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A Novel Form of Macrophage Function Inhibition by *Leishmania mexicana* Infection: Involvement in Inhibition of NF-kB Translocation

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Abstract: ***Manipulation of host cell signalling pathways and pro-inflammatory proteins expression by Leishmania is critical for Leishmania's survival and resultant pathology. Therefore, the characteristics of long time exposure of promastigotes mediated signalling to the MAP kinases, COX-2, iNOS, NO production and NF-κB pathways were investigated in comparison to LPS stimulation. Promastigotes caused no effect on phosphorylation of the three MAP kinases. Unlike outcome was observed on COX-2, iNOS and NO production using the same manner. Consequently, expressions of these proteins were also NF-κB dependent. Surprisingly, promastigotes inhibit macrophage NF-κB translocation into nucleus following LPS stimulation and caused a reversal of promastigotes mediated inhibition of macrophage COX-2, iNOS and NO production. These data demonstrated for the first time the role of NF-κB translocation in mediating the effect of L. mexicana promastigotes on pro-inflammatory proteins which these proteins play a crucial role in innate immunity against Leishmania infection.***

Keywords: *Leishmania Mexicana,* Promastigotes, NF-κB, COX-2, iNOS, NO

1. Introduction

Leishmaniasis is caused by protozoa of the genus *Leishmania*. It is transmitted by the bite of the infected female Phlebotomine sandfly in the old world and Lutzomyia in the new world. The sandfly vector is usually infected with one species of flagellate protozoa belonging to the genus *Leishmania*. Hosts are infected humans, animals such as rodents, and domestic animals such as dogs [1]. All *Leishmania* species have two main developmental stages in their life cycle: the amastigote, which reside inside the reticuloendothelial cell of the vertebrate hosts, and the promastigote, that replicate in the gut of sandfly. The life cycle begins when a vertebrate host is bitten by the infected sandfly [2, 3].

Leishmaniasis is an excellent example of a complex parasite-host interaction. *Leishmania* promastigotes bind to a number of surface molecules including complement receptors 1 and 3 (CR1& CR3) as well as to C3b of macrophages before they are internalized [4]. The effector function of macrophages in *Leishmania* infection and immune evasion mechanism is an important relation against this disease. Macrophages are the primary phagocyte that play host to *Leishmania* [5]. Activation of macrophages is a primary mechanism to eliminate the *Leishmania* parasite, presumably mediated by toxic metabolites of oxygen, which may include super oxide anion, hydrogen peroxide (H_2O_2) and nitric oxide (NO) [6]. A variety of stimuli can induce the morphological, biochemical and functional changes characteristic of activated macrophages. Therefore, activated macrophages produce different cytokines such as TNFα [7], IL-12 [8] and IFN-γ [9].

Studies of Leishmania's host subversion mechanisms may thus also provide important insight into the pathways by which macrophage function can be regulated under diverse physiological and pathological conditions. Macrophage infection typically leads to the induction of numerous cellular genes, several of which encode cytokines capable of stimulating both pathogen resistance and an inflammatory response. It seems that, to escape the host immune defenses, *Leishmania* developed sophisticated mechanisms, ultimately leading to the evasion and repression of such normal macrophage functions as inhibiting production of lethal antimicrobial agents like nitric oxide (NO) [10, 11]. Moreover, NO production is dependent on L-arginine and catalyzed by inducible nitric oxide synthase (iNOS) [12]. Similarly, NO synthesis has been shown to correlate with the killing of *Leishmania* parasites both in vitro and in vivo [13, 14] whereas mice deficient in iNOS fail to control *Leishmania major* infections in vivo [15]. In addition, after stimulation with LPS or some cytokines, many cell types, including endothelial cells and macrophages, express the inducible isoform COX-2 which is responsible for the production of large amounts of proinfammatory prostaglandins [16].

NF-κB is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types [17]. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are non-covalently associated with cytosolic inhibitory proteins, including IκB-α. Upon stimulation by a variety of pathogenic inducers like viruses, mitogens, bacteria, double-stranded RNA, agents providing oxidative stress and inflammatory cytokines, the NF-κB complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes [18].

Despite the wealth of information derived from studies on COX-2 and iNOS promoter gene, the potential regulatory role of NF-κB in COX-2, iNOS expression and nitric oxide production in inflammatory cells has not been fully investigated. Therefore, I have studied the effect of NF-κB translocation on COX-2, iNOS protein expression and NO production in LPS-stimulated bone marrow derived macrophages with promastigote of *Leishmania mexicana* infection.

These results show for the first time that NF-κB is involved in the expression of inflammatory proteins and their mediators in LPS-stimulated BMD macrophages and suggest that prevention of NF-κB translocation may represent a useful tool for the pharmacological control of inflammation.

2. Materials and Methods

2.1 Medium and reagents

All cell culture reagents were purchased from Invitrogen (Paisley, UK) and Cambrex BioScience (Veniers, Belgium). TC100 insect medium and LPS (from *Salmonella abortus*) were from Sigma (Poole, UK). MAP kinase and NF- kB (p65 iso-form) antibodies were from Santa Cruz Biotechnology (CA, USA) and rabbit polyclonal anti-iNOS and anti COX-2 antibodies from Cayman Chemicals (Michigan, USA).

2.2 Parasites

L. mexicana (MNYC/BZ/62/M379) promastigotes were cultured in 25 cm3 culture flasks (IBS) in TC100 insect medium supplemented with 10 % (v/v) FCS. The promastigotes were incubated at 26 ◦C for seven days, until the metacyclic stage was achieved.

2.3. Isolation of macrophages from bone marrow and infection

Bone marrow cells were obtained by flushing the femurs of C57BL/6 mice. Cells were cultured in DMEM, containing 10% (v/v) heat-inactivated FCS 30% (v/v) and L cell-conditioned medium. Once cells had become confluent after approximately 8–10 days, they were harvested by scraping into 5 ml of cold, sterile RPMI 1640 medium. The cell suspension was then washed three times; cells were diluted to the appropriate cell number using complete RPMI media and seeded in plates. These were then incubated at 33 ◦C/5% CO2 overnight to allow the cells to adhere to the plate. Cells were then stimulated, in a final volume of 500µl, with LPS, and with *L. mexicana* stationary phase metacyclic promastigotes a ratio of 5:1, parasite: macrophage was used except where indicated.

2.4. Western blotting

Cells were exposed to vehicle or appropriate agonists for the relevant period of time. They were then washed twice with ice cold PBS before adding 200 µl of pre-heated Laemmli's sample buffer. The cells were then harvested with a rubber policeman and the chromosomal DNA sheared by repeatedly passing through syringe with a 21 gauge needle in sterile Eppendorf tubes. The tubes were boiled for 5 min to denature proteins and samples were stored at -20 ◦C until use. Proteins were separated on a 10% (for detection of MAPKs), 8.5% (for detection of COX-2) or 7.5% (for detection of iNOS) SDS-PAGE gel. The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by electrophoretic blotting following a standard protocol [19]. Proteins were identified as outlined previously using specific antibodies [20].

2.5. Measurement of NO production

The supernatant of cell culture medium was collected for NO analysis. Quantification of NO production, by measuring nitrite (a stable metabolite of NO) levels, was as previously described (Tsai et al., 1999). To 50 µl of well supernatant, 50µl of Griess reagent (equal volumes of 2% (w/v) sulphanilamide in 5% (v/v) H_3PO_4 and 0.2% (w/v) naphylethylenediamine HCl in water) was added. After incubation for 10 min at room temperature in darkness, absorbance was read at 540 nm on a Spectromax 190 plate reader. Nitrite production was determined by comparison to a standard curve generated using known concentrations of $NaNO_2$.

2.6. Immunofluorescent staining

BMD-macrophages were cultured to ($1x10^6$/ml) in 24-well plates on sterile round glass coverslips in diameter 13 mm, thickness No.0 with complete RPMI 1640. Macrophages were exposed to inducers. To further detect the effect of *Leishmania* infections on NF-κB translocation, cells were pre-incubated with promastigotes expresses the m-Cherry tag and then cover-slips were washed two times in ice cold PBS (pH=7.4), prior to fixation for 10 minutes with 500µL/well of ice-cold methanol. After fixation, cells were washed again three times in cold PBS, and then cover-slips were incubated with permeabilisation buffer [(0.25%) Triton x-100 in PBS (pH=7.4)] 500µL/well for 10 min. Block buffer [1% BSA made up to 50ml in (0.25%) Triton x-100 in PBS (pH=7.4)] for 30 minutes to block unspecific binding of the antibodies. Cells were incubated in 25µL for each cover-slip with primary antibody [1:50 for polyclonal antibody in (0.25%) Triton x-100 in PBS (pH=7.4)] in a humidified chamber for overnight at 4°C. Then cells were incubated with 25µL for each cover-slip with secondary fluorescent antibody in different species such as Texas Red- conjugated or FITC-conjugated diluted 1:100 in [(0.25%) Triton X-100 in PBS (pH=7.4)] for 1 hr at room temperature in dark conditions. Cover-slips were incubated at room temperature with 4',6-diamidino-2-phenylindole DAPI (DNA stain) in volume of 500µL/well containing 100 ng/ml in PBS for 5 min in dark as well. The cover-slips then mounted on to glass microscope slides with 15 µL Mowiol (Fluka, sigma-aldrich company, Poole, UK) and visualisation by Nikon TE300-E upright epifluorescence microscope (Nikon, Kingston upon thames, UK). Cells were imaged at x100 or x40 magnification with an oil-immersion Plan Fluor objective lens. MetaMorph Imaging Series 7.0 (Molecular Devices Corp., Downington, PA, USA) was used for control of image acquisition, processing and modification of all image data. The background average statistical correction editing function in MetaMorph was used to produce background corrected images.

2.7 Statistical analysis

Densitometry data generated from immunoblots was expressed as mean ± SEM for at least 3 separate experiments. The statistical significance of differences between mean values from control and treated groups were determined by the one-way analysis of variance (ANOVA) using GraphPad Prism® Version 4.0 software or one tailed Student's Unpaired t-test. $p < 0.05$ was accepted as significant.

3. Results

3.1. *Regulation of MAP kinase signalling pathways following long time pre-treatment of Leishmania mexicana promastigote infection*

The characteristics of long time exposure of promastigotes mediated signalling to the MAP kinases pathways were investigated in comparison to LPS stimulation in the above primary cells. Having previously established a positive effect of short time pre-treatment of *L. mexicana* promastigotes mediated activation of MAP kinase signalling pathways (Shweash et al., 2011) it was necessary to further investigation of long time pre-treatment prior to stimulation with LPS in a stable manner. Therefore, macrophage cells were grown to ($1x10^6$/ml) in 12 well plates for overnight, after which cells were infected with promastigotes ($5x10^6$/MΦ) prior for 24 hours. Cells culture was stimulated with LPS (1µg/ml) for 30 minutes. Following preparation of samples, relative phosphorylation of MAP kinases was assessed by Western blotting, with total protein being used to determine equal protein loading. Stimulation by LPS alone demonstrated a strong phosphorylation of JNK1/2 (86.57 ± 15.24). Cells which underwent pre-incubation with promastigotes for 24 hours demonstrated the same activation of JNK1/2 phosphorylation (83.10 ± 12.67), therefore suggesting long time pre-incubation with promastigotes showed no effect and it is by this strategy that it mediates phosphorylation of JNK to maintain the host cell survival and to be suitable for transferring to tissue stages (**Fig.1A**). Again, results in LPS stimulated cells showed a high level of ERK phosphorylation (107.01 ± 10.75). However, whilst, like LPS, cells which were pre-incubated with promastigotes displayed a maximum response at 30 minutes after LPS stimulation (112.37 ± 11.77), suggesting a strong combination of promastigotes in association with activation of ERK (**Fig.1B**). LPS stimulated cell results demonstrated a highly induction of p38 MAP kinase phosphorylation (48.92 ± 5.21). Cells which underwent pre-incubation with promastigotes displayed a similar activation of p38 MAP kinase phosphorylation (53.75 ± 7.15), again suggesting a strong combination of promastigotes in association with activation of p38 MAP kinase (**Fig. 1C**).

3.2. *Long time pre-treatment of Leishmania mexicana promastigote inhibit COX-2, iNOS and NO production*

Having established the kinetics of signalling for both the LPS and promastigotes pathways in the BMD-macrophages, the following studies investigated a possible synergistic immune response between these two activators might be different in cause of long time pre-infection with parasite. It was hypothesised that using long time to activate these pathways a possible response may be present, where the two activators cooperate to cause a decreased or increased inflammatory response as measured through the COX-2, iNOS and NO production level.

Figure 2.A illustrates the effect of pre-incubating for 24 with promastigotes ($5x10^6$/MΦ) prior to the activation on the expression of COX-2. LPS mediated COX-2 expression in BMD-macrophages was measured over a 6 hours time period using the same concentration of LPS (1µg/ml). Results demonstrated that in LPS stimulated cells, LPS induced COX-2 expression with a peak at 6 hours (153.03 ± 11.57). Surprisingly, in cells which had been pre-incubated with promastigotes for 24 hours prior to treatment with LPS, a huge difference was evident due to LPS mediated expression of COX-2 was significantly inhibited in an inverse infecting with promastigotes for longer time period (35.09 ± 10.75, ***$p<0.001$). Again, in cells which had been infected with promastigotes alone did not show COX-2 expression.

To probably confirm this unexpected result on COX-2 protein expression, additional investigation on another pro-inflammatory markers protein was tested in this section. In the similar fashion of COX-2 protein, iNOS expression was carried out. **Figure 2.B** illustrates the effect of pre-incubating for 24 with promastigotes ($5x10^6$/MΦ) prior to the activation on the expression of iNOS. LPS mediated iNOS expression in BMD-macrophages was measured over a 6 hours time period using the same concentration of LPS (1µg/ml). Results demonstrated that in LPS stimulated cells, LPS induced iNOS expression with a peak at 6 hours (128.41 ± 5.81). The same findings were apparent, in cells which had been pre-incubated with promastigotes for 24 hours prior to treatment with LPS, a huge difference was evident due to LPS mediated expression of iNOS was significantly inhibited in an inverse infecting with promastigotes for longer time period (18.69 ± 6.30, ***$p<0.001$). Again, in cells which had been infected with promastigotes alone did not show iNOS expression.

My findings gave that host iNOS protein activity is inhibited by pre-incubated with longer time during intracellular promastigotes, it was necessary to test whether this inhibition influences NO production in response to LPS or promastigote infection. **Figure 2.C** shows representative graphs from the supernatants of experiments have done for iNOS enzyme activity with longer time period for LPS stimulation. Where BMD-macrophages were stimulated with the same design for promastigotes 24 hours prior to LPS treatment and the nos2 activity was measured over 48 hrs by analysed of NO production was made according to the procedure of Greiss assays. Results showed over the 48 hours time period, no nitrite was detectable in supernatants from unstimulated (control) or promastigotes alone cells. In contrast, nitrite production is induced after 48 hours of treatment with 1µg/ml of LPS (control = 0.74 ± 0.02 and LPS = 53.02 ± 1.29), although by comparison to the cells which had been pre-incubated with promastigotes for 24 hours prior to

treatment with LPS, as expected the levels of nitrite activity induced was significantly less more (18.39 ± 0.66, ***p<0.001).

3.3. *Inhibition of macrophages COX-2, iNOS and NO production by Leishmania mexicana promastigote is a result of prevention of NF-κB translocation into the nucleus*

I next examined whether NF-κB activity was involved in pathway which may mediate the action of promastigotes upon macrophage function expression of COX-2, iNOS and NO production (Fig. 3). In an attempt to induce NF-κB translocation into the nucleus, BMD-macrophages were treated with inflammatory stimuli, lipopolysaccharide (LPS). It was hypothesised that as these cells had been pre-treatment with promastigotes in culture they had possibly lost the NF-κB translocation stimuli which had previously kept these cells NF-κB activity in a limited state. For this purpose of experiments, cells were infected with promastigotes ($2.5x10^6$/ml, MOI= 5:1) which has been expressed mCherry protein tag for 24 hrs time points prior to stimulation with LPS (1µg/ml) for a time period of 0-60 minutes. **Figure 3 (1-8)** illustrates cells which have been stimulated with LPS alone and stained for translocation localization, using the T-NF-κB antibody. As expected, unstimulated cells (control) unsuccessful to show any translocation of interesting protein. In contrast, in cells had been stimulated with LPS alone, which show intense intracellular staining of NF-κB translocation into the nucleus. The translocation activity of NF-κB started according to the majority of NF-κB staining appeared distinct inside the nucleus from 15 min and still displayed a maximum response translocation between 30 and 60 minutes respectively. Application of cells which underwent pre-incubation with promastigotes carried out by immunofluorescent staining to ensure uptake of the promastigotes mcherry tag into macrophage cells. However, the absence of a distinct fluorescent staining inside of infected cell nucleus indicates that there was no nuclear translocation of NF-κB in these cells.

3.4. *Inhibition of macrophages NF-κB translocation by Leishmania mexicana promastigote is also NF-κB phosphorelation dependent manner*

To further support the theory that promastigote is a mediator of inhibition of NF-κB activation and to determine if the inhibition of COX-2, iNOS were related with the inhibition of this transcription factor, it was necessary to estimate by which mechanism promastigote has ability to prevent NF-κB translocation. For this purpose, it was important to investigate the findings of cytosolic phosphorylation of the NF-κB. Although these cells appeared to take up the parasite far better than previous cells and some parasite localization was evident, the infection efficiency was high to use these cells to characterize the intracellular interaction between the parasite and protein. **Figure 4** illustrates cells which have been stimulated with LPS alone and stained for p65 phosphorylation, using the T-NF-κB antibody. As expected, unstimulated cells (control) unsuccessful to show any activation of interesting protein. In contrast, in cells had been stimulated with LPS alone, which show intense intracellular staining of NF-κB phosphorylation and translocation into the nucleus. The phosphorylation and translocation activity of NF-κB started according to the majority of NF-κB staining appeared distinct around cytosolic and inside the nucleus from 15 min and still displayed a maximum response translocation between 30 and 60 minutes respectively. Surprisingly, in cells which had been infected with promastigotes, however all of the staining was internally with parasites as the parasite engulfed the protein. Significantly distinct bodies of parasite throughout the cytosol and this began to diffuse inside the parasite body by 15 up to 60 minutes, but with a lack of nuclear translocation. Again, NF-κB staining appeared to be more evenly distributed throughout the cytosol, with no any chance to translocation to the nucleus.

4. Discussion

It is believed that time of infection in macrophages greatly affects the outcome of classical macrophage signalling pathways and inflammatory proteins activity. During and after *Leishmania* infection there are several cellular outcomes that signify macrophage activation and destruction of invading pathogens. The interaction of pathogens leads to the activation of a series of intracellular signal transduction pathways which play a crucial role in mediating protection against these pathogens. These cascades are essential for the eventual destruction of *Leishmania* via the production of macrophage IL-12 which in turn drives naïve T cells to become differentiated Th1 cells and to produce IFN-γ. Both amastigote and promastigote infection stages use the macrophage as a host however, infection results in the regulation of intracellular signalling pathways in different ways, resulting in diverse effects upon inflammatory responses [20]. Furthermore, whilst amastigotes and promastigotes regulate COX-2 and iNOS expression differentially, the same effect is observed on these proteins in different time of infection in the same stage of the parasite, in particular, MAP kinases, COX-2, iNOS, expression and NO production. Therefore, the intracellular regulatory mechanisms regulated by this stage of *Leishmania* are of great interest and considerable medical importance.

In the present work I show that there is no increase in activation of MAP Kinases pathway including JNK, ERK and p38 phosphorylation following with LPS stimulation. I identify no significant changes in activation of MAPKs through long time prior of infection with promastigotes as a mechanisms by which this is achieved. These data are compared by previous work of [20] showing that the MAPKs pathway were significantly activated and regulated with short time of infection.

In many inflammations NF-kB activation in mammalian cells occurs in response to inflammatory inducer like microbial and others [21]. The development of modifying host signal pathway is a frequent and commonly problem outcome of Leishmaniasis. Consequently, COX-2, iNOS and IL-12 are stimulated by LPS and NF-κB mediated this induction, the possible role of promastigotes in the inhibition of NF-κB function via the inhibition of NF-κB

translocation it was expected. In this study, I tested the effect of promastigotes on the NF-κB translocation of macrophages. First the successful translocation of NF-κB was characterised in macrophage cells with LPS stimulation. This was conducted in order to find a suitable method to analysis the effect of promastigotes on LPS-induced NF-κB translocation to continue modifying host signaling studies of the intracellular pathogens, either following NF-κB endogenously or infecting cells with a *L. mexicana* m-Cherry tag protein (red fluorescent protein).

For *L. mexicana* promastigotes the relative effects of this stage, or their virulence, have been used as a means of understanding classical and alternative activation in various studies *in vitro* and in animal models of the disease. In the experiments designed in this study, I characterised the time-dependent stimulation or inhibition of MAP kinase, induction of COX-2, iNOS and NO production in BMD-macrophages. In addition, I correlated these effects with the NF-kB translocation. Again I found that long-time pretreatment of promastigotes has no effect on JNK, ERK and p38 MAP kinase in response to LPS stimulation. In contrast, another recent study has demonstrated the ability of *Leishmania mexicana* promastigotes with short-time pretreatment enhanced these proteins above in response to LPS stimulation [20]. These and other studies, including the work in this study, indicate that the regulation of MAP kinase pathways is species and specific time -dependent. It is intriguing that over the course of *Leishmania* evolution different species and time have acquired such diverse mechanisms of regulation and would be useful to construct some sort of framework regarding effects on kinase signalling. However, given the large variation in approaches used by different time scale of infection make this difficult. However, some studies are in disagreement and differences in stage of *Leishmania* infection have been reported. For example, infection of macrophages with *L. mexicana* amastigotes was completely abolished JNK, ERK, MEK but not p38 [20, 22].

In contrast, this study revealed for the first time, the strong inhibition of the COX-2 and iNOS expression in cultured murine macrophages infected by *Leishmania mexicana* promastigotes of prior for long time following with LPS stimulation. Furthermore, it was also demonstrated that NO production, a marker of classical macrophage activation, was also significantly reduced by promastigotes. These three markers were linked to regulation of IL-12 production and were induced by the activation of TLR4, again a novel finding by [20]. The co-regulation of all three inflammatory markers deserves some consideration. Down-regulation of COX-2 and iNOS is associated with inactivation of the immune response and inflammation. Therefore, paradoxically promastigotes may be seen to play role in disrupt inflammation, a response designed to fight infection. To support my mechanistic *in vitro* analysis, I analyzed macrophage samples with promastigotes infection for COX-2 expression and found that approximately more than 80% showed decreased expression of COX-2. Similar as I had observed it with iNOS protein expression. Importantly, in all these samples, NO production was also downregulated. It is possible that this subset of samples express the low amount of responsible enzyme of iNOS similar as previously described by [23]. This will need further modification in future studies.

However, products of these enzymes can clearly have divergent effects. For example, PGE_2 whilst induced during inflammation can nevertheless dampen down inflammatory responses as well as inhibit IL-12 and clearly the promastigotes are able to balance these outcomes. In addition, another issue to consider is how COX-2 activity is decreased in interpreting with effect of PGE_2, from our knowledge the activities of professional macrophages and dendritic cells are modulated by PGE_2 in a range of ways. Some of these modulation is PGE_2 appears to have a stimulatory effect on DCs, inducing their activation and migration to lymphoid organs [24]. Therefore, promastigotes assumes to inhibit COX-2 an inhibitory role, inhibiting the maturation of APCs and their ability to present antigen. This response was COX-2 inhibition dependent as a competitive inhibition of PGE_2, these results suggest that PGE_2 has an essential role for regulating DCs cytokines production during the immune response [25].

The findings that the expression of iNOS and NO production in response to LPS were also markedly reduced by *L. mexicana* promastigotes on macrophage (Figure 2B and 2C) provided further evidence for the functional impairment of these cells. Nevertheless, the mechanisms of action of NO as a final effector molecule on intracellular forms of *Leishmania* unknown. The exact mechanism of inhibition of iNOS and NO production by *Leishmania* remains to be identified. One potential mechanism to consider is that *Leishmania*-derived molecules may either directly or indirectly modulate both mediators activity. Although the results of this study provide strong evidence for deactivation of intracelluar kinase by a cellular dephosphorylation in *Leishmania*-infected cells, it was also possible that a *Leishmania*-derived inhibited of iNOS with activity towards of abolish of NO production was responsible for some of the effects observed. This possibility seems highly likely and similar evidence between Nandan et al. and my own study, there are significant differences in methodology [26].

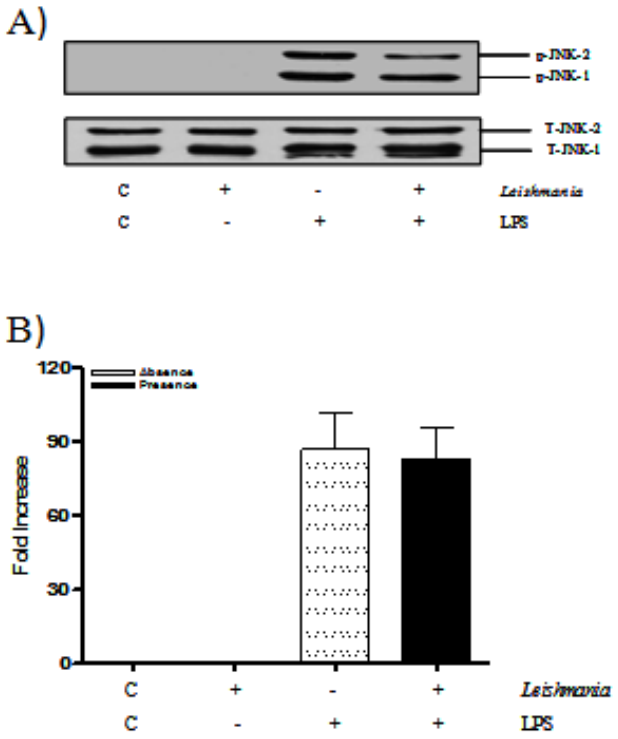
As indicated in the introduction, NF-κB has been extensively involved in inflammation. There is abundant evidence that abolishing NF-κB signalling reduce neutrophils invasion and decrease the expression of inflammatory genes [27, 28]. NF-κB is an essential mediator of several cellular functions. This is the first report to use the m-cheery *Leishmania* to investigate the effect of promastigote on NF-κB translocation. However, different pathogens have therefore developed many mechanisms for controlling its activity. For example, virus responsible of African swine fever encodes a dominant-negative IκB homologue which binds p65RelA and maintains it in the upstream side of cell cytoplasm [29]. E_7 protein of human papillomavirus prevents NF-κB activation by binding the IκB kinase (IKK) complex and inhibiting IKKα and IKKβ [30]. What is most convincing, however, is that my study also suggests that NF-κB is an

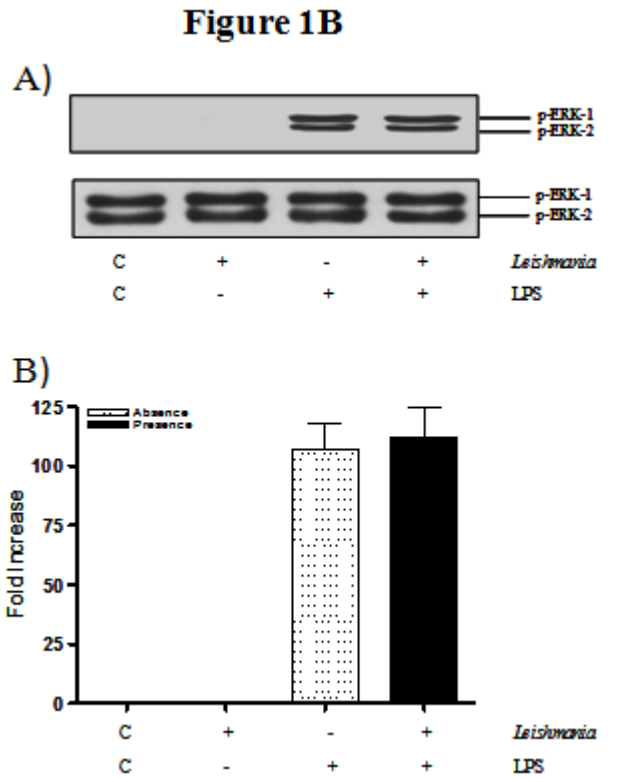
originator of inflammation through its direct activation and translocation into nucleus in the macrophages. Therefore, NF-κB functions as a trigger as well as an intermediate player in a sustained immune inflammatory cycle. Thereafter, several previous studies concentrated on the effect of different pathogens on NF-κB activity such as *Yersinia spp*. Which is bacteria showed inhibits NF-κB and IκB through the producing an ubiquitin hydrolase, that is injected into the cytoplasm of the macrophage [31]. Another study documents that cleavage of human p65RelA by directly secreting protease following with the infection of *Chlamydia trachomatis* [32]. In dissimilarity, the intracellular parasite *Theileria spp*. appears to activate NF-κB through directly interacting with the IKK complex [33]. Some researchers have suggested that *Toxoplasma gondii* induce phosphorylation of cellular IκB through IKK homologue at the membrane of parasitophorous vacuole [34, 35].

The exploitation of the *L. mexicana* model has revealed several features of the immune response responsible for either resistance or susceptibility. However, all mechanisms include a direct interaction between a pathogen molecule and components of the conventional NF-κB pathway, to either activate or inhibit translocation of NF-κB to the nucleus. In the present study, I have shown that *Leishmania* promastigotes modify NF-κB pathway by no effecting on cytosolic activation of p-p65 and IκB-α degradation (data not shown) but preventing translocation of NF-κB to the nucleus. This does not avoid the need for release of NF-κB from IκB, since promastigote infection prevents appearance of nuclear p65. Similar to these observations, other studies have shown that infection with *Leishmania* promastigotes has no effect on macrophage IκB-α [22, 36]. In contrast, a previous study by Calegari-Silva and his co-workers indicated that *L. amazonensis* promastigote infection activates the p50/p50 NF-κB complex and surprisingly *L. amazonensis* reduces the m RNA levels of the iNOS in addition to protein expression and the production of NO in LPS-stimulated macrophages [37]. Therefore, the situation during *Leishmania* infection is practically more related to that during stage and time of *Leishmania* infection. However, in that case the protease is virally encoded, whereas *Leishmania* promastigotes appear to service a host protease, without the need for direct interaction between the p65 and *Leishmania* molecules. Similarly, the infection with amastigotes of *L. mexicana* appears to be a direct effect of amastigote cysteine proteases on NF-κB component pathway [20, 22].

In summary, the results of this study show that *L. mexicana* promastigote prevents NF-kB translocation and inhibited the expression of both inflammatory proteins COX-2 and iNOS in macrophages. Attenuation of NF-κB translocation activity and inhibition of the expression of both iNOS and COX-2 appear to be accounted for by reducing the cellular defence activity. These findings suggest a possible mechanism for macrophage deactivation used by *Leishmania* and possibly by other intracellular pathogens. Therefore, I propose that *L. mexicana* employ a host signalling pathway to interfere transcriptionally with the expression of the COX-2 and iNOS genes. Almost certainly important for the establishment of macrophage infections, this process is part of an adaptive parasitic strategy of an important pathogenic New World *Leishmania* species. Hence, from this study it becomes seeming that the regulation of the intracellular MAP Kinases and NF-κB pathway in macrophage during *L. mexicana* infection might open up a route to enhance antiparasitic cellular responses and thereby restrict the progression of the disease.

Figure 1A





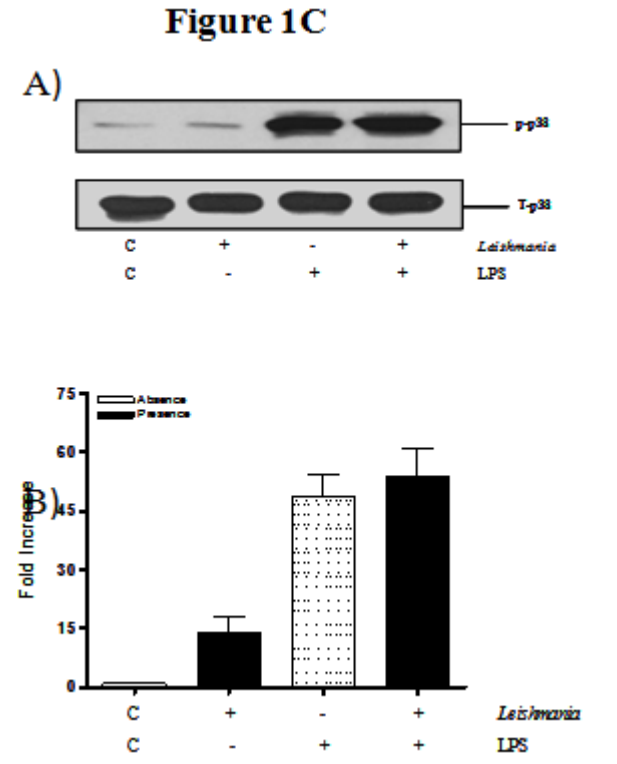
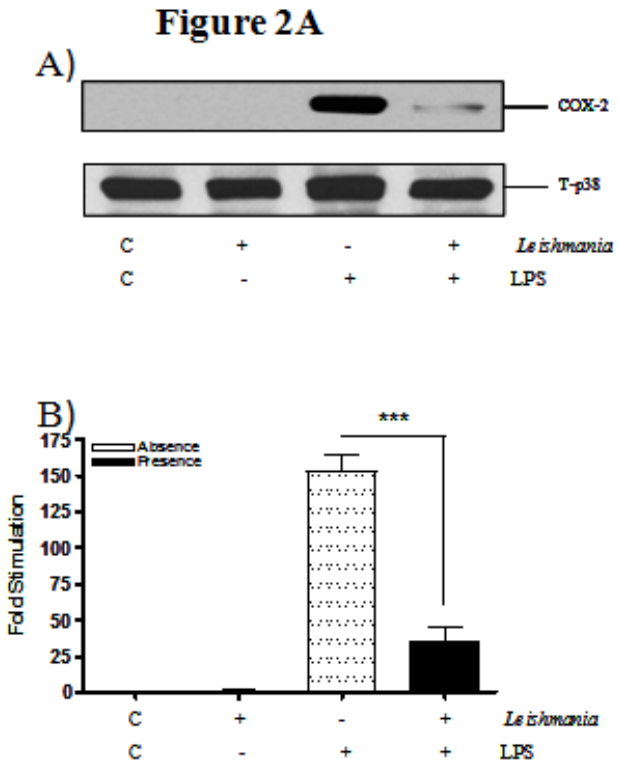
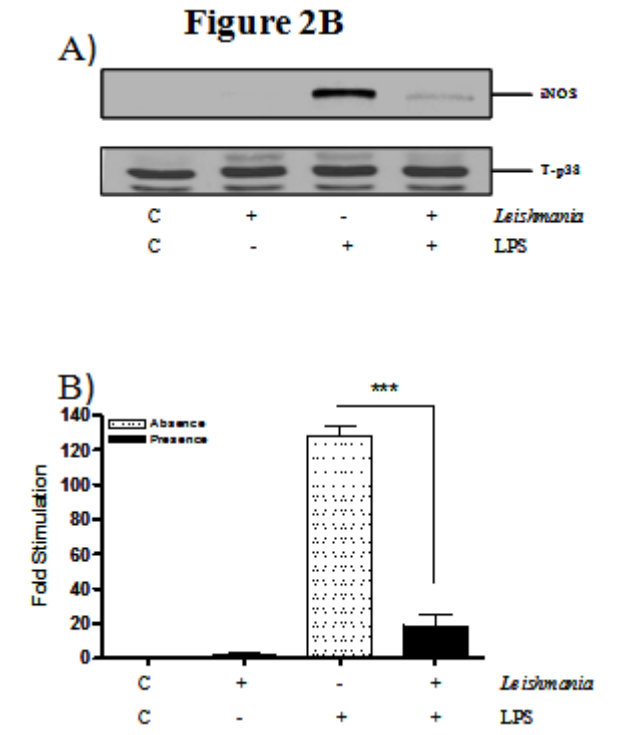


Figure 1A, 1B and 1C: Effect of long time pre-treatment of promastigotes on LPS mediated MAP Kinase phosphorylation in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* ($5x10^6$/MΦ) over a time period of 24 hrs prior to stimulation with (1µg/ml) of LPS for 30 minutes. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **Figure 1A:** (A) p-JNK (46/55 kDa) and JNK (46/55 kDa), **Figure 1B:** (A) p-ERK1/2 (42/44 kDa) and ERK (42/44 kDa) and **Figure 1C:** (A) p-p38 and p38 as outlined in Section 2.1.3. Blots were quantified for **(B)** fold stimulation by scanning densitometry; each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar finding.





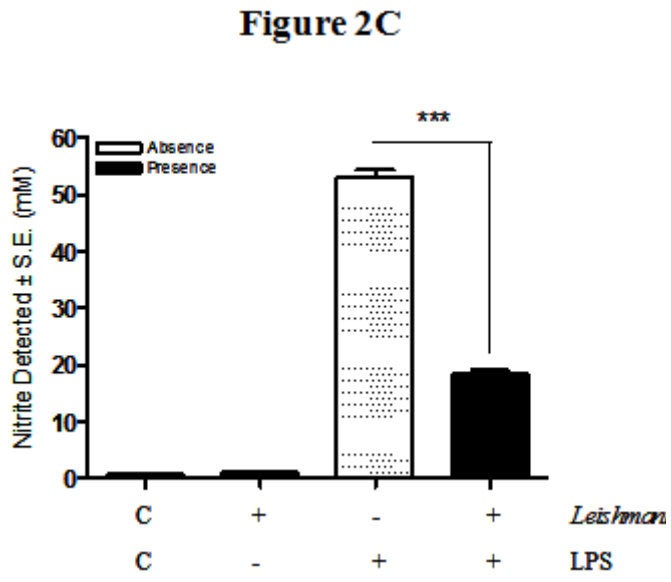
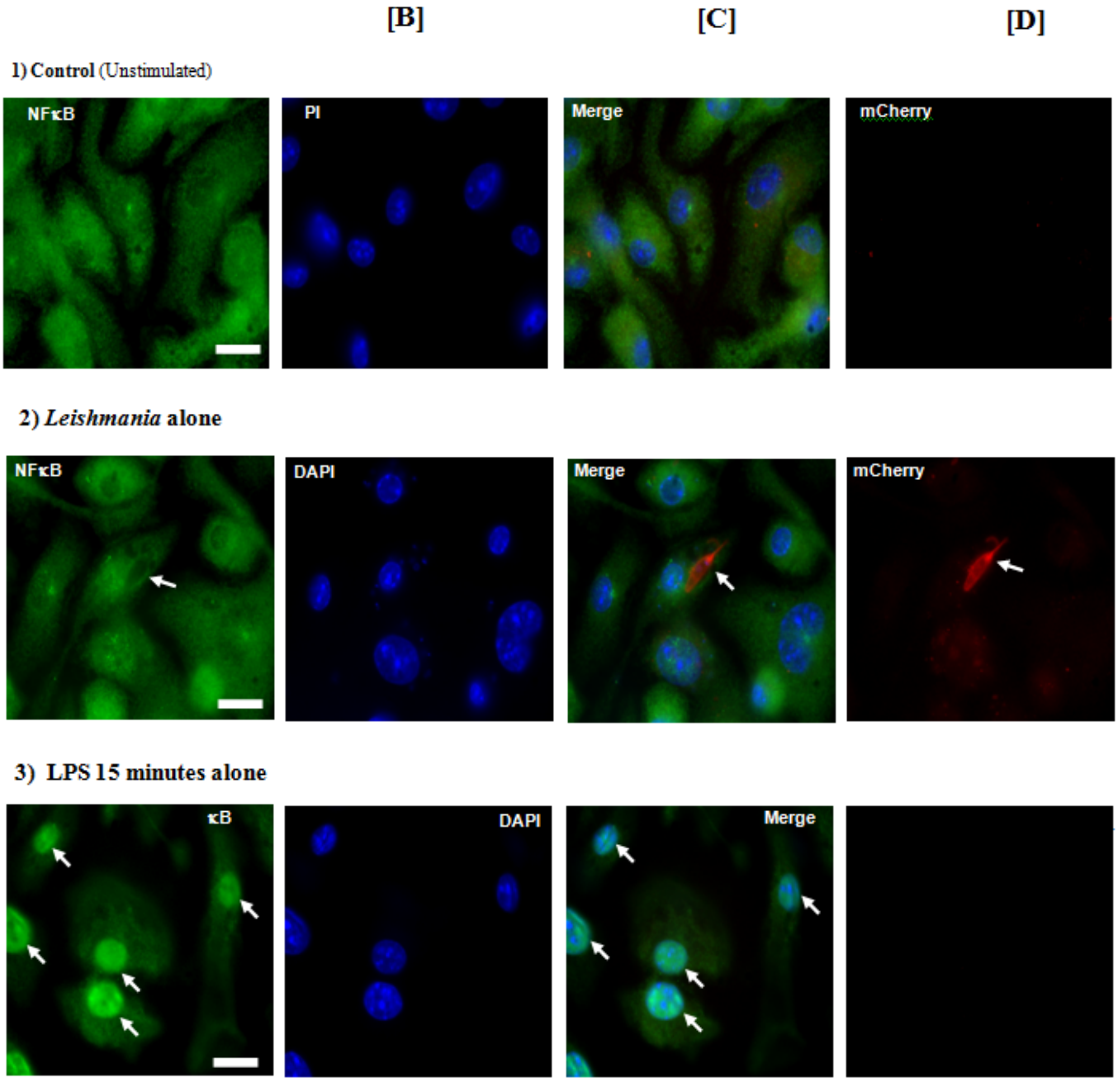
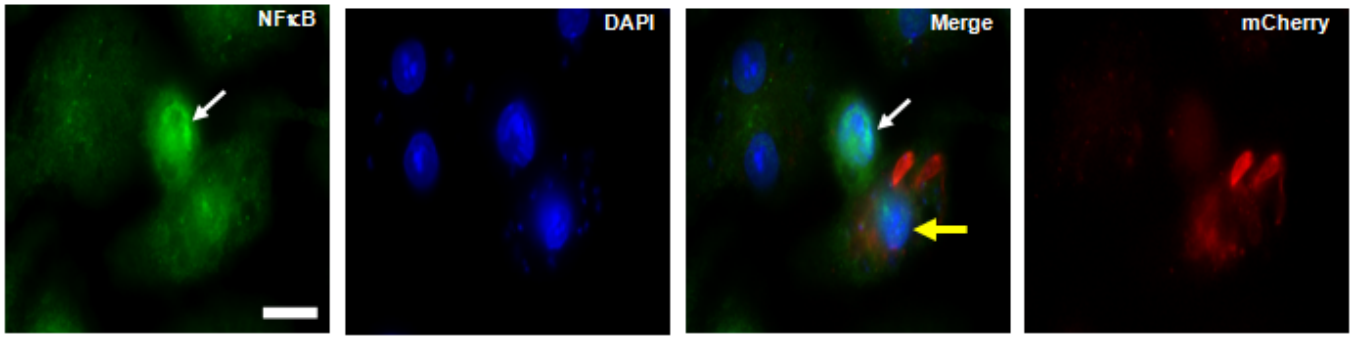


Figure 2: Effect of long time pre-treatment of promastigotes on LPS mediated COX-2, iNOS protein expression and NO production in macrophages. Cells ($1x10^6$/well) were infected with *Leishmania* ($5x10^6$/MΦ) over a time period of 24 hrs prior to stimulation with (1µg/ml) of LPS for 6 hours for COX-2 and iNOS**,** while 48 hrs for NO. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for **Figure 2A** (A) COX-2 (72 kDa) and T-p38 and **Figure 2B** (A) iNOS (130 kDa) and T-p38as outlined in Section 2. Blots were quantified for (B) fold stimulation by scanning densitometry. **Figure 2C**: supernatants were collected in fresh eppendorf tubes and subject to nitrite by Griess assay (as outlined in section 2). each value represents the mean ± S.E.M. The results are representative of 3 independent experiments with similar finding. ***p<0.001, compared with agonist-stimulated control.

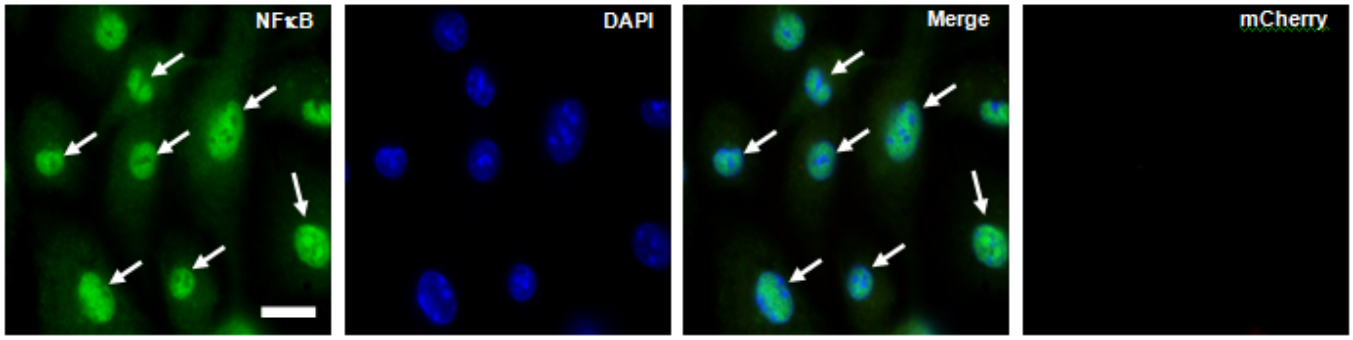


4) *Leishmania* + LPS 15 minutes

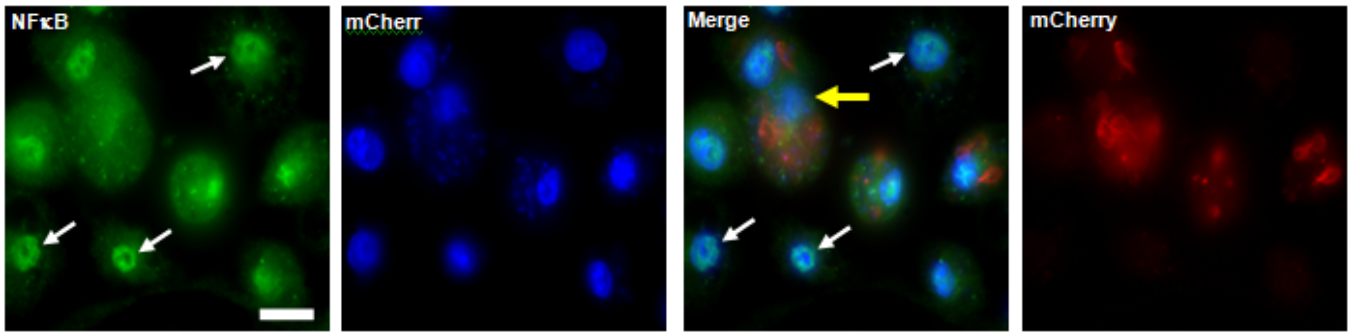


White Arrow Indicate Nuclear Translocation
Yellow Arrow Indicate Prevent Nuclear Translocation

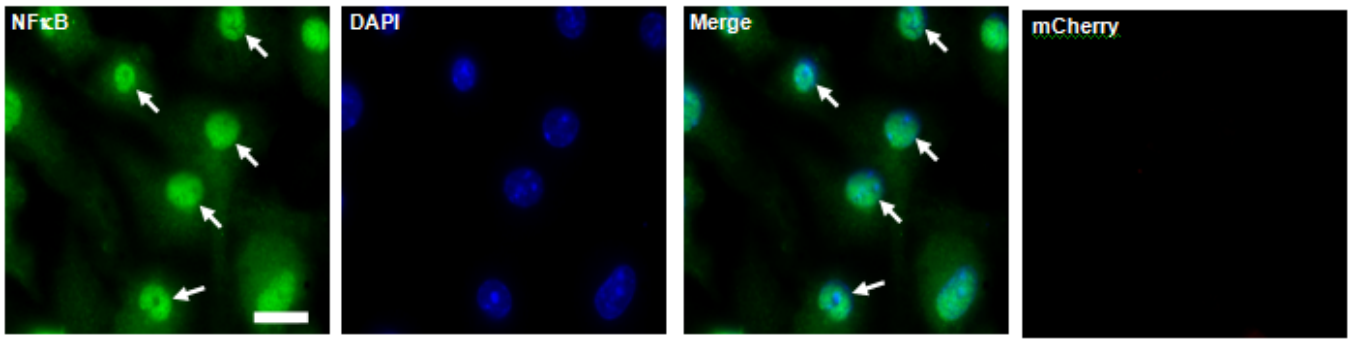
5) LPS 30 minutes alone



6) *Leishmania* + LPS 30 minutes



7) LPS 60 minutes alone



8) *Leishmania* + LPS 60 minutes

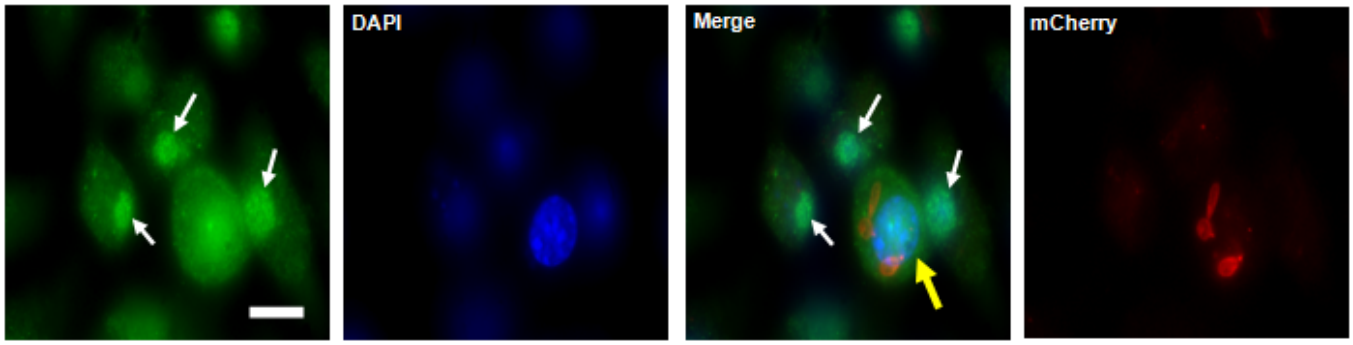
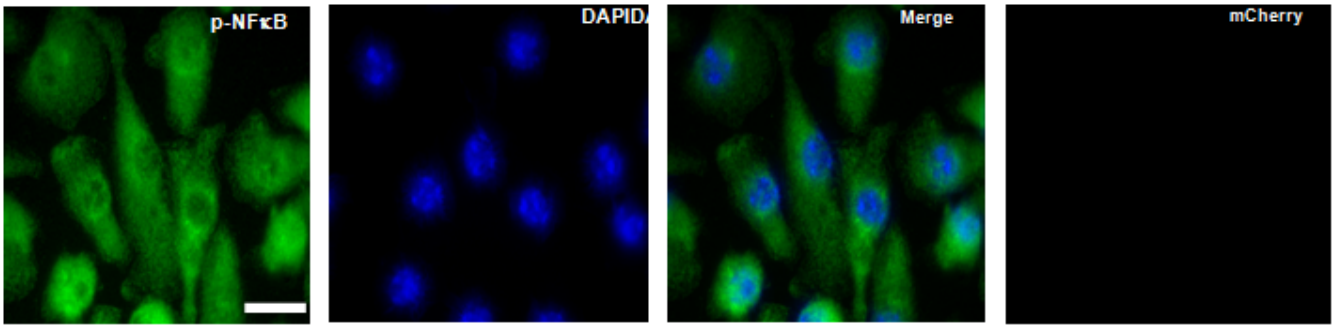
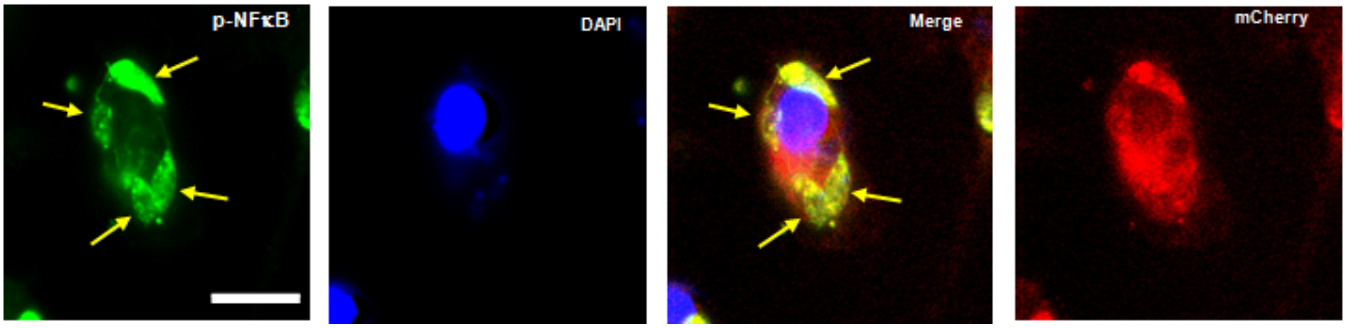


Figure 3: The effect of *Leishmania mexicana* promastigotes on LPS induced p65 (NF-κB) translocation into the nucleus. Macrophages were infected with pomastigotes expresses the mcherry protein ($5x10^6$/MΦ) for 24 hrs prior and then stimulated with LPS (1µg/ml) as indicated (1-8 above). Cells were fixed and stained as described in Section 2.1.4. **[A]** LPS induced p65 (NF-κB) translocation was measured by Epifluorescence microscopy using a 100x Plan Fluor oil objective lens. T-NF-κB localization was detected by indirect staining using a primary antibody specific for p-NF-κB and a secondary antibody conjugated with FITC. **[B]** Nuclei were visualized by DAPI staining, **[C]** Merged image demonstrating co-localisation of *Leishmania mexicana* promastigotes with T-p65 in nucleus localization and **[D]** m-cherry channel. The results are representative of 3 independent experiments (scale bar = 10 micron). Yellow arrows indicate prevent translocation of NF-κB while white arrows indicate nuclear translocation.

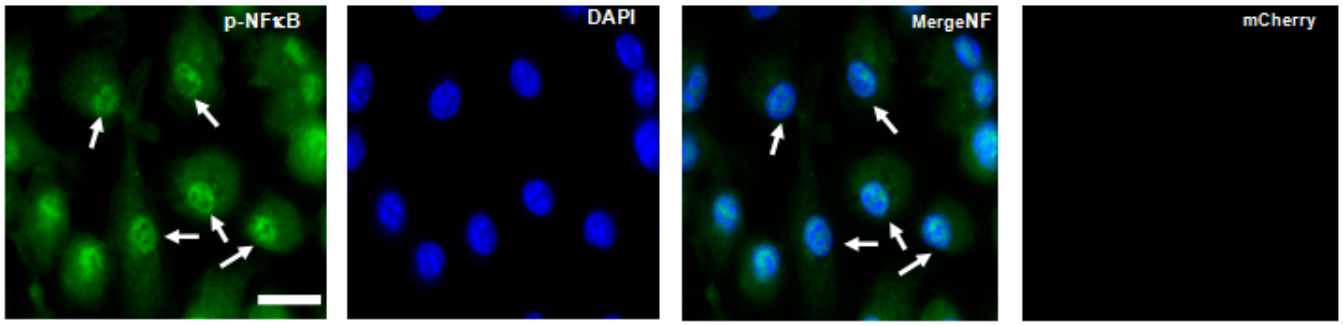
1) Control



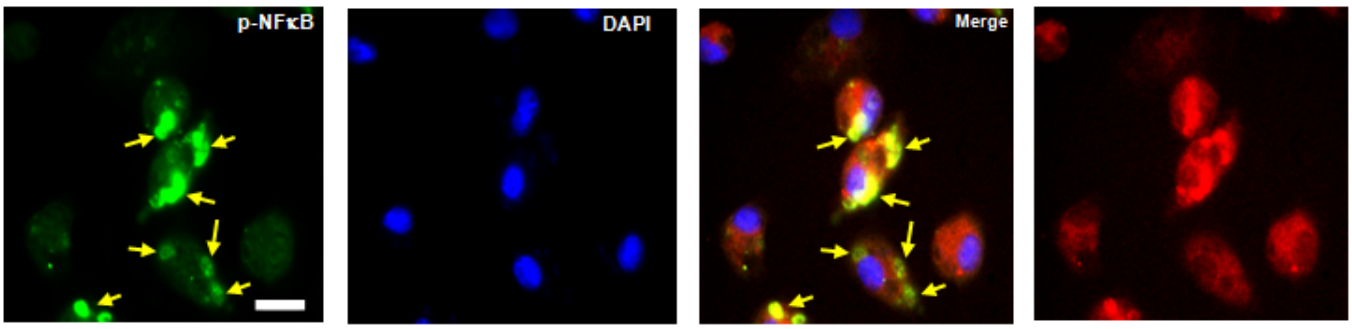
2) *Leishmania* alone



3) LPS 15 minutes alone

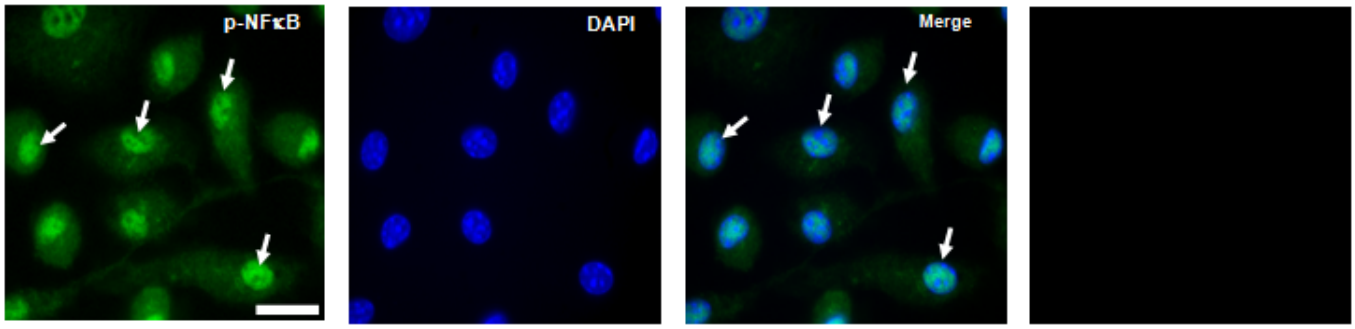


4) *Leishmania* + LPS 15 minutes

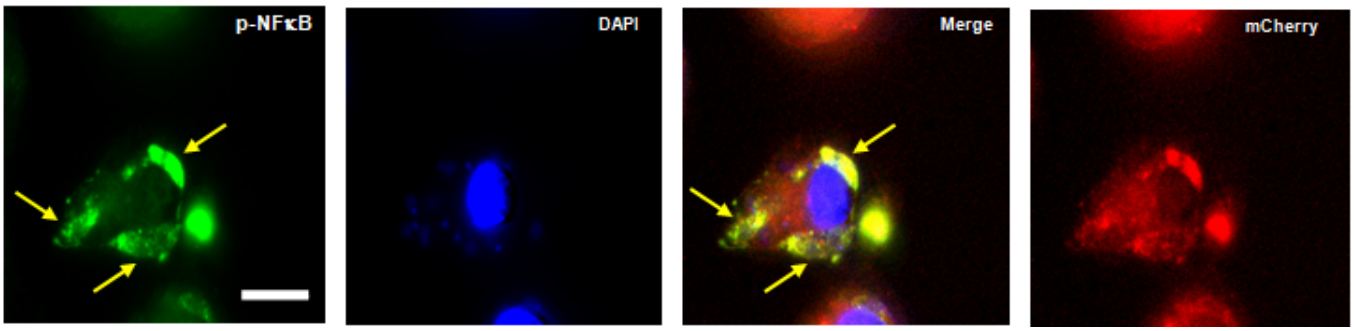


White Arrow Indicate Nuclear Translocation of NF-κB
Yellow Arrow Indicate Cytosolic phosphorylation of NF-κB

5) LPS 30 minutes alone



6) *Leishmania* + LPS 30 minutes



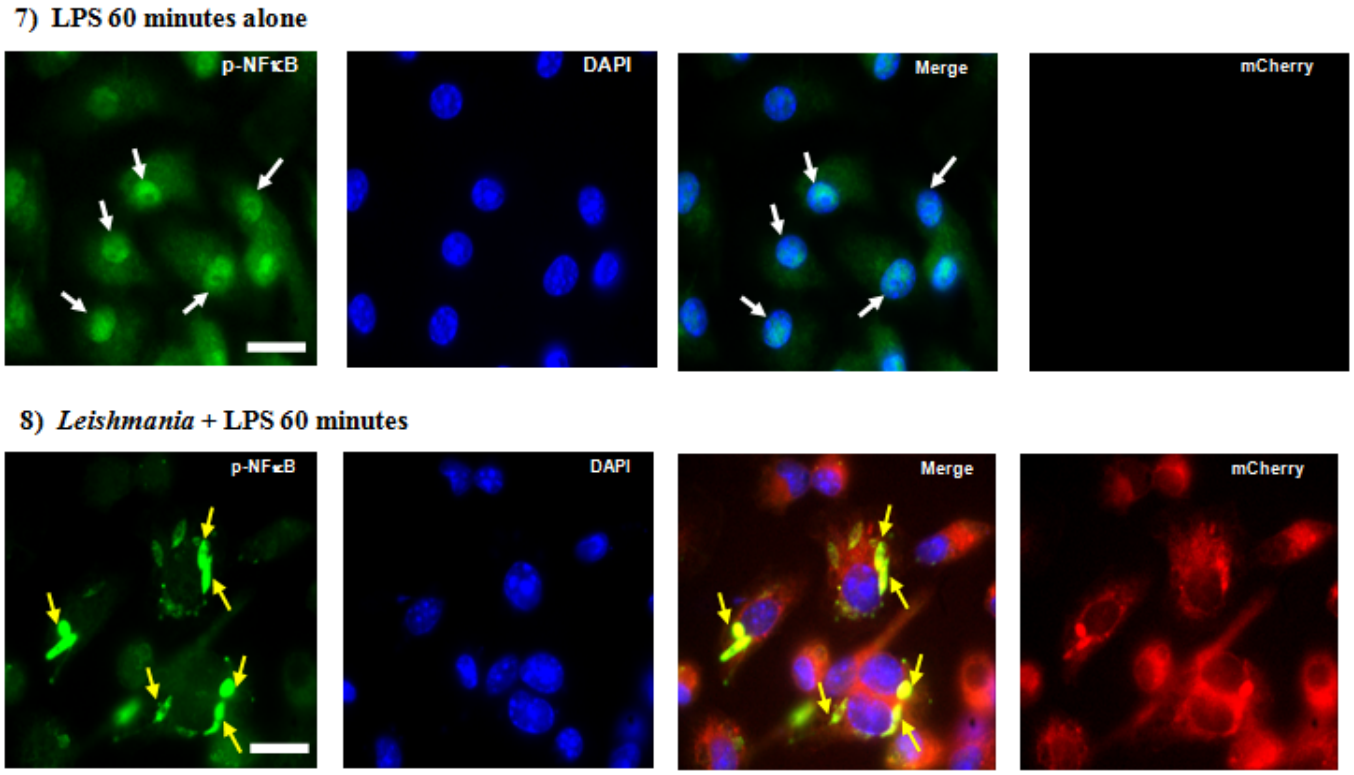


Figure 4: The effect of *Leishmania mexicana* promastigotes on LPS induced p65 (NF-κB) translocation into the nucleus. Macrophages were infected with pomastigotes expresses the mcherry protein ($5x10^6$/MΦ) for 2 hrs prior and then stimulated with LPS (1µg/ml) as indicated (1-8 above). Cells were fixed and stained as described in Section 2.1.4. **[A]** LPS induced p65 (NF-κB) phosphorylation and translocation was measured by Epifluorescence microscopy using a 100x Plan Fluor oil objective lens. p-NF-κB localization was detected by indirect staining using a primary antibody specific for p-NF-κB and a secondary antibody conjugated with FITC. **[B]** Nuclei were visualized by DAPI staining, **[C]** Merged image demonstrating co-localisation of *Leishmania mexicana* promastigotes with p-p65 in cytosolic localization and **[D]** m-cherry channel. The results are representative of 1 independent experiments (scale bar = 10 micron). Yellow arrows indicate cytosolic phosphorylation of NF-κB while white arrows indicate nuclear translocation.

5. Future Research Needs

The pathology and resolution of leishmaniasis is dependent to a large scope on the intracellular signalling pathway. Transduction factor pathways such as NF-κB do have a role in all cases either by initiating the development of a Th1 response or development of Th2 response in case of cutaneous leishmaniasis. The key cytokines in both cases are IL-12, IFN-γ, IL-10 and IL-4, the effects of which are mediated by specific transductions factor pathway are NF-κB and STATs. Therefore, IL-10 was demonstrated to play an essential role in the deactivation of macrophages function. However, the future work will requires further analysis on the specific role of STAT3 and STAT6 signalling in macrophages.

Acknowledgment

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