View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

European Journal of Pharmacology 670 (2011) 272–279

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

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

Immunopharmacology and Inflammation

Thalidomide modulates *Mycobacterium leprae*-induced NF-κB pathway and lower cytokine response

Maristela de Oliveira Hernandez [a,1], Tatiana de Oliveira Fulco [a,1], Roberta Olmo Pinheiro [a], Renata de Meirelles Santos Pereira [b], Paulo Redner [a,b], Euzenir Nunes Sarno [a], Ulisses Gazos Lopes [b], Elizabeth Pereira Sampaio [a,*]

[a] *Leprosy Laboratory, Oswaldo Cruz Institute, FIOCRUZ, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ, Brazil*
[b] *Laboratory of Molecular Parasitology, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

A R T I C L E I N F O

Article history:
Received 25 March 2011
Received in revised form 16 August 2011
Accepted 27 August 2011
Available online 14 September 2011

Keywords:
Thalidomide
NF-κB
TNF-α
M. leprae
Leprosy

A B S T R A C T

It is widely accepted that tumor necrosis factor alpha (TNF-α) plays a critical role in the development of tissue and nerve damage in leprosy and during the reactional episodes of acute inflammation. Thalidomide (N-α-phthalimidoglutarimide), a drug used to treat leprosy reaction, modulates immune response, inhibits inflammation and NF-κB activity. Here we investigated whether thalidomide inhibits NF-κB activation induced by *Mycobacterium leprae*, p38 and ERK1/2 MAPK activation. EMSA and supershift assays were performed to investigate NF-κB activation in response to *M. leprae* and its modulation following *in vitro* treatment with thalidomide. Luciferase assay was assayed in transfected THP-1 cells to determine NF-κB transcriptional activity. Flow cytometry and immunofluorescence were used to investigate p65 accumulation in the nucleus. Immunoblotting was used to investigate p38 and ERK1/2 phosphorylation. Following activation of PBMC and monocytes with *M. leprae*, the formation and nuclear localization of NF-κB complexes composed mainly of p65/p50 and p50/p50 dimers was observed. Induction of NF-κB activation and DNA binding activity was inhibited by thalidomide. The drug also reduced *M. leprae*-induced TNF-α production and inhibited p38 and ERK1/2 activation. Definition of the activation mechanisms in cells stimulated with *M. leprae* can lead to the development of new therapy applications to modulate NF-κB activation and to control the inflammatory manifestations due to enhanced TNF-α response as observed in leprosy and in leprosy reactions.

© 2011 Elsevier B.V. Open access under the Elsevier OA license.

1. Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* that affects the skin and peripheral nerves. The disease presents a clinical spectrum that correlates with the immune response of the host to the pathogen (Ridley and Jopling, 1966). It is widely accepted that tumor necrosis factor alpha (TNF-α) plays a critical role in the development of tissue and nerve damage in leprosy and in the pathogenesis of the episodes of acute inflammatory reaction (reversal reaction, RR, and erythema nodosum leprosum, ENL) (Kahawita and Lockwood, 2008; Sarno et al., 1991). These episodes may occur during the chronic course of the disease and are treated either with steroids and/or thalidomide. The clinical benefits of thalidomide in ENL have been attributed to its ability to inhibit TNF-α secretion both *in vitro* and *in vivo* (Sampaio et al., 1991, 1993), among others (Rafiee et al., 2010; Teo et al., 2002).

Nuclear factor-κB (NF-κB) constitutes a group of dimeric transcription factors composed of various combinations of members of the NF-κB/Rel family. NF-κB proteins are usually found in the cytoplasm in association with inhibitory proteins (inhibitory κB factors or simply IκBs). Degradation of IκBs allows NF-κB to translocate to the nucleus, binds to DNA binding sites, and regulates the transcription of a large number of genes including cytokines, chemokines, and antimicrobial peptides (Li and Verma, 2002). Mycobacteria and their cell wall components, such as lipoarabinomannan, have been described to induce NF-κB nuclear translocation (Means et al., 2001) and MAP kinase activation, as important events for both cytokine production and cell activation. In our previous work we showed that *M. leprae* also induces activation of NF-κB complexes in a human Schwannoma cell line (Pereira et al., 2005).

Thalidomide (N-α-phthalimidoglutarimide) is a known anti-angiogenic, anti-tumor, and antiproliferative agent, widely used for the treatment of several immunological disorders, mainly leprosy reaction (ENL) and cancer (Erlinger, 2010; Sampaio et al., 1993;

* Corresponding author at: Leprosy Laboratory - Oswaldo Cruz Foundation, Av. Brasil, 4365 - Manguinhos, Rio de Janeiro, RJ, 21045-900, Brazil. Tel./fax: +55 21 2270 9997.
E-mail address: esampaio@ioc.fiocruz.br (E.P. Sampaio).
[1] These authors contributed equally to this work.

0014-2999 © 2011 Elsevier B.V. Open access under the Elsevier OA license.
doi:10.1016/j.ejphar.2011.08.046

Teo et al., 2002). The drug is also known to inhibit pro-inflammatory cytokines while its TNF-α modulating property seems to be greatly related to drug activity (De Sanctis et al., 2010). Thalidomide inhibits TNF-α via enhanced degradation of the TNF mRNA (Kim et al., 2004; Moreira et al., 1993). In addition, thalidomide has broad activity with a clear effect on the inflammatory cascade including modulation of p38 MAPK (Bartlett et al., 2004; Noman et al., 2009) and NF-κB signaling pathway in response to different stimuli and in a variety of cell types (Keifer et al., 2001; Majumdar et al., 2002).

In the present study, we sought to detail how thalidomide modulates *M. leprae*-induced response *in vitro* as it can correlate to the improvement in patient clinical responses. We demonstrate that thalidomide suppresses NF-κB transcriptional activity, DNA binding activity and activation induced by *M. leprae* in primary human cells and consequently leading to reduced cytokine production. Moreover,

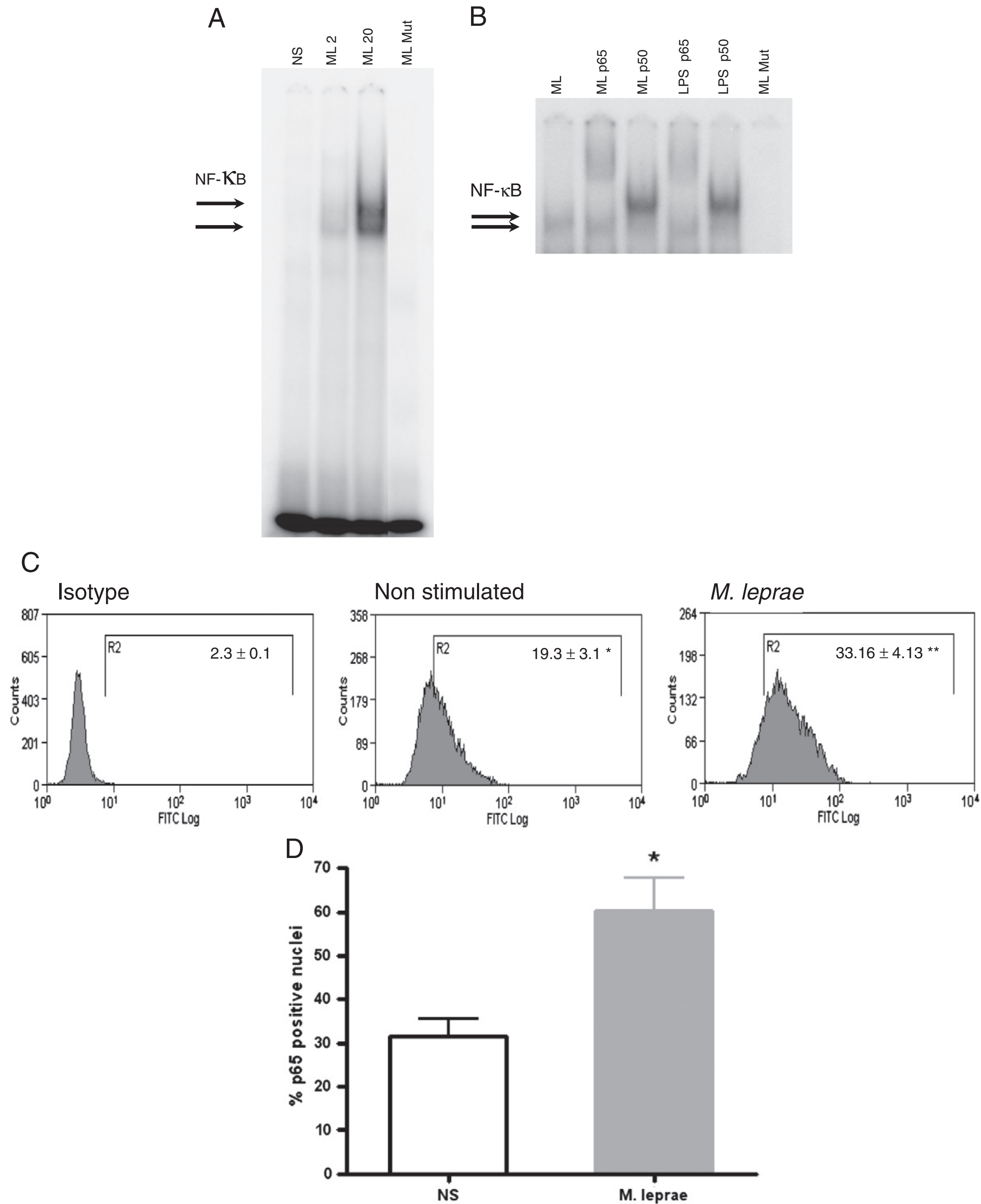

Fig. 1. *M. leprae* induces NF-κB nuclear translocation in primary human cells. (A) PBMC isolated from lepromatous leprosy patients were stimulated or not (NS) with *M. leprae* (ML), MOI 2 and 20, for 30 min when nuclear proteins were extracted, incubated with a DNA probe containing a wild-type or mutant (Mut) NF-κB binding site and analyzed by EMSA; (B) Nuclear extracts obtained from the stimulated cells (ML, MOI 20; LPS, 1 μg/ml) were pre-incubated with antibodies against the p65 and p50 subunits of the NF-κB complex and analyzed in the supershift assays; (C) Flow cytometry analysis of p65 subunit was assayed in isolated nuclei from patients' monocytes. Histograms are representative of 5 individual experiments: left panel, isotype control antibody; middle panel, nonstimulated cells; right panel, *M. leprae*-stimulated wells. Numbers are mean (± S.E.M.) fluorescence intensity. **Indicate significant difference ($P = 0.001$) when in the presence of the mycobacteria; (D) Graph shows mean percentage of p65 positive labeled cells ($n = 5$; *$P = 0.03$).

thalidomide interferes with p38 and ERK1/2 MAPK activation induced by the mycobacteria.

2. Material and methods

2.1. Studied population

A total of 13 leprosy patients (11 males, 2 females) who attended the Leprosy Out-Patient Unit, Leprosy Laboratory, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were enrolled into the study. Patients were diagnosed according to Ridley and Jopling classification (1966), were all multibacillary (bacillary index >0), and were treated with multidrug therapy as recommended by the World Health Organization. Healthy donors (n = 5) from the Hemotherapy Unit, Clementino Fraga Filho Hospital, Federal University of Rio de Janeiro were also included. The study was approved by the Ethical Committee of FIOCRUZ and following informed consent, blood was withdrawn from all individuals and experiments performed as described below.

2.2. Cell isolation and culture condition

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood under endotoxin free conditions through Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation. PBMC were suspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% FCS (Gibco BRL, Gaithersburg, MD), subsequently cultured in 24-well plates (Costar Corporation, Cambridge, MA), at 37 °C for 2 h, when cultures were washed for removal of non-adherent cells. For *in vitro* stimulation, whole *M. leprae* irradiated (2×10^9 bacteria/ml), provided by Dr. P. Brennan (Microbiology Department, Colorado State University, Fort Collins, CO), and lipopolysaccharide (LPS) from *Salmonella Minnesota* Re 595 (Sigma Chemical Co., St. Louis, MO) were used. In parallel experiments, thalidomide (25 μg/ml; Calbiochem, Cambridge, MA) was added to the cultures 1 h prior to cell stimulation.

2.3. Electrophoretic mobility shift assay (EMSA) and supershift assay

PBMC (10^7 cells) were cultured in Teflon beakers (Thomas Scientific, Swedesboro, NJ) and stimulated with *M. leprae* (multiplicity of infection, MOI 2 or 20) or LPS (1 μg/ml) for 30 min. After the stimulation period, cells were washed, nuclear protein extracts were obtained and EMSA and supershift assays were performed as detailed elsewhere (Pereira et al., 2005).

2.4. Detection of NF-κB translocation by flow cytometry

Cultured monocytes or PBMC were stimulated with *M. leprae* (MOI 20) or TNF-α (1 μg/ml), and after 1 h, nuclei from the cells were obtained by using Pipes–Triton buffer (10 mM Pipes, 0.1 M NaCl, 2 mM $MgCl_2$ and 0.1% Triton X-100; Sigma). Nuclei samples were washed, stained with anti-p65 monoclonal or isotype control antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), subsequently incubated with FITC-conjugated goat anti-mouse Ig antibody and counterstained with propidium iodide (1 mg/ml; Sigma) (Cognasse et al., 2003). In some experiments, cells were treated with thalidomide, the NF-κB inhibitor SN50, its non-active mutant, SN50m (18 μM; Calbiochem), or the proteasome inhibitor MG132 (4 μM; Calbiochem) followed by mycobacteria stimulation. For evaluation of NF-κB translocation, samples were analyzed by flow cytometry on a FACS Calibur (BD Biosciences, San Jose, CA). A total of 10^4 events were recorded for each experimental condition.

2.5. Cytokine detection

For detection of TNF-α, culture supernatants were harvested 20–24 h after cell stimulation and stored at −20 °C until use. Cytokine levels were assayed by a commercial specific enzyme-linked immunosorbent assay (ELISA) processed according to the manufacturer's specifications (R&D Systems Inc., Minneapolis, MN). Detection limit of the assay was 8 pg/ml.

2.6. Immunofluorescence and confocal microscopy

Monocytes settled onto glass coverslips were stimulated with the mycobacteria (MOI 20) for 1 h, washed and fixed in paraformaldehyde 3.7%. Cells were permeabilized with PBS-0.5% Triton X-100, labeled with a primary antibody against phospho-p65 (Santa Cruz Biotechnology), followed by incubation with the secondary antibody (Chemicon International, Temecula, CA). Nuclei were stained with DAPI and images were acquired on a Laser Scanning Confocal Microscopy LSM 510-META (Zeiss, Thornwood, NY).

2.7. Luciferase assay

To measure NF-kB transcriptional activity, THP-1 cells were co-transfected using FUGENE 6 reagent (Roche, Madison, WI) with a

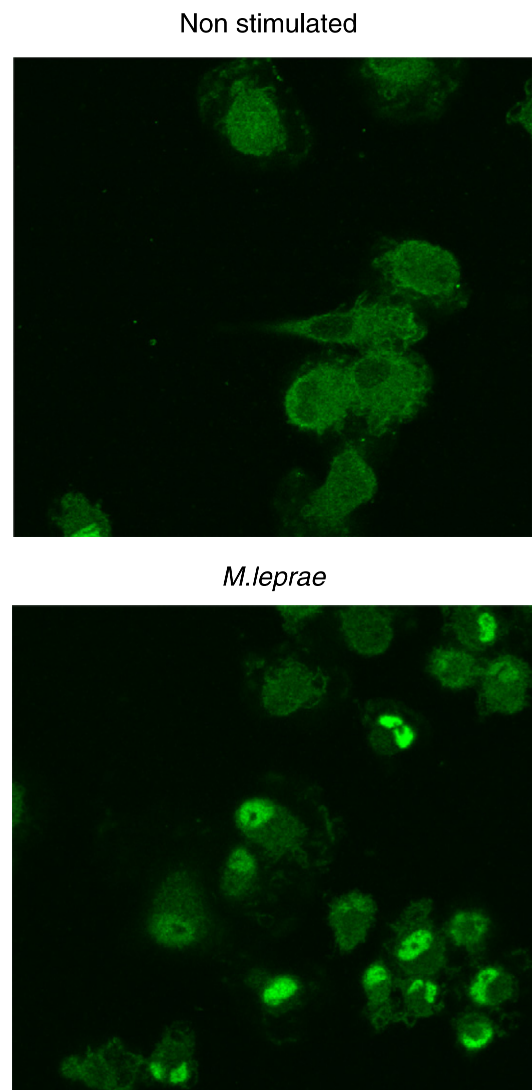

Fig. 2. Immunofluorescence staining and confocal microscopy show translocation of NF-κB p65 subunit to the nucleus in monocyte cultures after stimulation with *M. leprae* (lower panel, MOI 20) as opposed to the nonstimulated wells (upper panel). Visualization of cells was carried out by confocal microscopy using a 40× oil immersion objective and a 2× zoom magnification. One experiment out of four is presented.

reporter plasmid containing NF-κB consensus-binding sites upstream of firefly luciferase reporter gene (750 ng IgK-IFNLUC, kindly provided by Dr. David Baltimore, Massachusetts Institute of Technology, Cambridge) and a constitutive active reporter containing Renilla luciferase gene (250 ng pRLCMV plasmid; Promega, Madison, WI). After 48 h, cultures were washed, medium was replaced and cells stimulated with *M. leprae* or TNF-α, in the presence or absence of thalidomide for 6 h. Cells were resuspended in lysis buffer and luciferase activity evaluated using the dual luciferase assay (Promega), detected in a luminometer TD-20/20 (Turner Designs, Sunnyvale, CA). Experiments were done in triplicate and values normalized to Renilla activity. Data are expressed as fold increase of luciferase activity over the nonstimulated samples.

2.8. Immunoblotting

For Western blot analysis, cell lysates were obtained from cultured monocytes in cell lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors (Calbiochem). Proteins (30 μg) were resolved on a 12% SDS-polyacrylamide gel blotted onto nitrocellulose membrane (Bio-Rad). The membranes were blocked with BSA 5% and 0.1% Tween, washed and incubated with the primary anti-phospho-p38 or anti-pERK1/2 antibodies (1:1000 dilution; Cell Signaling). Following washing, the blots were incubated for an additional hour in horseradish peroxidase conjugated secondary antibody (eBioscience, San Diego, CA), and the bands detected by an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

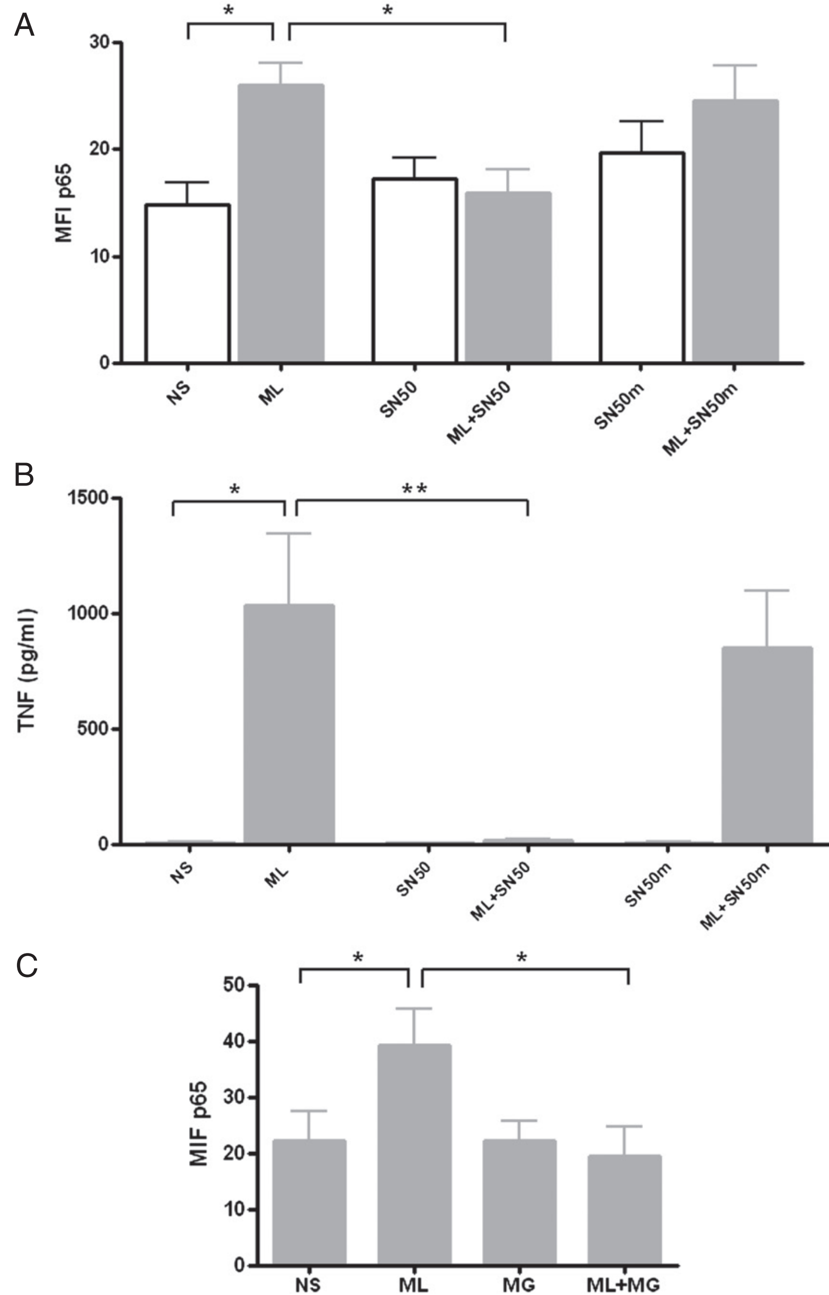

Fig. 3. *M. leprae*-induced NF-κB is linked to proteasome activity and TNF-α release. (A) Monocytes were pre-incubated with the NF-κB inhibitor SN50 or its mutant SN50m and cells stimulated with *M. leprae* (ML, MOI 20). Flow cytometry analysis for detection of nuclear p65 subunit shows decreased mean fluorescence intensity (MFI) in the stimulated cells as compared to the nonstimulated samples (NS); (B) TNF-α levels were evaluated in the 20 h culture supernatants. The NF-κB inhibitor but not its mutant significantly abolished cytokine response in the stimulated cells; (C) Monocytes pre-incubated with the proteasome inhibitor MG132 (MG) were stimulated with the mycobacteria and assayed for nuclear p65 staining as above (MFI). Inhibition of proteasome activity interferes with *M. leprae*-induced NF-κB activation. Data are mean of 5 individual experiments (*$P<0.05$; **$P<0.01$).

Blots were stripped and re-probed with anti-p38 or anti-pERK antibody (Cell Signaling).

2.9. Statistical analysis

Results are expressed as mean ± standard error (S.E.M.). Statistical analysis was performed by using one-way ANOVA test. Flow cytometry data were analyzed using a paired *t* test (InStat/Prism software; GraphPad Software, San Diego, CA). Differences were significant at $P<0.05$.

3. Results

3.1. *M. leprae* induces NF-κB nuclear translocation in primary human cells in vitro

NF-κB activation was investigated through EMSA in freshly isolated PBMC obtained from leprosy patients (Fig. 1A) and healthy donors, following *in vitro* stimulation with *M. leprae*. Enhanced NF-κB binding was observed in nuclear cell extracts from stimulated cultures when compared to nonstimulated cells. NF-κB/DNA binding induced by the mycobacteria showed to be specific as bands were not observed in the presence of the mutant probe. Results were similar when using cells from patients or controls.

To characterize the protein composition of the NF-κB complex, specific antibodies against the NF-κB subunits were used in the supershift assay. As observed in Fig. 1B, antibodies against p65 and p50 subunits led to slower migrating complexes in the gels. We observed the formation and nuclear localization of NF-κB complexes composed mainly of p65/p50 and p50/p50 dimers. Addition of anti-p50 antibody shifted both complexes in the cells stimulated either with *M. leprae* or LPS, whereas antibody against the p65 protein shifted the top complex only (Fig. 1B). Antibodies against p52 or c-Rel proteins did not modify the pattern of complex migration (not shown).

We then evaluated NF-κB p65 nuclear translocation in adherent patients' monocytes by flow cytometry. Following p65 staining in isolated nuclei (Fig. 1C), mean florescence intensity (MFI) was markedly induced by *M. leprae* (33 ± 4.1) when compared to the nonstimulated

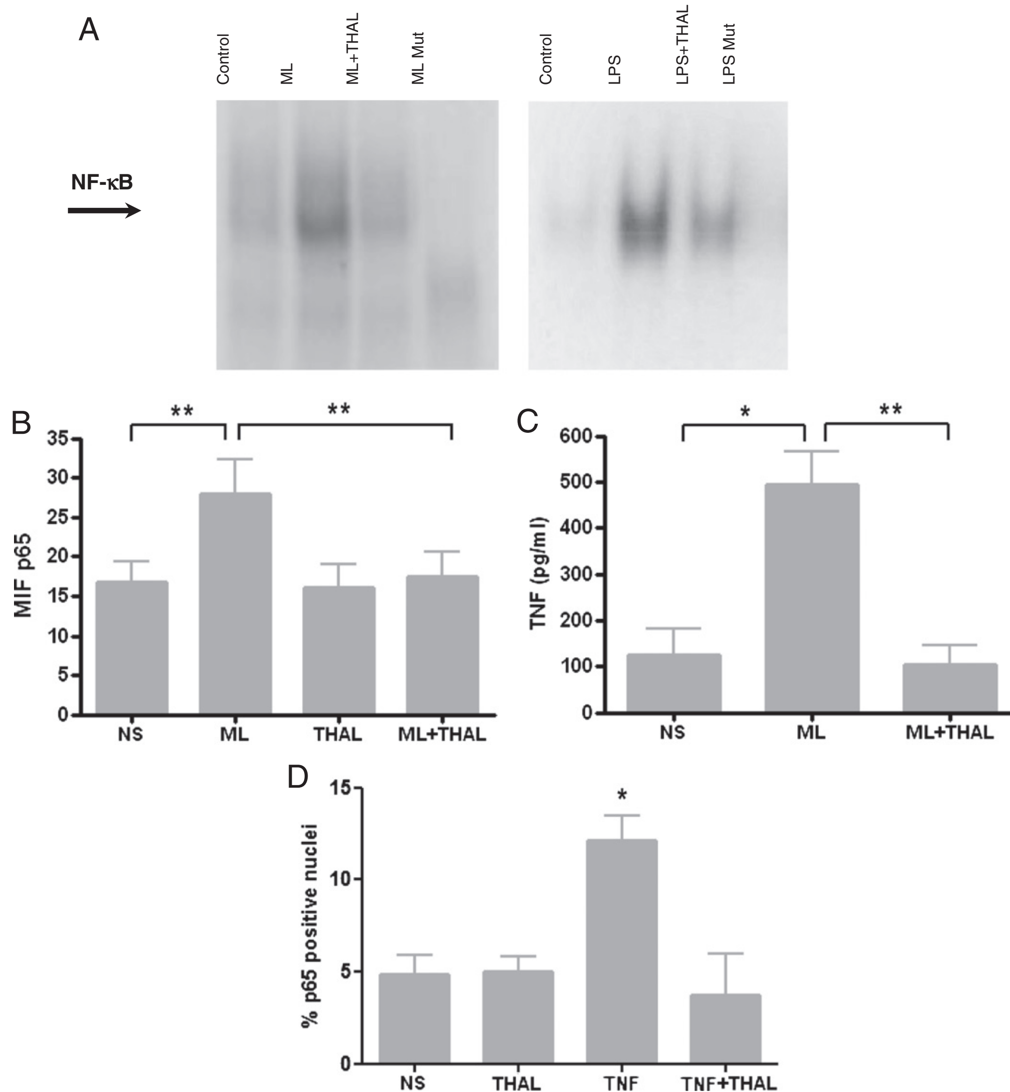

Fig. 4. Thalidomide down-regulates *M. leprae*-induced NF-κB activation and TNF-α production. (A) PBMC obtained from leprosy patients were pre-incubated with thalidomide (THAL) and stimulated with *M. leprae* (MOI 20). Nuclear proteins were extracted after 30 min stimulation and analyzed by EMSA. Mut = mutant NF-κB binding site. Data are representative of 4 experiments; (B) Flow cytometry analysis of p65 NF-κB was performed in patients' PBMC pre-incubated with THAL, stimulated with *M. leprae* and in (C) PBMC from healthy donors treated with THAL and TNF-α (1 μg/ml) for 1 h. Results showed decreased MFI following thalidomide treatment; (D) Monocytes stimulated with ML in the presence or absence of THAL were assayed for TNF-α levels (pg/ml) in the 20 h culture supernatants; *$P<0.05$; **$P<0.01$, indicate significant differences when compared to the other culture conditions (n = 4). NS = nonstimulated cells.

cells (19.3 ± 3; $P = 0.001$). Accordingly, rate of p65 labeled cells was 60.2 ± 7.4 vs. 31.3 ± 4%, respectively (Fig. 1D; $P < 0.05$). Confocal microscopy confirmed, at a single cell level, the nuclear translocation of the p65 protein triggered by the mycobacteria (Fig. 2).

3.2. Activation of NF-κB is essential for M. leprae-induced TNF-α production

Initially, the ability of SN50 to impair NF-κB activation was confirmed in cultured monocytes from healthy donors and patients (not shown). Cells pre-treated with SN50, but not the mutated peptide SN50m, showed impaired p65 nuclear staining in response to *M. leprae* (Fig. 3A; $P < 0.05$) as MFI returned to baseline levels when in the presence of the inhibitor.

The involvement of NF-κB in *M. leprae*-induced TNF-α response was then assayed in cells pre-incubated with SN50 and stimulated with the mycobacteria for 20–24 h. TNF-α values (1150 ± 44 pg/ml) were significantly lower ($P < 0.001$) in the presence of the NF-κB inhibitor (15 ± 4.1 pg/ml), whereas addition of SN50m showed no effect (Fig. 3B).

Previous work demonstrated that inhibition of the proteasome interfered with *M. leprae*-induced cytokine release (Fulco et al., 2007). Therefore, in this study, we pre-incubated the cells with MG132 and observed through flow cytometry reduced p65 staining in the treated mycobacteria stimulated wells (Fig. 3C; $P = 0.03$).

3.3. Thalidomide (THAL) reduces NF-κB activation and the production of TNF-α in vitro

To determine if THAL can interfere with NF-κB binding activity induced by *M. leprae*, PBMC obtained from leprosy patients were stimulated with the mycobacteria in the presence of the drug and assayed by EMSA. Cultures pre-treated with thalidomide showed decreased NF-κB nuclear translocation generated both in response to *M. leprae* and LPS (Fig. 4A). Viability of the cells in culture was not affected by thalidomide (not shown).

Analysis by flow cytometry also showed enhanced nuclear p65 staining induced by *M. leprae* (MFI = 27.9 ± 4.4) and such response was down-regulated (16 ± 3.1; $P = 0.007$) in the presence of THAL to the levels observed in the unstimulated cells (Fig. 4B). Accordingly, percentage of p65 labeled cells was diminished in the treated stimulated cultures (67.2 ± 6.9 vs. 45.9 ± 6%, respectively; $P = 0.015$). The effect of thalidomide was also evident since the drug abrogated NF-κB activation (% p65 positive) induced by TNF-α in PBMC from healthy donors (Fig. 4C) or patients. As previously described, levels of TNF-α induced by *M. leprae* in the 20 h cultures were reduced by THAL (Fig. 4D; $P < 0.01$).

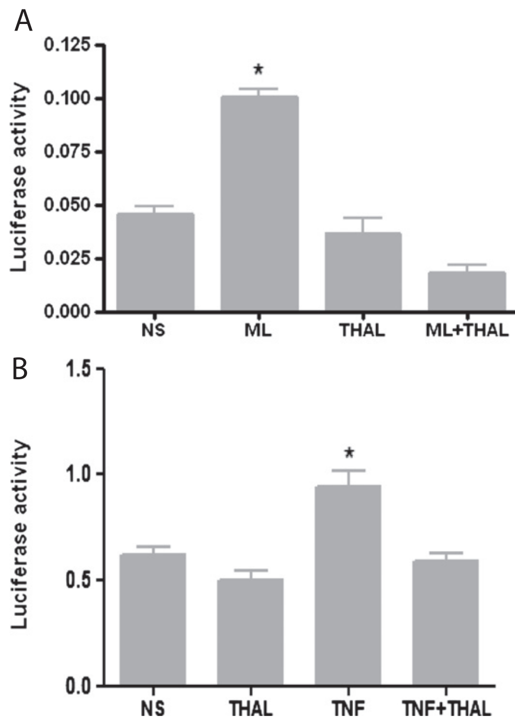

Fig. 5. NF-κB transcriptional activity. (A) Whole cell lysates were obtained from transiently transfected THP-1 cells with a NF-κB luciferase reporter plasmid introduced along with a Renilla luciferase expression plasmid, stimulated for 6 h with (A) *M. leprae*, ML, or (B) TNF-α in the presence or absence of thalidomide and analyzed for luciferase activity. Data are mean of 3 individual experiments; *$P < 0.05$. NS = nonstimulated cells.

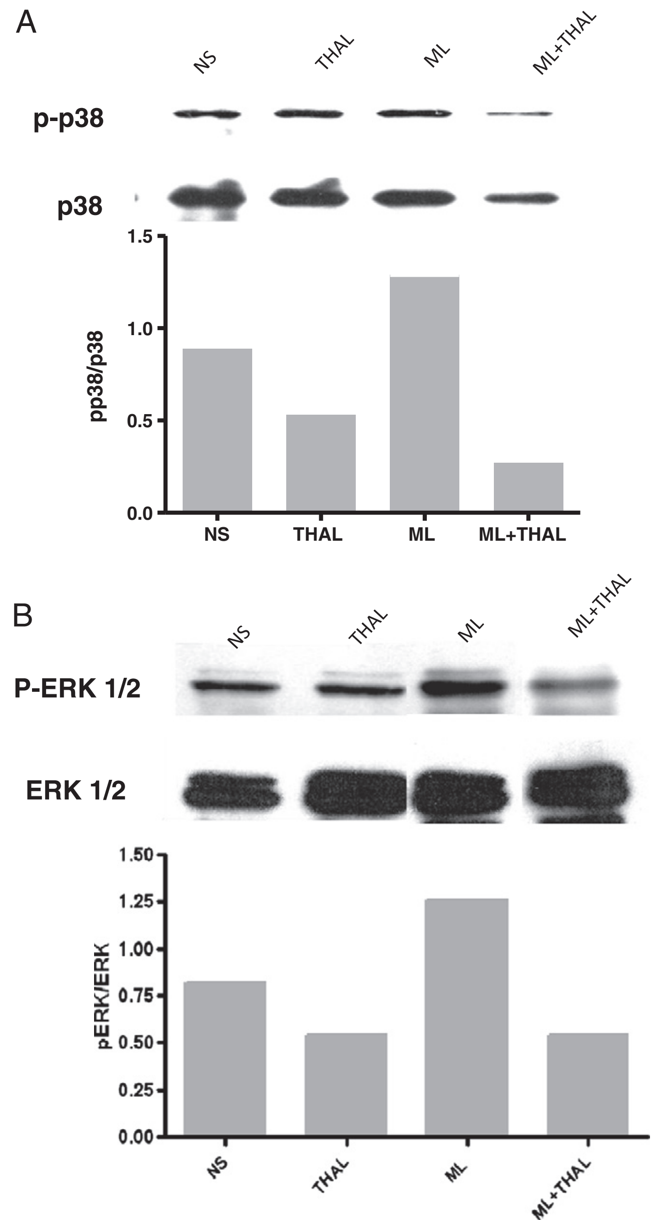

Fig. 6. Thalidomide modulates *M. leprae*-induced activation of p38 MAPK. Monocytes from healthy donors were pre-treated with THAL (25 μg/ml) and stimulated with *M. leprae* for 30 min. Phosphorylation of (A) p38 and (B) ERK1/2 was detected by immunoblotting with the specific antibodies as described in the Material and methods section. Densitometry of one representative experiment is presented.

To evaluate NF-κB transcriptional activity, we transfected THP-1 cells with a luciferase reporter assay using a plasmid that contains binding sites for NF-κB and a constitutively active Renilla plasmid for normalization. In accordance to the above described results, stimulation of THP-1 with *M. leprae* (Fig. 5A) or TNF-α induced (Fig. 5B) NF-κB mediated response was repressed when cells were treated with thalidomide.

3.4. Thalidomide treatment inhibits MAPK activation induced by *M. leprae*

Previous studies have described that thalidomide inhibits the phosphorylation of the MAPK pathway induced by LPS (Noman et al., 2009). We then evaluated whether THAL interfered with the upstream pathways involved in the *M. leprae*-induced NF-κB response. Analysis by immunoblotting revealed that phosphorylation of p38 observed in monocytes isolated from healthy individuals (Fig. 6A) or leprosy patients, and phosphorylation of ERK1/2 assayed 30 min after stimulation with *M. leprae* (Fig. 6B) was inhibited by THAL.

4. Discussion

Accumulating evidence suggests that production of TNF-α in response to *M. leprae* infection can contribute to the pathology of leprosy (Hernandez et al., 2003; Kahawita and Lockwood, 2008; Scollard et al., 2006). However, the intracellular mechanisms leading to mycobacteria-induced cytokine response are not yet fully characterized. Previous results demonstrate that *M. leprae* triggers the TLR2 signaling pathway (Krutzik et al., 2003) and as shown here, it enhances both the nuclear translocation and the DNA binding activity of NF-κB p65/p50 and p50/p50 complexes in primary human cells. Our results also reinforce the involvement of NF-κB on *M. leprae*-induced TNF-α release *in vitro*.

The development of an inflammatory response is a key event during mycobacterial infections. Several groups have reported activation of NF-κB in response to both intact mycobacteria and mycobacterial cell wall components (Lee and Schorey, 2005; Means et al., 2001), macrophages infected with *Mycobacterium smegmatis* (Gutierrez et al., 2008), and in cells from patients infected with *M. tuberculosis* (Ameixa and Friedland, 2002). NF-κB activation seems to be essential for mycobacterial killing since when NF-κB is blocked *M. smegmatis* survived (Gutierrez et al., 2008). On the other hand, some studies have described inhibition of NF-κB activation by *M. tuberculosis* components (Pathak et al., 2005, 2007).

Here we demonstrate that *M. leprae* induces NF-κB activation in primary human cells (PBMC and monocytes) and that thalidomide can suppress mycobacteria-induced NF-κB *in vitro*. We found that both homo and heterodimer forms of NF-κB were inhibited and led to the reduced TNF-α response. Thalidomide also inhibited NF-κB activation induced by TNF-α in Schwann cells (Pereira et al., 2005) and in PBMC (Fig. 4) *in vitro*. Overall, the results support the ability of *M. leprae* to induce TNF-α and NF-κB activation; TNF sustains NF-κB response and thalidomide interferes in this positive feedback loop.

The ability of thalidomide to down-regulate *M. leprae*-induced NF-κB activity poses a picture for the potent inhibitory effect this drug has over controlling the inflammatory manifestations of leprosy. Previous data showed that thalidomide also inhibited the complex p50/p50 and abolished *M. leprae*-induced gene transcriptional repression in a human Schwann cell line (Pereira et al., 2005). These results suggest that the inhibitory effect of thalidomide over *M. leprae* stimulation is not cell-type specific.

It has been suggested that the drug's ability to modulate gene expression through suppression of NF-κB might be related to the pathway that is activated by the inducer (Majumdar et al., 2002). Recent studies have described that thalidomide inhibits LPS-induced TNF-α production and NF-κB activation in RAW 264.7 cells by preventing phosphorylation of IkBα and IkB kinases (IKK-α and -β), and via down-regulation of MyD88 (Noman et al., 2009).

The ubiquitin–proteasome pathway controls various cellular processes and modulates NF-κB regulation through proteolytic processing of NF-κB precursors, polyubiquitination, and degradation of the inhibitory protein IkBα. We have also shown herein that inhibition of proteasome reduces NF-κB activity in response to *M. leprae*, and that this inhibition may impact into the lower induced cytokine production as reported previously (Fulco et al., 2007). A recent data suggest that the inhibition of NF-κB-dependent gene expression by bortezomib, a proteasome inhibitor, is gene specific and depends on the subunit composition of NF-κB dimers recruited to NF-κB-responsive promoters (Juvekar et al., 2011). More interestingly, one recent study identified a thalidomide-binding protein, cereblon (CRBN), as a primary target for thalidomide teratogenicity and the drug inhibits its ubiquitin ligase activity (Ito et al., 2011).

It is clear that many pathways are to be involved in leprosy and other diseases in which thalidomide is effective. Further experiments are still necessary to clarify the molecular mechanisms by which thalidomide modulates *M. leprae*-induced NF-κB.

Work by several groups has established that MAPKs play a critical role in the pathogenesis of various malignancies. One recent work described that *M. leprae* inhibited the phosphorylation of either p38 or ERK1/2 in T cells (Dagur et al., 2010). We observed herein that *M. leprae* was able to increase activation of ERK1/2 and to a lesser extent of p38 in primary human monocytes. Thalidomide was able to decrease the *M. leprae*-induced phosphorylation of both p38 and ERK1/2 MAPKs, implying that the effect of thalidomide on NF-κB activity and on TNF-α secretion might involve MAPKs deactivation.

Our data show that thalidomide inhibits both p38/ERK and NF-κB activation in the stimulated cells. Definition of the mechanisms triggered in human cells challenged with mycobacteria can lead to the development of new therapy applications to control the inflammatory manifestations as observed in leprosy and in leprosy reactions.

Conflict of interest

The authors declare no competing conflict of interest.

Acknowledgments

We are grateful to J.A.C. Nery and A.M. Sales, the Leprosy Outpatient Unit, FIOCRUZ, to E.B. Oliveira and R. Teles for technical assistance, and P.A. Manso for assistance with the confocal experiments. This study was supported by IOC/FIOCRUZ, in part by CNPq Research Grant and CNPq Fellowship Program, Brazil.

References

Ameixa, C., Friedland, J.S., 2002. Interleukin-8 secretion from *Mycobacterium tuberculosis* — infected monocytes is regulated by protein tyrosine kinases but not ERK 1/2 or p38 mitogen-activated protein kinases. Infect. Immun. 70, 4743–4746.

Bartlett, J.B., Dredge, K., Dalgleish, A.G., 2004. The evolution of thalidomide and its IMiD derivatives as anticancer agents. Nat. Rev. Cancer 4, 314–322.

Cognasse, F., Sabido, O., Béniguel, L., Genin, C., Garraud, O., 2003. A flow cytometry technique to study nuclear factor-kappa B (NFkappaB) translocation during human B cell activation. Immunol. Lett. 90, 49–52.

Dagur, P.K., Sharma, B., Kumar, G., Khan, N.A., Katoch, V.M., Sengupta, U., Joshi, B., 2010. Mycobacterial antigen(s) induce anergy by altering TCR- and TCR/CD28-induced signaling events: insights into T-cell unresponsiveness in leprosy. Mol. Immunol. 47, 943–952.

De Sanctis, J.B., Mijares, M., Suárez, A., Compagnone, R., Garmendia, J., Moreno, D., Salazar-Bookaman, M., 2010. Pharmacological properties of thalidomide and its analogues. Rec. Pat. Inflamm. Allergy Drug Discov. 4, 144–148.

Erlinger, S., 2010. The thalidomide renaissance. Gastroenterol. Clin. Biol. 34, 426–428.

Fulco, T.O., Lopes, U.G., Sarno, E.N., Sampaio, E.P., Saliba, A.M., 2007. The proteasome function is required for *Mycobacterium leprae*-induced apoptosis and cytokine secretion. Immunol. Lett. 110, 82–85.

Gutierrez, M.G., Mishra, B.B., Jordao, L., Elliott, E., Anes, E., Griffiths, G., 2008. NFkappa B activation controls phagolysosome fusion-mediated killing of mycobacteria by macrophages. J. Immunol. 181, 2651–2663.

Hernandez, M.O., Neves, I., Sales, J.S., Carvalho, D.S., Sarno, E.N., Sampaio, E.P., 2003. Induction of apoptosis in monocytes by *Mycobacterium leprae* in vitro: a possible role for tumour necrosis factor-alpha. Immunology 109, 156–164.
Ito, T., Ando, H., Handa, H., 2011. Teratogenic effects of thalidomide: molecular mechanisms. Cell. Mol. Life Sci. 68, 1569–1579.
Juvekar, A., Manna, S., Ramaswami, S., Chang, T.P., Vu, H.Y., Ghosh, C.C., Celiker, M.Y., Vancurova, I., 2011. Bortezomib induces nuclear translocation of I{kappa}B{alpha} resulting in gene-specific suppression of NF-{kappa}B-dependent transcription and induction of apoptosis in CTCL. Mol. Cancer Res. 9, 183–194.
Kahawita, I.P., Lockwood, D.N., 2008. Towards understanding the pathology of erythema nodosum leprosum. Trans. R. Soc. Trop. Med. Hyg. 102, 329–337.
Keifer, J.A., Guttridge, D.C., Ashburner, B.P., Baldwin Jr., A.S., 2001. Inhibition of NFkappa B activity by thalidomide through suppression of IkappaB kinase activity. J. Biol. Chem. 276, 22382–22387.
Kim, Y.S., Kim, J.S., Jung, H.C., Song, I.S., 2004. The effect of thalidomide on the stimulation of NF-kappaB activity and TNF-alpha production by lipopolysaccharide in a human colonic epithelial cell line. Mol. Cells 17, 210–216.
Krutzik, S.R., Ochoa, M.T., Sieling, P.A., Uematsu, S., Ng, Y.W., Legaspi, A., Liu, P.T., Cole, S.T., Godowski, P.J., Maeda, Y., Sarno, E.N., Norgard, M.V., Brennan, P.J., Akira, S., Rea, T.H., Modlin, R.L., 2003. Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. Nat. Med. 9, 525–532.
Lee, S.B., Schorey, J.S., 2005. Activation and mitogen-activated protein kinase regulation of transcription factors Ets and NF-kappa B in *Mycobacterium*-infected macrophages and role of these factors in tumor necrosis factor alpha and nitric oxide synthase 2 promoter function. Infect. Immun. 73, 6499–6507.
Li, Q., Verma, I.M., 2002. NF-kappaB regulation in the immune system. Nat. Rev. Immunol. 2, 725–734.
Majumdar, S., Lamothe, B., Aggarwal, B.B., 2002. Thalidomide suppresses NF-kappa B activation induced by TNF and H_2O_2, but not that activated by ceramide, lipopolysaccharides, or phorbol ester. J. Immunol. 168, 2644–2651.
Means, T.K., Jones, B.W., Schromm, A.B., Shurtleff, B.A., Smith, J.A., Keane, J., Golenbock, D.T., Vogel, S.N., Fenton, M.J., 2001. Differential effects of a Toll-like receptor antagonist on *Mycobacterium tuberculosis*-induced macrophage responses. J. Immunol. 166, 4074–4082.
Moreira, A.L., Sampaio, E.P., Zmuidzinas, A., Frindt, P., Smith, K.A., Kaplan, G., 1993. Thalidomide exerts its inhibitory action on tumor necrosis factor alpha by enhancing mRNA degradation. J. Exp. Med. 177, 1675–1680.
Noman, A.S., Koide, N., Hassan, F., I-E-Khuda, I., Dagvadorj, J., Tumurkhuu, G., Islam, S., Naiki, Y., Yoshida, T., Yokochi, T., 2009. Thalidomide inhibits lipopolysaccharide induced tumor necrosis factor-alpha production via down-regulation of MyD88 expression. Innate Immun. 15, 33–41.
Pathak, S.K., Basu, S., Bhattacharyya, A., Pathak, S., Kundu, M., Basu, J., 2005. *Mycobacterium tuberculosis* lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin 12p40 production in macrophages. J. Biol. Chem. 280, 42794–42800.
Pathak, S.K., Basu, S., Basu, K.K., Banerjee, A., Pathak, S., Bhattacharyya, A., Kaisho, T., Kundu, M., Basu, J., 2007. Direct extracellular interaction between the early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* and TLR2 inhibits TLR signaling in macrophages. Nat. Immunol. 8, 610–618.
Pereira, R.M., Calegari-Silva, T.C., Hernandez, M.O., Saliba, A.M., Redner, P., Pessolani, M.C., Sarno, E.N., Sampaio, E.P., Lopes, U.G., 2005. *Mycobacterium leprae* induces NF-kappaB-dependent transcription repression in human Schwann cells. Biochem. Biophys. Res. Commun. 335, 20–26.
Rafiee, P., Stein, D.J., Nelson, V.M., Otterson, M.F., Shaker, R., Binion, D.G., 2010. Thalidomide inhibits inflammatory and angiogenic activation of human intestinal microvascular endothelial cells (HIMEC). Am. J. Physiol. Gastrointest. Liver Physiol. 298, G167–176.
Ridley, D.S., Jopling, W.H., 1966. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. Other Mycobact. Dis. 34, 255–273.
Sampaio, E.P., Sarno, E.N., Galilly, R., Cohn, Z.A., Kaplan, G., 1991. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. J. Exp. Med. 173, 699–703.
Sampaio, E.P., Kaplan, G., Miranda, A., Nery, J.A.C., Miguel, C.P., Viana, S.M., Sarno, E.N., 1993. The influence of thalidomide on the clinical and immunological manifestation of erythema nodosum leprosum. J. Infect. Dis. 168, 408–414.
Sarno, E.N., Grau, G.E., Vieira, L.M., Nery, J.A., 1991. Serum levels of tumour necrosis factor-alpha and interleukin-1 beta during leprosy reactional states. Clin. Exp. Immunol. 84, 103–108.
Scollard, D.M., Joyce, M.P., Gillis, T.P., 2006. Development of leprosy and type 1 leprosy reactions after treatment with infliximab: a report of 2 cases. Clin. Infect. Dis. 43, e19–22.
Teo, S.K., Resztak, K.E., Scheffler, M.A., Kook, K.A., Zeldis, J.B., Stirling, D.I., Thomas, S.D., 2002. Thalidomide in the treatment of leprosy. Microbes Infect. 4, 1193–1202.