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Biochemical and Biophysical Research Communications 335 (2005) 20–26
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Mycobacterium leprae induces NF-κB-dependent transcription repression in human Schwann cells

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Received 12 July 2005
Available online 25 July 2005

Abstract

Mycobacterium leprae, the causative agent of leprosy, invades peripheral nerve Schwann cells, resulting in deformities associated with this disease. NF-κB is an important transcription factor involved in the regulation of host immune antimicrobial responses. We aimed in this work to investigate NF-κB signaling pathways in the human ST88-14 Schwannoma cell line infected with *M. leprae*. Gel shift and supershift assays indicate that two NF-κB dimers, p65/p50 and p50/p50, translocate to the nucleus in Schwann cells treated with lethally irradiated *M. leprae*. Consistent with p65/p50 and p50/p50 activation, we observed IκB-α degradation and reduction of p105 levels. The nuclear translocation of p50/p50 complex due to *M. leprae* treatment correlated with repression of NF-κB-driven transcription induced by TNF-α. Moreover, thalidomide inhibited p50 homodimer nuclear translocation induced by *M. leprae* and consequently rescues Schwann cells from NF-κB-dependent transcriptional repression. Here, we report for the first time that *M. leprae* induces NF-κB activation in Schwann cells and thalidomide is able to modulate this activation.
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Keywords: NF-κB; Leprosy; *Mycobacterium leprae*; Thalidomide; Transcription repression; Schwann; IκB-α; p105; p65; p50

The nuclear transcription factor-κB (NF-κB) plays a pivotal role in the regulation of the host innate antimicrobial response. Several microbial pathogens trigger cellular signal transduction pathways that induce NF-κB activation or subvert these pathways to overcome the innate immune response. NF-κB regulates the expression of many immunological mediators, including cytokines, their receptors, and components of their signal transduction [1,2].

In mammals, the NF-κB family of transcription factors comprises five members containing the Rel homology domain: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel. Functional NF-κB is formed by homo- or heterodimers which are sequestered in the cytoplasm via non-covalent interactions with a class of inhibitory proteins called IκBs (NF-κB inhibitors). Signals that induce NF-κB activity usually lead to IκB phosphorylation by the IκB kinase (IKK) complex, and subsequent multiubiquitination and degradation of this protein via proteasome, allowing NF-κB dimers' translocation to nucleus [1,3–5].

Proteolytic processing of p105 and p100 precursors, respectively, generates the p50 and p52 subunits. In both precursors, the N-terminus contains the Rel homology domain, whereas the C-terminal IκB-like domain functions as a covalently attached IκB inhibitor protein. Both precursors are processed in vivo and in vitro by a mechanism requiring ubiquitination and partial degradation by proteasome 26S. The precursor proteins p105

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.061

and p100 can control NF-κB activation by dimerization with individual members of the family and, in addition to IκB degradation, removal of the C-terminal ankyrin region of the precursors also leads to NF-κB activation [5]. Accordingly, activation of p50 homodimer can be generated by stimulus-coupled processing of the p105 precursor to p50 [6]. However, a number of observations demonstrate that following cellular stimulation with ligands, such as TNF-α, p105 is phosphorylated and ubiquitinated, leading predominantly to its complete degradation by the proteasome [7,8]. The p50/p50 complex is thought to maintain basal transcription or act as a repressor by blocking the κB elements in promoter regions [9], whereas p65/p50 heterodimer is the mediator of induced transactivation [10].

Pathogens, such as *Salmonella*, *Shigella*, *Listeria*, *Helicobacter* [11], *Mycobacterium tuberculosis*, and *Mycobacterium avium* [12,13], trigger NF-κB activation, leading to modulation of gene expression. However, little is known about the role of NF-κB in leprosy disease.

Leprosy is a chronic granulomatous infection caused by *Mycobacterium leprae* that affect approximately 700,000 individuals each year [14]. *M. leprae* presents a tropism for peripheral nerves, where it resides within Schwann cells [15]. Infection of nerves can result in disabilities and deformities, observed in over 25% of affected individuals [16]. However, the mechanisms of nerve damage in leprosy and the Schwann cells' signaling in response to *M. leprae* infection are not well understood.

The erythema nodosum leprosum (ENL) is an acute inflammatory complication occurring in lepromatous patients, usually associated to initiation of multidrug therapy (MDT). Thalidomide has been selectively used in various autoimmune or inflammatory disorders and it is the drug of choice for the treatment of ENL. TNF-α seems to play a critical role in ENL damage [17] and thalidomide selectively inhibits the production of TNF-α by human monocytes stimulated in vitro with both LPS and mycobacterial products [18]. The immunomodulatory effect of thalidomide can rely on the inhibition of NF-κB activity through suppression of IκB kinase activity [19].

In this work, we investigated the nuclear translocation and transcriptional activity of NF-κB in Schwann cells treated with lethally irradiated *M. leprae* and its modulation by thalidomide.

Materials and methods

Bacteria and Schwann cells. Irradiated *M. leprae* derived from armadillo [20] was kindly provided by Dr. Patrick Brennan, Department of Microbiology, Colorado State University, Fort Collins, CO, through NIAID contract NO155262. The ST88-14 Schwannoma cell line was isolated from a patient with neurofibromatosis type I [21] (obtained from Jonathan Fletcher, Harvard University, Boston), and cultured in RPMI medium (Invitrogen, Rockville, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator at 37 °C with 5% CO_2.

Electrophoretic mobility shift assay and supershift assay. ST88-14 cells (4×10^6 cells) were treated with *M. leprae* at a multiplicity of infection (MOI) of 100:1 for 30 min, 1 μg/ml LPS (Sigma) for 30 min, and 25 ng/ml TNF-α (Sigma) for 4 h, pre-treated with 50 mM sodium salicylate (Fluka Chemika), and then treated with *M. leprae* at a MOI of 100:1 for 30 min. Alternatively, ST88-14 cells were co-treated with 25 μg/ml thalidomide (Calbiochem) and *M. leprae* for 30 min. HeLa cells were treated with 10 μg/ml LPS for 30 min. Nuclear cell extracts were obtained as described previously [22]. Electrophoretic mobility shift assay (EMSA) was performed by incubating 3 μg nuclear protein extract with 40,000 CPM of ^{32}P-end-labeled double-stranded NF-κB consensus oligonucleotide (Santa Cruz Biotechnologies) for 30 min at 25 °C. The binding mixture included 1 μg poly(dI–dC):poly(dI–dC) in binding buffer (10 mM Hepes, pH 7.9, 4% glycerol, 1 mM DTT, 1 mM EDTA, and 0.1 μg BSA). The DNA–protein complex was separated from free probe on 4% native polyacrylamide gel, and then dried and visualized by PhosphoImage analysis (Molecular Dynamics, Amersham). A double-stranded mutated oligonucleotide (Santa Cruz Biotechnologies) was used to examine the specificity of binding of NF-κB to DNA. In supershift assays, nuclear cell extracts were incubated with 0.5 μg antibodies against the NF-κB subunits p65 (c-20) and p50 (H-119) (Santa Cruz Biotechnologies) for 1 h on ice before incubation with the probe.

Western blot assay. After the treatment with *M. leprae* at a MOI of 100:1, ST88-14 cells (2×10^6 cells) were washed twice with ice-cold PBS and then lysed in 60 μl 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, 0.1% Triton X-100, 1 mg/ml BSA, and 1:1000 of protease inhibitor cocktail II (Calbiochem)) for total protein extracts. Nuclear and cytoplasmatic cell extracts were obtained as described previously [22]. Total, cytoplasmatic or nuclear protein (15 μg) was subjected to electrophoresis in 12% SDS–polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membrane (Amersham). After blocking with 5% non-fat dried milk in TBS added with 0.15% Tween (TBS-T), blots were incubated with 1 μg/ml antibody against IκB-α (c-21), p105/p50 (H-119) or Actin (H-300) (Santa Cruz Biotechnologies), followed by anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (1:3000), with three washings with TBS-T after each incubation. The proteins were detected by ECL chemiluminescent detection system (Amersham).

Luciferase assay. For measurement of NF-κB transcriptional activity, ST88-14 cells were co-transfected using LipofectAMINE 2000 reagent (Invitrogen) with 1 μg p6κB-LUC (kindly provided by Dr. Patrick Baeuerle) and 40 ng pRL-CMV plasmids (Promega). After incubation with *M. leprae* at a MOI of 100:1, TNF-α (25 ng/ml) or thalidomide (25 μg/ml) cells were then washed with PBS, lysed according to Dual Luciferase System protocol (Promega), and analyzed in TD-20/20 Luminometer (Turner Designs).

Statistical analyses. Data were analyzed by Student's t test for independent samples using the software Statistica 6.0. Data are expressed as an average of three determinations and significant differences were indicated for $p < 0.05$.

Results and discussion

Since the nuclear transcription factor NF-κB is an important factor involved in the host response to pathogens [2], and the infection of Schwann cells by *M. leprae* is fundamental in leprosy pathogenesis [15], we sought to investigate the effect of the treatment of Schwann cells with irradiated *M. leprae* on the activation of this nuclear factor. Our model consisted of the human Schwanno-

ma cell line ST88-14, which has been used in a number of studies as a model for *M. leprae* infection [23–25].

Mycobacterium leprae induces nuclear translocation of NF-κB in Schwann cells

ST88-14 cells were incubated with *M. leprae* at a MOI of 100:1, while nuclear extracts were prepared and assayed by EMSA for their ability to bind to a NF-κB consensus probe. To determine the conditions of NF-κB activation, we assayed ST88-14 cells for different times (30 min to 24 h) with different MOI (10:1, 50:1 or 100:1) (data not shown). NF-κB activation was stronger in 30 min at a MOI of 100:1 and two complexes were observed (Fig. 1A). To evaluate the response of ST88-14 cells to distinct NF-κB activators, cells were treated with either 1 μg/ml LPS (Fig. 1B) or 25 ng/ml TNF-α (Fig. 1C) and analyzed by EMSA. TNF-α treatment induced strong NF-κB activation, in contrast, LPS induced poor activation of this transcriptional factor. These data are expected since ST88-14 cells express TNF-RI and TNF-RII [26], but not CD14 (personal communication).

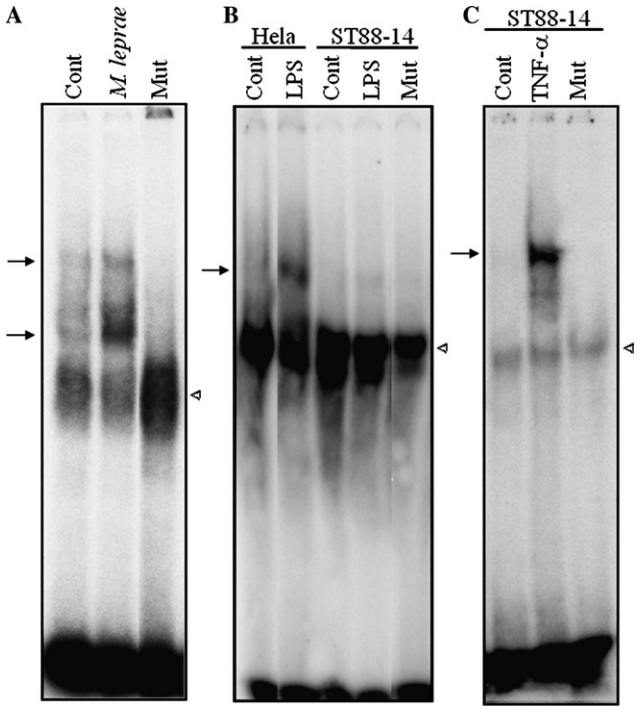

Fig. 1. Effects of *M. leprae*, LPS, and TNF-α on NF-κB activation in Schwann cells. (A) ST88-14 cells were untreated (Cont) or exposed to *M. leprae* (MI) at a MOI of 100:1 for 30 min. (B) ST88-14 cells were untreated (Cont) or treated with 1 μg/ml LPS for 30 min and HeLa cells were untreated (Cont) or treated with 10 μg/ml LPS for 30 min. (C) ST88-14 cells were untreated (Cont) or treated with 25 ng/ml TNF-α for 4 h. Nuclear extracts were analyzed by EMSA. Mut, NF-κB oligonucleotide mutant. The specific NF-κB bands are indicated by arrow and unspecific bands are indicated by open triangles.

Mycobacterium leprae treatment of Schwann cells leads to repression of NF-κB-driven transcription associated to p50/p50 activation

To characterize NF-κB-binding complexes induced by *M. leprae*, nuclear protein extracts of ST88-14 cells were submitted to supershift assays. As shown in Fig. 2A, the p65 and p50 antibodies produced a slow migrating complex. Moreover, antibodies against c-rel, p52, and RelB did not produce a supershift band (data

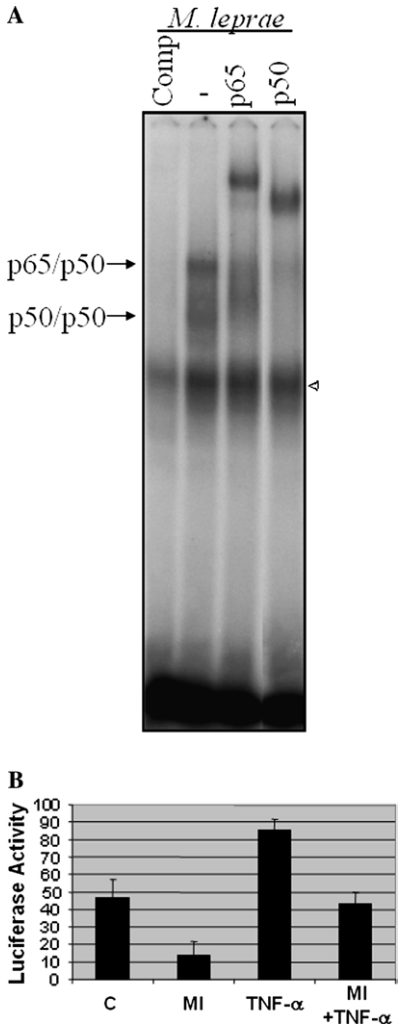

Fig. 2. *Mycobacterium leprae* induces p65/p50 and p50/p50 NF-κB nuclear translocation and inhibits NF-κB-dependent transcription induced by TNF-α in Schwann cells. (A) Nuclear extracts of ST88-14 cells treated with *M. leprae* at a MOI of 100:1 were pre-incubated for 1 h with antibodies anti-p65 and p50, as indicated, and then analyzed by EMSA. Comp, 100× cold NF-κB competitor probe. The specific NF-κB bands are indicated by arrow and unspecific bands are indicated by open triangles. (B) ST88-14 cells were transiently transfected using a reporter plasmid containing six NF-κB consensus binding sites upstream of luciferase reporter gene. Twenty-four hours post-transfection, cells were either not treated (C), treated with *M. leprae* (MI) alone at a MOI of 100:1, 25 ng/ml TNF-α alone or *M. leprae* plus TNF-α for 24 h. Whole cell lysates were analyzed for luciferase activity.

not shown). Our results reveal that the NF-κB complexes p65/p50 and p50/p50 are activated by *M. leprae* treatment of Schwann cells.

To determine the NF-κB-dependent transcriptional activity, we transiently transfected ST88-14 cells with a NF-κB luciferase reporter construction, which contains six consensus sites for NF-κB binding (κB sites), and the pRL-CMV plasmid, that has a constitutive cytomegalovirus promoter, used for luciferase activity normalization. The day after transfection, cells were treated with *M. leprae*, TNF-α or *M. leprae* plus TNF-α during 24 h and then assayed for luciferase activity. The treatment with *M. leprae* reduced the NF-κB transcription activity induced by TNF-α ($p < 0.05$) and the basal level expressed in untreated cells ($p < 0.05$) (Fig. 2B). The same result was obtained when we used the plasmid pRL-TK (Promega), that has a herpes simplex thymidine kinase promoter, as internal control (data not shown). *M. leprae* inhibition of NF-κB-dependent transcription induced by TNF-α may have important implications in the maintenance of nerve integrity in leprosy, since this cytokine is secreted by immune cells in the infection site and has been implicated in skin and peripheral nerve damage [27].

This transcription repression of NF-κB-dependent promoter is probably due to p50 homodimer activation *by M. leprae* [9]. A possible p50/p50 inducer is the cell wall-associated glycolipid ManLAM, produced by *M. leprae* and other slow-growing mycobacteria. It has been established in murine macrophages that ManLAMs induce nuclear translocation of p50/p50 complex [28]. There is now an emerging consensus that ManLAMs are anti-inflammatory molecules and are largely responsible for the inhibition of apoptosis in *M. tuberculosis*-infected macrophages [29–31].

Consistent with the idea that *M. leprae* can favor the environment for infection, it has previously been shown that this microbe induces demyelination which contributes to Schwann cell proliferation and increases the number of non-myelinating Schwann cells, the preferred niche for *M. leprae* [32,33]. However, a more complex scenario is emerging since recent observations have demonstrated that *M. leprae* is able to promote apoptosis of ST88-14 Schwann cells [26]. The activation of NF-κB in Schwann-neurons' co-culture and its involvement in peripheral damage induced by *M. leprae* should be addressed, since this transcription factor is required for peripheral myelin formation [34] and regulation of cell cycle and survival factors.

Mycobacterium leprae induces IκB-α degradation and p105 level reduction in Schwann cells

Since the degradation of IκB-α proteins is required for p65/p50 complex activation, we monitored the levels of this protein by Western blot analysis. The treatment of ST88-14 cells for 5 min with *M. leprae* caused a reduction of IκB-α levels, as shown in Fig. 3A. To confirm that the activation of p65/p50 heterodimer requires IκB degradation, we pre-treated cells with sodium salicylate for 30 min followed by incubation with *M. leprae*. Sodium salicylate is an anti-inflammatory drug that inhibits NF-κB activation by preventing the phosphorylation and consequent degradation of IκBs [35]. Our results showed that pre-treatment with salicylate inhibited the heterodimer p65/p50 activation by *M. leprae* in

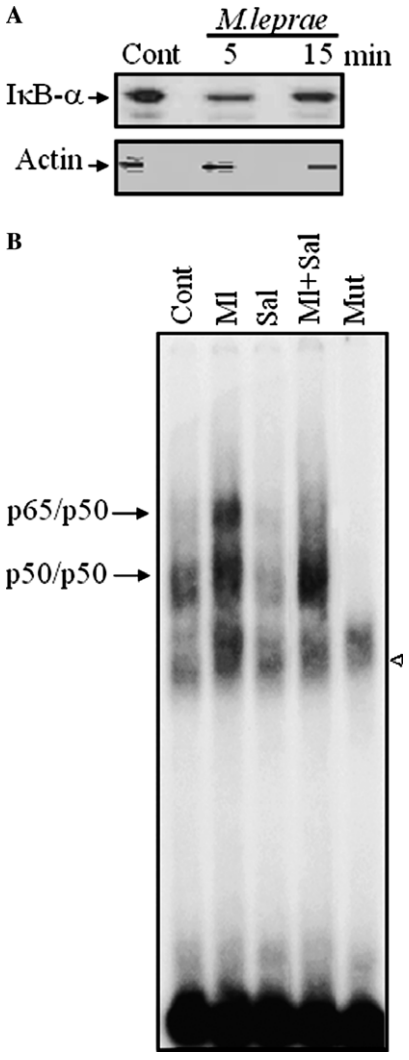

Fig. 3. p65/p50 NF-κB activation by *M. leprae* in Schwann cells depends on IκB-α degradation. (A) ST88-14 cells were either not treated (Cont) or treated with *M. leprae* at a MOI of 100:1 for the indicated times. Total protein extracts were prepared, analyzed by Western blot using antibody anti-IκB-α, and the membranes were reprobed with anti-actin, as indicated. (B) ST88-14 cells were either not treated (Cont), treated with *M. leprae* (MI) at a MOI of 100:1 for 30 min, treated with sodium salicylate (Sal) alone at 50 mM for 1 h, pre-treated with salicylate for 30 min, and then treated with *M. leprae*. Nuclear extracts were analyzed by EMSA. A mutant NF-κB oligonucleotide (Mut) was used for the determination of unspecific bands. The specific NF-κB bands are indicated by arrow and unspecific bands are indicated by open triangles.

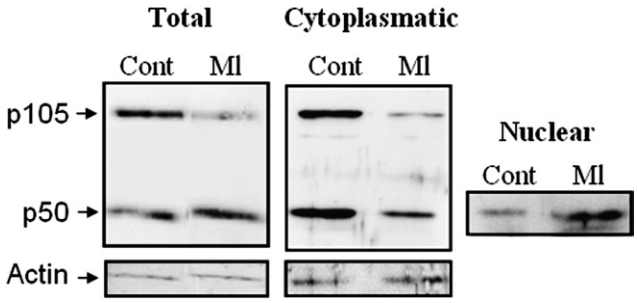

Fig. 4. *Mycobacterium leprae* induces reduction of p105 levels in Schwann cells. ST88-14 cells were either not treated (Cont) or treated with *M. leprae* at a MOI of 100:1 for 30 min. Total, cytoplasmatic or nuclear protein extracts were analyzed by Western blot using anti-p50/105 antibody and the membranes were reprobed with anti-actin, as indicated.

ST88-14 but not the activation of p50 homodimer (Fig. 3B).

The activation of p50 homodimer can be coupled to p105 processing or degradation. Consistent with this notion, *M. leprae* infection of ST88-14 cells led to a reduction of p105 and an increase in p50 levels, as observed in total protein extracts (Fig. 4). The reduction of p105 levels is more evident than the increase in p50 levels. However, we could not discriminate between processing and degradation of p105 precursor. When the analysis was carried out with cytoplasmatic extracts of cells treated with *M. leprae*, we observed a reduction of both p105 and p50, probably due to nuclear translocation of the generated p50 subunits (Figs. 1A and 2A). In fact, we observed increased levels of p50 in nuclear extracts of *M. leprae* treated cells (Fig. 4).

Thalidomide inhibits p50/p50 activation and rescues Schwann cells from NF-κB-dependent transcription repression induced by M. leprae

Thalidomide is a drug used in the treatment of ENL in leprosy. As this drug is able to inhibit NF-κB activation through suppression of IKK activity, we sought to investigate its effect on *M. leprae* induced activation of this transcriptional factor in ST88-14 cells. Cells were treated simultaneously with thalidomide and *M. leprae*, and then nuclear extracts were analyzed by EMSA. Fig. 5a shows that thalidomide treatment leads to a strong inhibition of p50/p50 complexes induced by *M. leprae*. In addition, thalidomide was able to rescue Schwann cells from NF-κB-dependent transcription repression induced by *M. leprae*, enhancing transcriptional activity ($p < 0.05$), as observed in lucifer-

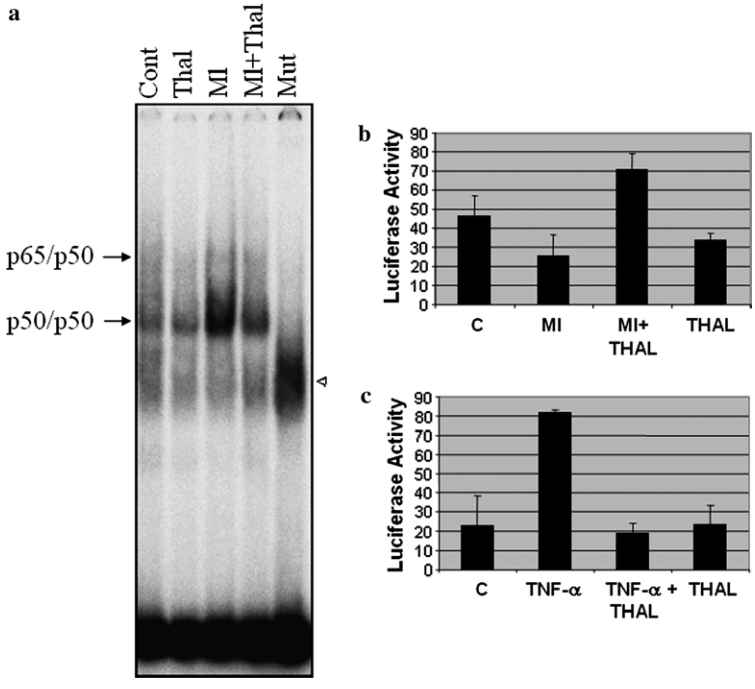

Fig. 5. Effect of thalidomide on NF-κB-dependent transcription repression induced by *M. leprae* in Schwann cells. ST88-14 cells were either not treated (Cont), treated with thalidomide (Thal) alone at 25 μg/ml, *M. leprae* (MI) at a multiplicity of 100:1 for 30 min, or thalidomide plus *M. leprae*. Nuclear extracts were analyzed by EMSA. A mutant NF-κB oligonucleotide was used for the determination of unspecific bands (Mut). Specific NF-κB bands are indicated by arrow and unspecific bands are indicated by open triangles (a). ST88-14 cells were transiently transfected using a reporter plasmid containing six NF-κB consensus-binding sites upstream of luciferase reporter gene. Twenty-four hours post-transfection, the cells were either not treated (C), treated with *M. leprae* (MI) alone at 100:1, with thalidomide (THAL) alone at 25 μg/ml or *M. leprae* plus thalidomide (b) or cells were not treated (C), treated with 25 ng/ml TNF-α, with thalidomide (THAL) alone at 25 μg/ml or TNF-α plus thalidomide (c) for 24 h. Whole cell lysates were prepared and analyzed for luciferase activity.

ase assay (Fig. 5b). However, this drug represses NF-κB-dependent transcription activation induced by TNF-α in Schwann cells ($p < 0.05$) (Fig. 5c), as previously demonstrated in other models [19,36]. Thus, although thalidomide inhibits NF-κB-driven transcription in different models, our data demonstrated that this drug can repress p50/p50 activation leading to NF-κB-dependent transcriptional activation.

Our results suggest that thalidomide acts in leprosy not only modulating the activity of immune cells, as previously described, but also the response of peripheral nerves' Schwann cells to *M. leprae* infection.

In summary, we have shown that two NF-κB complexes, p65/p50 and p50/p50, are activated by *M. leprae* in the human Schwann cell line ST-8814, but *M. leprae* induces repression of the basal and TNF-α induced NF-κB-dependent transcription, probably due to the transcriptional inhibitory complex p50/p50. We also showed that this NF-κB homodimer activation can be inhibited by thalidomide. These findings have important implications to elucidate how Schwann cell signaling can be modulated by *M. leprae*, which can contribute to the understanding of nerve pathogenesis in leprosy.

Acknowledgments

We thank Dr. José Augusto A. Pereira and Dr. Maria Bellio for critically reviewing the manuscript. We also thank Renato Ferreira and Paulo Cordeiro for technical assistance. This work was supported by FAPERJ, Fogarty Foundation, and Instituto Milênio. Renata M.S. Pereira is the recipient of CNPq (Brazilian National Research Council) fellowship for M.Sc. program.

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