stain. Parasite load was determined by counting the number of amastigotes in 100 cells under the light microscope. Cells after 24h post-infection were used for the estimation of cytokines, chemokines, and MAPK induced ERK1/2 and p38.

2.4. Quantitative estimation of cytokine and chemokines

Cells were harvested using a cell scraper (SPL, Germany) in Dulbecco's phosphate-buffered saline (DPBS Gibbco, USA) and washed twice in DPBS. Total RNA was extracted from cells using an RNase kit (Qiagen, Germany) following the manufacturer's instructions. RNA concentration was determined photometrically using Nanodrop, (Thermo-Scientific US). Complementary DNA (cDNA) was synthesized in a total reaction volume of 20 μl using 1 μg RNA with an iScript synthesis kit (Biorad US). Quantitative PCR was conducted in a total reaction volume of 25 μl with 1 μl of cDNA using 12.5 μl Maxima SYBR Green qPCR Master with Mix (2X) (Fermentas, US) using Step-One Real-Time PCR system (Applied Biosystem Foster City, CA). PCR was conducted as initial denaturation at 95 °C for 10 min followed by 40 cycles, each consisting of denaturation at 95 °C for 15s, annealing at 60 °C for 30s, and extension at 72 °C for 1 min per cycle. $^{\Delta\Delta}$Ct method was used to calculate the results and the beta-actin (β-Actin) gene was used as the internal reference gene. The primer sequences for measuring the expression of selected genes are provided in Table 1 and 2.

2.5. Assessment of the level of MAPK phosphorylation or activation of ERK1/2 and p38 by western blotting

Cells (10^6) were washed twice with ice-cold PBS and lysed in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 10 mM EGTA; 50 mM NaF; 20 mM β-glycerophosphate; 250 mM NaCl and 0.1% Triton X-100) to which contains a 1:100 dilution of protease inhibitor cocktail (Sigma, USA). Proteins were quantified by the Bradford assay kit (Bio-Rad US). Proteins were subjected to electrophoresis in 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Subsequently, the membrane was blocked with 5% BSA in TBS (50 mM Tris, 150 mM NaCl) with 0.1% of Tween-20 (TBST) buffer. Further blots were incubated for 2h with diluted primary antibodies p38 (Phosphorylated p38 MAPK and p38 MAPK antibody, CST, USA) and ERK1/2 (phosphorylated ERK1/2 and ERK1/2 antibody, CST, USA) separately in a blocking solution (TBST+5% skimmed milk). Finally, the membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugate IgG coupled secondary antibody for 1h. The membranes were then washed three times with TBST buffer, and proteins were detected by the ECL chemiluminescent detection system (BioRad).

2.6. Densitometric Analysis

Densitometric analyses for all experiments were carried out using ImageLab (Bio-Rad US). Band intensities were quantitated densitometrically, and the values were normalized to endogenous control and expressed in arbitrary units. The ratios of optical density of particular bands/endogenous control are indicated as bar graphs adjacent to figures.

Table 1
List of primers of *Mus musculus* gene.

Gene Description	Forward Prime 5′-3′	Reverse Primer 5′-3′
GBP1	ACAACTCAGCTAACTTTGTGGG	TGATACACAGGCGAGGCATATTA
GBP2	CTGCACTATGTGACGGAGCTA	GAGTCCACACAAAGGTTGGAAA
GBP3	CTGACAGTAAATCTGGAAGCCAT	CCGTCCTGCAAGACGATTCA
GBP4	GGAGAAGCTAACGAAGGAACAA	TTCCACAAGGGAATCACCATTTT
GBP5	CAGACCTATTTGAACGCCAAAGA	TGCCTTGATTCTATCAGCCTCT
GBP6	GTTCCAGGAAGTAACAAAGGCT	ATCCCTAGTCTATTCCCAGTGAC
GBP7	TCCTGTGTGCCTAGTGGAAAA	CAAGCGGTTCATCAAGTAGGAT
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IFN-γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
CxCl-9	GGAGTTCGAGGAACCCTAGTG	GGGATTTGTAGTGGATCGTGC
CxCl-10	CCAAGTGCTGCCGTCATTTTC	TCCCTATGGCCCTCATTCTCA
CxCl-11	TGTAATTTACCCGAGTAACGGC	CACCTTTGTCGTTTATGAGCCTT
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-4	ATCATCGGCATTTTGAACGAGG	TGCAGCTCCATGAGAACACTA
IL-12b	TGGTTTGCCATCGTTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
H2-Aa	TGGGAGTCTTGACTAAGAGGTC	CTGACTTGCTATTTCTGAGCCAT
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
PKR	CCAAAGAGAAAGGCAGGCTCC	TTCCTCCCTCCCTCCTCCATTC
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC

Table 2
List of primers for *Homo sapiens* gene.

Gene Description	Forward Prime 5′-3′	Reverse Primer 5′-3′
GBP1	AGGAGTTCCTTCAAAGATGTGGA	GCAACTGGACCCTGTCGTT
GBP2	CATCCGAAAGTTCTTCCCCAA	CTCTAGGTGAGCAAGGTACTTCT
GBP3	ATTCCCTGAAGCTAACGCAAG	GGGCAGATCGAAGACAAAACATT
GBP4	AGGCTGCTAAAACACAAGCTG	CCCCAGGTAGAGTGACAATCAT
GBP5	TGCTATCGACCTACTGCACAA	GCAGGATCTTCAACCCTGTCA
GBP6	GTGGAGCGACTCCTTGTCTG	GTGGGGAATCTCACTTGCTGG
GBP7	AACCATCTGGCAGGACAGAAT	TCACCCTTTTCCACATCGCC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IFN-γ	TCGGTAACTGACTTGAATGTCCA	TCGCTTCCCTGTTTTAGCTGC
CxCl-9	CCAGTAGTGAGAAAGGGTCGC	AGGGCTTGGGGCAAATTGTT
CxCl-10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
CxCl-11	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
IL-4	CCAACTGCTTCCCCCTCTG	TCTGTTACGGTCAACTCGGTG
IL-12b	ACCCTGACCATCCAAGTCAAA	TTGGCCTCGCATCTTAGAAAG
H2-Aa	GAGCAGGTTAAACATGAGTGTCA	CTCTCCACAACCCCGTAGT
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
PKR	TGGAAAGCGAACAAGGAGTAAG	CCAAAGCGTAGAGGTCCACTT
iNOS	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC

2.7. Statistical analysis

Data processing and analysis were performed using GraphPad Prism 5.2. An unpaired Student's t-test was used for statistical analysis between the two groups. One-way analysis of variance was performed for multiple comparisons between different groups. In all situations, a P-value of, 0.05 was considered statistically significant.

3. Results

3.1. Gbp1to 7 induced upon L. donovani infection and IFN-γ treatment

Mouse macrophage cell line RAW264.7 and PMA differentiated human monocyte THP1 cell line can be readily infected by *L. donovani* and used as an *in vitro* model for the study of the host-parasite immune response. By using a quantitative RT-PCR (qRT-PCR), we measured the expression of GBP1to 7 in *L. donovani* infected and IFN-γ treated THP1 cells and RAW264.7 cells. Post-infection, time-dependent induction of GBP1 (up to 17 fold), GBP2 (up to 8 fold), GBP3 (up to 5 fold), GBP4 (up to 17 fold), GBP5 (up to 6 fold), GBP6 (up to 9 fold), and GBP7 (up to 20 fold) were observed in THP1 cells. In RAW264.7 cells, induction of GBP1 (up to 15 fold), GBP2 (up to 22 fold), GBP3 (up to 2 fold), GBP4 (up to 2 fold), GBP5 (up to 0.8 fold), GBP6 (up to 1.5 fold), and GBP7 (up to 5 fold) were reported (Fig. 1). IFN-γ treated THP1 cells and RAW264.7 cells also showed time-dependent induction of GBPs. The expression of GBP1 (up to 25 fold), GBP2 (up to 6 fold), GBP3 (up to 3fold), GBP4 (up to 9 fold), GBP5 (up to 11 fold), GBP6 (up to 7 fold), and GBP7 (up to 15 fold) were observed in THP1 cells. In RAW264.7 cells, induction of GBP1 (up to 24 fold), GBP2 (up to 12 fold), GBP3 (up to 3 fold), GBP4 (up to 3 fold), GBP5 (up to 7 fold), GBP6 (up to 3 fold), and GBP7 (up to 5 fold) were reported. (Fig. 2).

3.2. Gbp1 has anti-parasitic activity

To establish the role of GBP1 during *L. donovani* infection, RNA interference-based study with GBP1 specific siRNA was performed in both mouse RAW264.7 cells and human THP1 cells. The level of GBP1 transcript and protein were determined by qRT-PCR and Western blot respectively. The β-actin was used as an internal standard for each experiment. The level of Gbp1 transcript and protein were significantly reduced in PMA derived knockdown THP1 cell line (>90% at mRNA level and >52% at protein level) and in mouse macrophage RAW264.7 cell line (>80% at mRNA level and up to 70% at protein level) respectively as compared to scrambled control (Fig. 3A and B). Both macrophage cells, PMA derived THP1 and RAW264.7 were infected *in vitro* with *L. donovani* promastigote at a parasite-to-host cell ratio of 20:1.

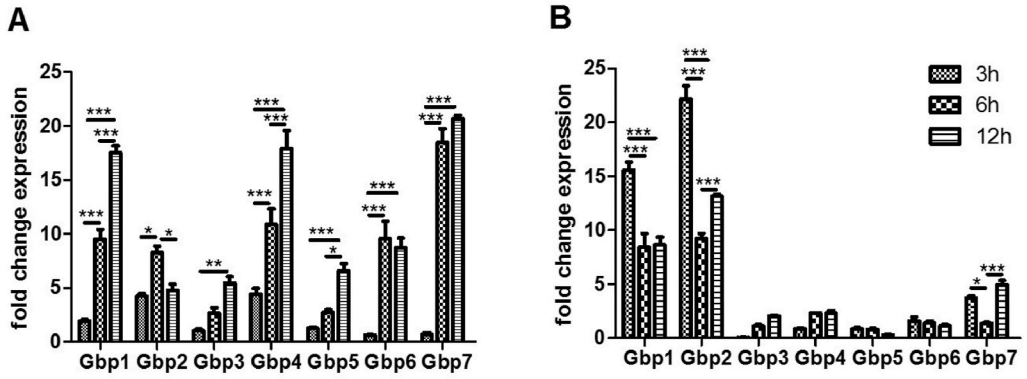


Fig. 1. Time-dependent quantification of GBP1to 7 expression in the PMA differentiated THP-1 and RAW 264.7 cells infected with *L. donovani*. A) Induction of GBP1to 7 in PMA differentiated THP1 cells quantified by q-RT-PCR. B) Induction of GBP1to 7 in PMA differentiated RAW 264.7 cells quantified by RT-PCR.

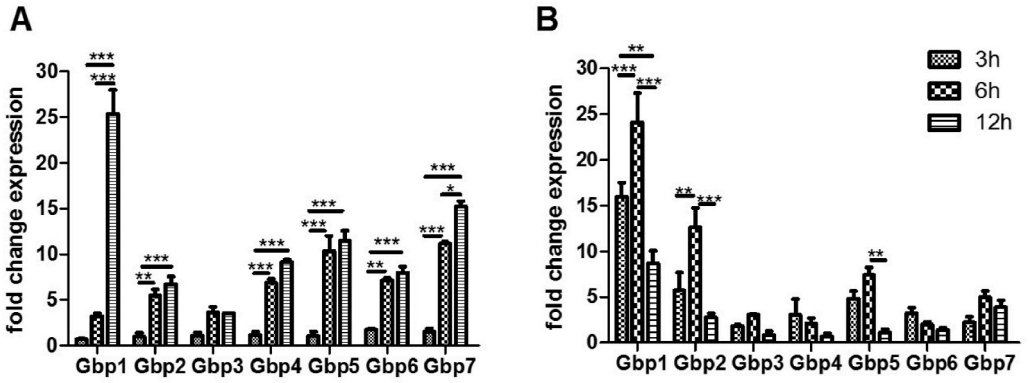


Fig. 2. Time-dependent quantification of GBP1to 7 expression in the PMA differentiated THP-1 and RAW 264.7 cells stimulated with IFN-γ (10 ng/ml). A) Induction of GBP1to 7 in PMA differentiated THP1 cells quantified by q-RT-PCR. B) Induction of GBP1to 7 in PMA differentiated RAW 264.7 cells quantified by RT-PCR.

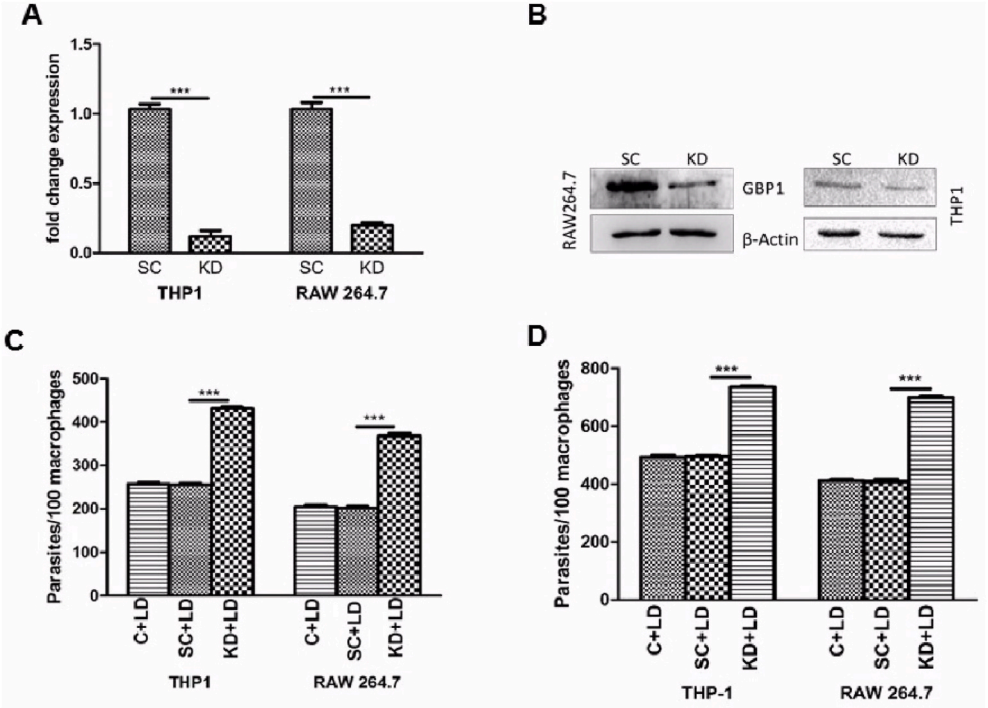


Fig. 3. Silencing/knock down of Gbp1 and parasite load in macrophages. (A & B) RNAi efficiency for Gbp1 was shown by RT-PCR and Western blot respectively in both the cell lines. (C&D) The PMA differentiated THP-1 and RAW 264.7 cells were transfected with scrambled control or GBP1 siRNA and infected with the parasite for 24 and 48h. Parasite load was determined by Giemsa staining. SC = scrambled control, KD = siRNA treated cells/knockdown cells, C = control, LD = *Leishmania donovani*.

After 24hr of post-infection the parasite load was reported to be increased ~2 fold in Gbp1 silenced cells compared with control cells in both the cell lines. Up to a two-fold increase in parasite load in knockdown THP1 cells (mean parasite load of 250 in control and 552 in knockdown) and RAW 264.7 cells (mean parasite load of 200 in control ad 415 knockdown cell line) were observed after 24h post-infection as compared to control. The parasite load was further increased after 48h post-infection in knock down cells of both the cell line as compared to control cells. (Fig. 3C and D).

3.3. Expression levels of selective cytokines and chemokines are altered in Gbp1 silenced cells upon L. donovani infection

Macrophages are proposed primary host cells for *Leishmania* but the role of these cells has been well characterized neither in disease prevention nor in progression independent of T cell. It has been reported that cytokines and chemokines play a vital role in the activation of macrophages and overall pathogenesis and immunity against intracellular pathogens including *L. donovani* [36].To investigate whether the silencing/knockdown of Gbp1 would affect the expression of some crucial cytokines (IFN-γ, TNF-α, IL-4, IL-10, IL-12b) and chemokines (CXCL9, CXCL10, CXCL11) which play important role in the final outcome of VL, the expressions of some typical cytokines and chemokines and IFN effector molecules (iNOS, Protein kinase R (PKR) along with histocompatibility 2, class II antigen A (H2-Aa) were measured in Gbp1 siRNA or scrambled siRNA treated cells after *L. donovani* infection. After infection, the knockdown RAW264.7 cells showed a significant upregulation in the mRNA expression level of H2-Aa (19.55fold), PKR (8.24fold), IL-10 (8.06fold), CXCL11 (7.37fold), CXCL10 (6.56fold), and CXCL9 (2.57fold) whereas the mRNA expression level of IFN-γ (0.92fold) and TNF-α (0.89fold) remained constant as compared to infected control cells. Moreover, in the knockdown THP-1 cells, all cytokines and chemokines were found to be significantly increased i.e., H2Aa (9.96fold), PKR (8.83fold), IL-4 (14.19fold), IL-10 (17.77fold), IL-12b (11.7fold), iNOS (13.97fold), IFN-γ (27.77fold), TNF-α (4.66fold), CXCL9 (11.04fold), CXCL10 (5.98fold), CXCL11 (7.5fold) as compared to the infected control cells (Figure. 4 A and B). These data suggested GBP1 can modulate some pro-inflammatory cytokines/chemokines and effector molecules during *L. donovani* infection.

3.4. Gbp1 gene silencing modulates the MAPK induced activity of ERK/12 and p38 MAPK

The expression levels of some cytokines/chemokines were increased and some of them almost remained unchanged in Gbp1 silenced cells after *Leishmania* infection. ERK1/2 and p38 MAPK have been reported to be activated upon a broad range of pathogen infections including *Leishmania donovani* and play a crucial role in its pathogenesis, [37]. Hence, we measured the effect of GBP1 knock down the activities of these transcription factors in both the THP-1 and RAW264.7 cell lines. Both cell lines were infected by *L. donovani*, and the transcriptional factor activity was measured using a Western blot assay. The relative activity of ERK1/2 and p38 were found to be increased in GBP1 knockdown infected cells as compared to infected control cells. After the densitometric analysis of the western blots, we found more than a 3-fold and a 2-fold increase in the level of phospho-ERK1/2 in THP-1 differentiated macrophage and RAW 264.7 cell line respectively. Moreover, the level of phospho-p38 MAPK was increased 1.6-fold and 1.8-fold in THP-1 differentiated macrophage and RAW 264.7 cell line respectively (Figure. 5 A and B). These data suggested that GBP1 may influence the activity of ERK1/2 and p38, thereby contributing to the production of pro-inflammatory cytokines/chemokines.

4. Discussion

Visceral leishmaniasis, caused by *L. donovani* and endemic in more than 60 countries of the world, is the most serious systemic disease with diverse clinical manifestations, ranging in severity from self-healing skin lesions to life-threatening visceral diseases, which may have fatal consequences if left untreated [1,38]. Macrophage and dendritic cells are the major cells that play important role in innate immunity against intracellular pathogens including *L. donovani.* Activated macrophages are a heterogeneous collection of cells with very diverse functions such as secretion of pro- or anti-inflammatory mediators, intracellular pathogen killing, induction of T-helper 1 or T-helper 2 cell responses, and others [36]. The effector functions of macrophages for *Leishmania* have always been described in a T-dependent manner. The fate of infected macrophages in the pre-T cell phase is not well known. It is possible that the parasite modulates its host in terms of signaling or antigen presentation for its benefit and induces factors that provide a disease progressive environment and prime T cells for Th2 differentiation. Moreover, parasites may also start modulating the macrophages at the time of entry, and later on modulated parasitized macrophages interact with T cells and may induce IL-4 and disease-inducing factors from T cells that help in disease progression and parasite survival in susceptible hosts [39]. IFN – inducible GBPs are reported to play important role in the containment of these pathogens. Therefore, this study was carried out to establish the role of GBPs in *L. donovani* pathogenesis. By using quantitative real-time PCR, we measured the expression level of GBPs (GBP1-7). The induction of GBPs (GBP1-7) was found to be increased significantly in a time-dependent manner in PMA differentiated THP1 macrophages whereas only GBP1, GBP2 and GBP7 were significantly expressed in RAW264.7 cells. Since GBPs are IFN inducible effector molecules, therefore we have also measured the induction of GBP1to7 in

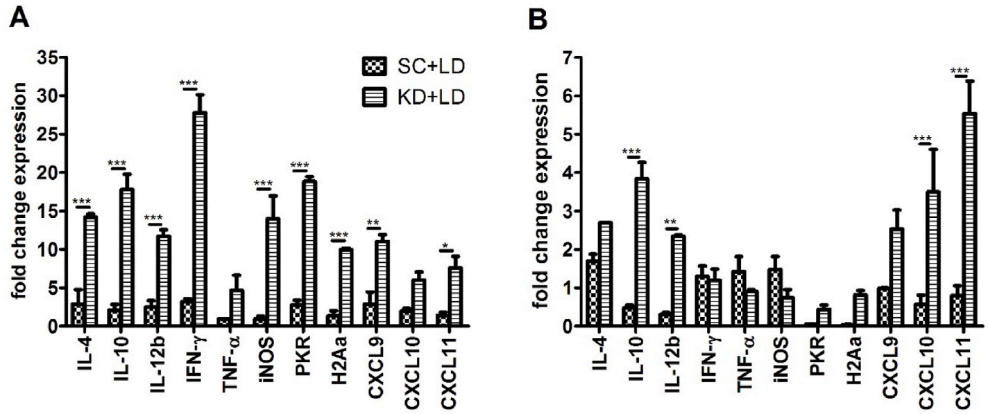


Fig. 4. Effect of Gbp1 RNAi silencing on parasite-induced cytokines, chemokines, and IFN inducible effector molecules iNOS and PKR in (A) PMA differentiated THP-1 and (B) RAW 264.7 macrophages.

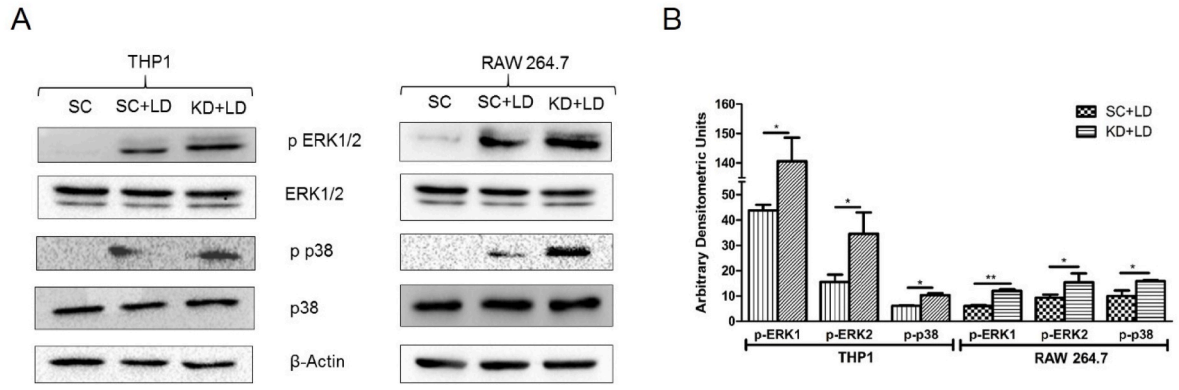


Fig. 5. Effect of Gbp1 RNAi silencing on ERK1/2 and p38 phosphorylation levels in PMA differentiated THP-1 and RAW 264.7 macrophages. (A) Both the cell lines (THP1 and RAW264.7) were infected with the parasite for 24h, whole-cell lysate was prepared and analyzed by western blotting. (B) Densitometric analysis of THP1 and RAW 264.7 Western blot showed the ratio of the intensity of protein expression to β-Actin expression per unit.

both macrophage cell lines. Here the time-dependent expression of GBP1to7 was reported in THP1 cells and GBP1, GBP2, GBP5, and GBP7 were significantly induced in RAW264.7 cells. Further, the induction GBP1 and GBP2 expression have been also reported in *L. donovani* strains AG83 and DD8 infection in human epithelial A549 cells [31], *Listeria monocytogenes,* and *Toxoplasma gondii* infection *in vivo* and *in vitro* [40], and dengue virus infection [41]. A siRNA-based RNA interference study was performed to study the specific role of GBP1 during *L donovanai* infection, in both mouse RAW264.7 cells and PMA differentiated human monocyte THP1 cells. The parasite load was found to be increased in Gbp1 silenced cells compared with control cells. These data suggested the anti-parasitic role of Gbp1 against *L. donovani* in both the macrophage cells. A balance between Th1 (IL-12b, IFN-γ, and, TNF-α) and Th2 (IL-4, and TGF beta) cytokines along with IL-10 determines the outcome of VL. Th1 cytokines IFN-γ, TNF-α, and IL-12 are involved in the protection or natural resistance against VL, wherein IFN-γ induces NO production and blocks IL-10 production by macrophages and monocytes [42]. NO has been reported to be anti-parasitic activity [42,43]. Similarly, TNF-α synergize with IFN-γ to kill the parasite, and IL-12 plays an essential role in the polarization of Th1-associated immunity as it induces IFN-γ production and allows differentiation and proliferation of naive T-cells into Th1 cells [36]. Conversely, Th2 cytokines, TGF-β, IL-4, and IL-10 play a crucial role in parasite survival, and disease progression, by inhibiting oxidative burst and IFN-γ production by macrophages [36]. These cytokines are also involved in the suppression of Th1-associated immune response in murine VL [44,45]. So the level of some of the representative cytokines was analyzed in Gbp1 silenced/-knockdown macrophage cells. A significant alteration in the level of these cytokines were observed in Gbp1 knock down cells as compared to control cells. It has been revealed that there is a cross-talk between IFN-γ and CXCR3-cognate chemokines and has beneficial role in *Leishmania* infection. In *Leishmania* infection these chemokines were found to be involve in protection and disease pathogenesis [18]. Therefore we also tried to investigate the correlation between GBP1 and CXCR3 chemokines if any. We found that the level of these chemokines are also significantly altered in Gbp1 knock down macrophage cells as compared to control cells. Further this has been shown that some intracellular pathogens inhibit the level of MHC class II molecules for their prolonged survival inside macrophages [36]. Alteration in the level of histocompatibility 2, class II antigen A was evident in both the knock down macrophage cells as compared to control cells. Double-stranded, RNA-activated protein kinase R (PKR) activation has been reported to have antiviral and antiproliferative effects for many years (17). This has been shown to involve in both the survival and immunity against *Leishmania* depending on the species [46,47]. In our study the expression level of PKR was significantly changed in Gbp1 knock down macrophages as compared to control cells. Intracellular pathogens interfere with host cell signaling in order to prevent or modulate cellular activation in their favor. *Leishmania* interferes with host protein kinase and phosphatase activities, thereby preventing macrophage activation. It was shown that *Leishmania* parasite modulates the level of JAK-STAT and MAP kinase signaling for its survival inside macrophages [36]. In light of these facts we also measured the level of MAPK inducible ERK1/2 and p38 MAPK in knock down macrophage cells after infection with *L. donovani.* Interestingly, the level of these MAP kinases were considerably increased in knock down macrophage cells as compared to control cells.

In a nutshell, this study confirmed that GBP1 limits the growth of *L. donovani*, influences the activity of ERK1/2 and p38 MAPK, and further modulates the induction of pro-inflammatory cytokine/chemokines along with IFN inducible effector molecules iNOS and PKR. This makes it an interesting molecule that needs further study to explore its role in *L. donovani* infection and immunity.

Conflict of intereset

Authors declare no competing interests.

CRediT authorship contribution statement

Ravindra Kumar: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Pramod Kumar Kushawaha:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability

The data that has been used is confidential.

Acknowledgments

This work was supported by a grant under the DST-INSPIRE Faculty award to PKK (IFA-13-LSBM-80) and the Central University of Punjab RSM Grant (CUPB/CC/16/cc/13). The authors are thankful to Dr. Amogh A. Sahasrabuddhe, CSIR-CDRI, Lucknow, for sharing the *Leishmania donovani* parasite. We would also like to thank the DST-FIST grant sanctioned to the Department of Biochemistry and Microbial Sciences and the Central University Punjab, Bhatinda (CUPB), India for providing the necessary research facilities. RK acknowledges the fellowship from CUPB and DST-INSPIRE grant.

References

[1] WHO. Leishmaniasis. http://wwwwhoint/mediacentre/factsheets/fs375/en/2022.

[2] T. Scharton-Kersten, L. Afonso, M. Wysocka, G. Trinchieri, P. Scott, IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis, J. Immunol. 154 (1995) 5320–5330.
[3] U. Sharma, S. Singh, Immunobiology of Leishmaniasis, 2009.
[4] N.E. Reiner, Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection, Immunol. today 15 (1994) 374–381.
[5] J. Liese, U. Schleicher, C. Bogdan, The innate immune response against Leishmania parasites, Immunobiology 213 (2008) 377–387.
[6] H.W. Murray, C.F. Nathan, Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani, J. Exp. Med. 189 (1999) 741–746.
[7] L.E. Perez, B. Chandrasekar, O.A. Saldarriaga, W. Zhao, L.T. Arteaga, B.L. Travi, et al., Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen, J. Immunol. 176 (2006) 5519–5528.
[8] J.K. White, P. Mastroeni, J.F. Popoff, C.A. Evans, J.M. Blackwell, Slc11a1-mediated resistance to Salmonella enterica serovar Typhimurium and Leishmania donovani infections does not require functional inducible nitric oxide synthase or phagocyte oxidase activity, J. Leukoc. Biol. 77 (2005) 311–320.
[9] R. Birnbaum, N. Craft, Innate immunity and Leishmania vaccination strategies, Dermatol. Clin. 29 (2011) 89–102.
[10] O.P. Singh, S. Sundar, Immunotherapy and targeted therapies in treatment of visceral leishmaniasis: current status and future prospects, Front. Immunol. 5 (2014) 296.
[11] A. Dayakar, S. Chandrasekaran, S.V. Kuchipudi, S.K. Kalangi, Cytokines: key determinants of resistance or disease progression in visceral leishmaniasis: opportunities for novel diagnostics and immunotherapy, Front. Immunol. 10 (2019) 670.
[12] I. Contreras, M.A. Gómez, O. Nguyen, M.T. Shio, R.W. McMaster, M. Olivier, Leishmania-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63, PLoS Pathog. 6 (2010), e1001148.
[13] G. Pearson, F. Robinson, T. Beers Gibson, B-e Xu, M. Karandikar, K. Berman, et al., Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions, Endocr. Rev. 22 (2001) 153–183.
[14] A.B. Figueiredo, M.C. Souza-Testasicca, T.W.P. Mineo, L.C.C. Afonso, Leishmania amazonensis-induced cAMP triggered by adenosine A2B receptor is important to inhibit dendritic cell activation and evade immune response in infected mice, Front. Immunol. 8 (2017) 849.
[15] A. Ruhland, N. Leal, P.E. Kima, Leishmania promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis, Cell Microbiol. 9 (2007) 84–96.
[16] A. Rojas-Bernabe, O. Garcia-Hernandez, C. Maldonado-Bernal, J. Delegado-Dominguez, E. Ortega, L. Gutierrez-Kobeh, et al., Leishmania mexicana lipophosphoglycan activates ERK and p38 MAP kinase and induces production of proinflammatory cytokines in human macrophages through TLR2 and TLR4, Parasitology 141 (2014) 788–800.
[17] C. Bogdan, M. Röllinghoff, How do protozoan parasites survive inside macrophages? Parasitol. Today 15 (1999) 22–28.
[18] P.E. Kima, L. Soong, Interferon gamma in leishmaniasis, Front. Immunol. 4 (2013) 156.
[19] I. Vouldoukis, P.A. Bécherel, V. Riveros-Moreno, M. Arock, O. Da Silva, P. Debré, et al., Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation, Eur. J. Immunol. 27 (1997) 860–865.
[20] V. Iniesta, L. Carlos Gómez-Nieto, I. Molano, A. Mohedano, J. Carcelén, C. Mirón, et al., Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular Leishmania parasites, Parasite immun. 24 (2002) 113–118.
[21] U. Boehm, T. Klamp, M. Groot, J. Howard, Cellular responses to interferon-γ, Annu. Rev. Immunol. 15 (1997) 749–795.
[22] A.R. Shenoy, B.-H. Kim, H.-P. Choi, T. Matsuzawa, S. Tiwari, J.D. MacMicking, Emerging themes in IFN-γ-induced macrophage immunity by the p47 and p65 GTPase families, Immunobiology 212 (2008) 771–784.
[23] A.P. Taylor, H.W. Murray, Intracellular antimicrobial activity in the absence of interferon-γ: effect of interleukin-12 in experimental visceral leishmaniasis in interferon-γ gene-disrupted mice, J. Exp. Med. 185 (1997) 1231–1240.
[24] B.-H. Kim, A.R. Shenoy, P. Kumar, R. Das, S. Tiwari, J.D. MacMicking, A family of IFN-γ–inducible 65-kD GTPases protects against bacterial infection, Science 332 (2011) 717–721.
[25] P. Tripal, M. Bauer, E. Naschberger, T. Mörtinger, C. Hohenadl, E. Cornali, et al., Unique features of different members of the human guanylate-binding protein family, J. Interferon Cytokine Res. 27 (2007) 44–52.
[26] S.M. Man, D.E. Place, T. Kuriakose, T.-D. Kanneganti, Interferon-inducible guanylate-binding proteins at the interface of cell-autonomous immunity and inflammasome activation, J. Leukoc. Biol. 101 (2017) 143–150.
[27] Y. Cheng, M.F. Becker-Manley, T.P. Chow, D.C. Horan, Affinity purification of an interferon-induced human guanylate-binding protein and its characterization, J. Biol. Chem. 260 (1985) 15834–15839.
[28] T. Gu, D. Yu, L. Xu, Y.-L. Yao, Y.-G. Yao, Tupaia GBP1 interacts with STING to initiate autophagy and restrict herpes simplex virus type 1 infection, J. Immunol. 207 (2021) 2673–2680.
[29] M. Kutsch, L. Sistemich, C.F. Lesser, M.B. Goldberg, C. Herrmann, J. Coers, Direct binding of polymeric GBP1 to LPS disrupts bacterial cell envelope functions, EMBO J. 39 (2020), e104926.
[30] J.C. Santos, D. Boucher, L.K. Schneider, B. Demarco, M. Dilucca, K. Shkarina, et al., Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria, Nat. Commun. 11 (2020) 1–15.
[31] A.K. Haldar, U. Nigam, M. Yamamoto, J. Coers, N. Goyal, Guanylate binding proteins restrict Leishmania donovani growth in nonphagocytic cells independent of parasitophorous vacuolar targeting, mBio 11 (2020) e01464-20.
[32] A. Qin, D.-H. Lai, Q. Liu, W. Huang, Y.-P. Wu, X. Chen, et al., Guanylate-binding protein 1 (GBP1) contributes to the immunity of human mesenchymal stromal cells against Toxoplasma gondii, Proc. Natl. Acad. Sci. Unit. States Am. 114 (2017) 1365–1370.
[33] R. Garg, J.K. Srivastava, A. Pal, S. Naik, A. Dube, Isolation of integral membrane proteins of Leishmania promastigotes and evaluation of their prophylactic potential in hamsters against experimental visceral leishmaniasis, Vaccine 23 (2005) 1189–1196.
[34] A. Dube, N. Singh, S. Sundar, Refractoriness to the treatment of sodium stibogluconate in Indian kala-azar field isolates persist in in vitro and in vivo experimental models, Parasitol. Res. 96 (2005) 216–223.
[35] H. Schwende, E. Fitzke, P. Ambs, P. Dieter, Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1, 25-dihydroxyvitamin D3, J. Leukoc. Biol. 59 (1996) 555–561.
[36] E.P. Thi, U. Lambertz, N.E. Reiner, Sleeping with the enemy: how intracellular pathogens cope with a macrophage lifestyle, PLoS Pathog. 8 (2012), e1002551.
[37] M. Junghae, J.G. Raynes, Activation of p38 mitogen-activated protein kinase attenuates Leishmania donovani infection in macrophages, Infect. Immun. 70 (2002) 5026–5035.
[38] S. Burza, S.L. Croft, M. Boelaert, Leishmaniasis–authors' reply, Lancet 393 (2019) 872–873.
[39] A. Awasthi, R.K. Mathur, B. Saha, Immune response to Leishmania infection, Indian J. Med. Res. 119 (2004) 238–258.
[40] D. Degrandi, C. Konermann, C. Beuter-Gunia, A. Kresse, J. Würthner, S. Kurig, et al., Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense, J. Immunol. 179 (2007) 7729–7740.
[41] W. Pan, X. Zuo, T. Feng, X. Shi, J. Dai, Guanylate-binding protein 1 participates in cellular antiviral response to dengue virus, Virol. J. 9 (2012) 1–6.
[42] S. Ratnapriya, N.K. Yadav, A. Dube, A.A. Sahasrabuddhe, A chimera of Th1 stimulatory proteins of Leishmania donovani offers moderate immunotherapeutic efficacy with a Th1-inclined immune response against visceral leishmaniasis, BioMed Res. Int. (2021) 2021.
[43] C.F. Nathan, J.B. Hibbs Jr., Role of nitric oxide synthesis in macrophage antimicrobial activity, Curr. Opin. Immunol. 3 (1991) 65–70.
[44] J. Joshi, S. Kaur, To investigate the therapeutic potential of immunochemotherapy with cisplatin+ 78 k D a+ MPL-A against L eishmania donovani in BALB/c mice, Parasite Immunol. 36 (2014) 3–12.
[45] C.B. Palatnik-de-Sousa, Vaccines for canine leishmaniasis, Front. Immunol. 3 (2012) 69.
[46] M.S. Faria, T.C. Calegari-Silva, AdC. Vivarini, J.C. Mottram, U.G. Lopes, A.P. C. Lima, Role of protein kinase R in the killing of Leishmania major by macrophages in response to neutrophil elastase and TLR4 via TNFα and IFNβ, Faseb. J. 28 (2014) 3050–3063.
[47] R.M. Pereira, K.L. Dias Teixeira, V. Barreto-de-Souza, T.C. Calegari-Silva, L.D. De-Melo, D.C. Soares, et al., Novel role for the double-stranded RNA-activated protein kinase PKR: modulation of macrophage infection by the protozoan parasite Leishmania, Faseb. J. 24 (2010) 617–626.