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Signaling events leading to the curative effect of cystatin on experimental visceral leishmaniasis: Involvement of ERK1/2, NF-κB and JAK/STAT pathways

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Curative effect of cystatin, a natural cystein protease inhibitor, on experimental visceral leishmaniasis was associated with strong upregulation of iNOS. The transductional mechanisms underlying this cellular response was investigated in the murine macrophage cell line RAW 264.7 and in the BALB/c mouse model of visceral leishmaniasis. Cystatin synergizes with IFN-γ in inducing ERK1/2 phosphorylation and NF-κB DNA-binding activity. Pretreatment of cells with specific inhibitors of NF-κB or ERK1/2 pathway blocked the cystatin plus IFN-γ-inducible NF-κB activity and markedly reduced the expression of iNOS at both mRNA and protein levels. Silencing of mitogen- and stress-activated protein kinase 1 significantly reduced cystatin-mediated NF-κB-dependent iNOS gene transcription suggesting the involvement of mitogen- and stress-activated protein kinase 1 activation in ERK1/2 signaling. DNA binding as well as silencing experiments revealed the requirement of IFN-γ-mediated JAK-STAT activation even though cystatin did not modulate this signaling cascade by itself. In the *in vivo* situation, key steps in the activation cascade of NF-κB, including nuclear translocation of NF-κB subunits, IκB phosphorylation and IκB kinase, are all remarkably enhanced in *Leishmania*-infected mice by cystatin. Understanding the molecular mechanisms through which cystatin modulates macrophage effector responses will contribute to better define its potential for macrophage-associated diseases, in general.

Key words: Cystatin · Leishmaniasis · Macrophage · Nitric oxide · Signal transduction

Introduction

The fatal disease of visceral leishmaniasis is caused by the protozoan parasite, *Leishmania donovani*, which resides and multiplies within the phagolysosomes of the host macrophages. In order to establish infection, *Leishmania* invariably develop mechanisms to neutralize the macrophage microbicidal machinery and the outcome of infection depends on the balance between the host's ability to activate macrophage killing and the parasite's ability to escape or evade this host immune response. Previous studies showed that the expression of iNOS and generation of NO in response to IFN-γ and TNF-α is important in the control of infection in different *Leishmania* species [1, 2]. The LPS and/or IFN-γ-dependent iNOS induction is mostly controlled by two regulatory regions present in the iNOS promoter, which contains binding sequences for two transcription factors, NF-κB and IFN regulatory factor 1 (IRF-1) [3]. The synergistic role of IFN-γ is due to its ability to induce the expression of STAT1 and IRF-1, transcriptional complexes that can also bind to the IFN-γ-activating sequence and IRF response element sequences, respectively, at the 5′-flanking region of the iNOS promoter. Of all the signaling pathways involved in the production of NO in response to IFN-γ, the JAK/STAT and NF-κB signaling pathways seem most important [4]. The NF-κB family includes five

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members of which p50, p65 (Rel A) and c-Rel have been detected in macrophages. Homo- and hetero-dimers can form between any of the Rel proteins, giving rise to protein complexes with different DNA-binding sites and activities [5]. Most of the dimers, of which p50–p65 is probably the most common, are retained in the cytoplasm complexed with its inhibitory subunit IκBα. Agonist stimulation promotes serine phosphorylation of IκBα, which triggers its proteasomal degradation and subsequent activation of NF-κB. MAPK signaling pathways have been identified as the upstream kinases that induce NF-κB activation through the phosphorylation of its inhibitor, IκBα [6]. The pathway mainly consists of three subfamilies, ERK, JNK and p38 MAPK. These kinases are activated by conserved upstream protein kinase signaling modules, MEK, which activate the MAPK by dual phosphorylation on threonine and tyrosine residues within a Thr-Xaa-Tyr motif located in the protein kinase subdomain VIII. ERK are activated predominantly by growth factors or phorbol esters [7], but activation by TNF or IL-1 has also been documented [8, 9]. Once activated, ERK phosphorylate a number of cellular substrates that can trigger diverse signal cascades, and accumulating evidences suggest a significant role of ERK in the regulation of inducible NO generation in macrophage *via* NF-κB-dependent pathways [10, 11]. *Leishmania*-induced macrophage deactivation may be linked to dephosphorylation of ERK1/2 by protein tyrosine phosphatases, which in turn might facilitate the parasites' infection and propagation within the cell [12, 13].

One promising strategy to develop antileishmanial drugs has been to target the parasite's cysteine proteases (CP) [14, 15]. CP of *Leishmania* have been shown to be important for growth, differentiation, replication and infectivity [15, 16], and play crucial roles in host–parasite interactions [15]. Knockout studies in *Leishmania* have established these enzymes not only as virulence factors but also as modulators of host immune responses [16]. The ability to genetically manipulate *Leishmania* has opened up new avenues for studying CP structure and function in the host–parasite interaction. Our previous work has demonstrated that cystatin, a natural CP inhibitor, can synergize with IFN-γ in inducing favorable cytokine responses and generation of NO for effective treatment of experimental visceral leishmaniasis [17], and this effect is unrelated to its inhibition of CP activity [18, 19]. One of the mechanisms that operates in such synergism may be attributed to the level of transcription of the target genes and in the present study we set out to explore the possible signaling mechanisms and the transcription factors involved in cystatin plus IFN-γ-mediated iNOS induction in macrophage culture as well as in animal model of visceral leishmaniasis.

Results

Therapeutic effects of cystatin through the upregulation of NO

Our earlier studies demonstrated that cystatin could upregulate NO in peritoneal macrophages along with IFN-γ (100 U/mL) in a concentration-dependent manner with an optimal effect at 0.5 μM. Although IFN-γ was required for cystatin to induce NO production in peritoneal macrophages (Fig. 1A), in an *in vivo* situation, IFN-γ was not a prerequisite. Thus, peritoneal macrophages isolated from BALB/c mice given i.v. injections of cystatin produced significantly higher levels of NO_2^- (15.02 ± 1.43 nmol/10^6 cells) than those from mice that did not receive cystatin (1.89 ± 0.21 nmol/10^6 cells) (Fig. 1B). Moreover, macrophages isolated from mice given combined regimen of cystatin plus IFN-γ produced much higher levels of NO_2^- (24.56 ± 2.62 nmol/10^6 cells) (Fig. 1B) compared with their *in vitro* counterparts (12.78 ± 1.18 nmol/10^6 cells) (Fig. 1A). The increase in NO production was paralleled by an increase in the antileishmanial activity of these cells (Fig. 1A and B). Almost complete reversal of NO stimulation as well as antileishmanial effect was observed upon addition of N^G-monomethyl-L-arginine (NMMA), a specific NOS inhibitor, suggesting the involvement of NO in cystatin-mediated antileishmanial activity. The *in vitro* as well as *ex vivo* effects of cystatin on NO production were also reflected in the iNOS mRNA expression analyzed by RT-PCR (Fig. 1C and D). Preincubation of cystatin with polymyxin B, an LPS inhibitor, did not alter NO production suggesting that the upregulation of NO by cystatin is not due to LPS contamination (Fig. 1A). In a mouse model of visceral leishmaniasis, the administration of cystatin at a dose of 20 mg/kg/day for 4 consecutive days beginning 15 days after infection could cause a substantial suppression of parasite burden ($\log_{10}$ Leishman–Donovan units (LDU) of 1.06 ± 0.13 compared with 2.53 ± 0.11 for the untreated controls for spleen and $\log_{10}$LDU of 1.20 ± 0.14 compared with 3.79 ± 0.18 for the untreated controls for liver, $p<0.001$). However, when a suboptimal dose of IFN-γ (5×10^5 U/kg/day) was coadministered with cystatin, a much more pronounced effect (complete suppression of spleen and liver parasite burden) was obtained at a much lower dose of cystatin of 5 mg/kg/day (Fig. 1E). A protective immunity for cystatin therapy was indicated by reinfection of animals after 45 days, which resulted in only a slight and transient increase in organ parasite burdens (Fig. 1F). The introduction of NMMA (50 mM), 1 wk after reinfection resulted in reversal of parasite suppressive effect suggesting the involvement of NO in cystatin-mediated antileishmanial activity. The infection was under control again after NMMA was withdrawn (Fig. 1F). To evaluate the type of immune response in infected mice after cystatin treatment, mRNA transcription levels for TNF-α, IFN-γ and IL-12 were determined on isolated spleen cells every 15 days after infection. There were very low levels of all the cytokines in infected mice, which were significantly increased after cystatin treatment (Fig. 1G and H). iNOS was also found to be upregulated in infected cystatin-treated cells at both mRNA and protein levels (Fig 1I and J). The role of Th1 cytokines in iNOS regulation was also assessed by applying various anticytokine antibodies along with cystatin. Anti-IFN-γ, anti-TNF-α, and anti-IL-12 greatly reduced cystatin-mediated splenocyte iNOS induction at both mRNA and protein levels (Fig 1I and J). Control antibodies had no effect suggesting the cooperation of other immune effector cells.

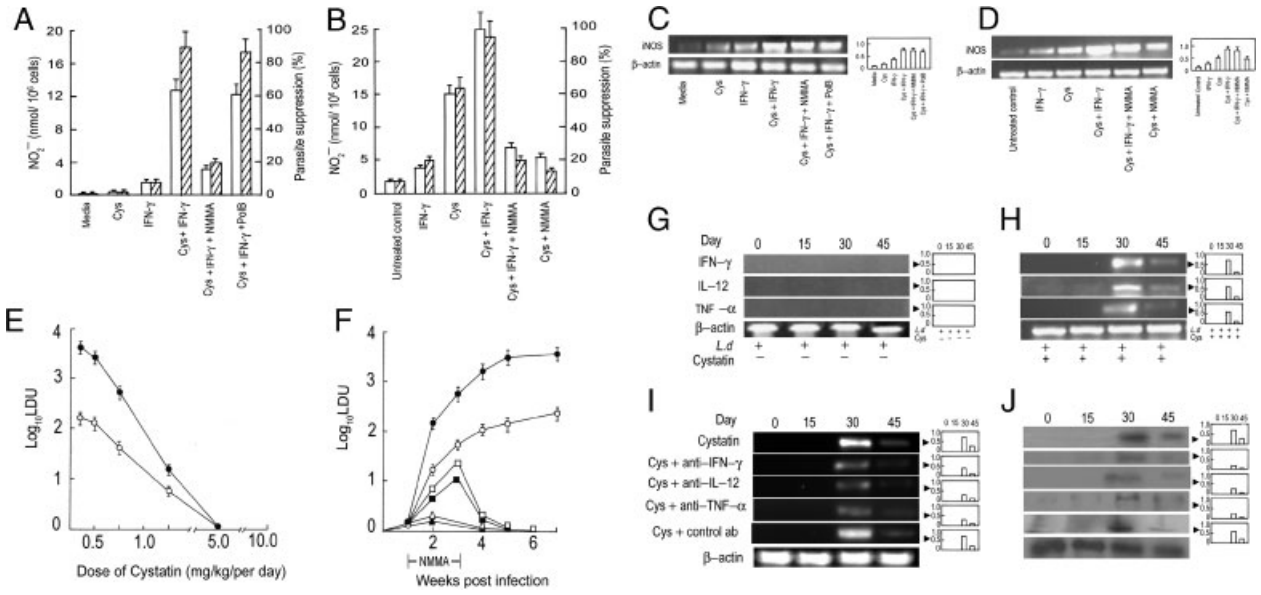

Figure 1. Antileishmanial activity of cystatin mediated by NO. NO_2^- production of (blank bar) and intracellular parasite suppression by (hatched bar) mouse peritoneal macrophages. (A) *L. donovani*-infected macrophages were incubated for 24 h with cystatin (0.5 μM), IFN-γ (100 U/mL), IFN-γ plus cystatin ± NMMA (2.5 μM) or polymyxin B preincubation (10 U/mL for 2 h). Intracellular parasite number was determined by Giemsa staining. (B) Peritoneal macrophages were isolated from mice treated with cystatin (5 mg/kg/day) or IFN-γ (5×10^5 U/kg/day) or both for 4 consecutive days, 10 h after the last injection and then infected with *L. donovani*. NMMA (5 mM) was coinjected with cystatin and was also present in the culture medium of macrophages. (C and D) iNOS expression from macrophages as described above was determined by RT-PCR of its mRNA transcript ($n = 4$). (E) *In vivo* antileishmanial activity of cystatin plus IFN-γ. Cystatin (0.1–10 mg/kg/day) along with IFN-γ (5×10^5 U/kg/day) was given i.v. daily for 4 consecutive days after 15 days of infection. The parasite burden in liver (●) and spleen (○) were then determined at 45 days after infection. (F) Cystatin plus IFN-γ-treated and completely cured mice were reinfected 45 days after primary infection (liver (△), spleen (▲)). As control, a set of naïve, age-matched mice was also infected (liver (●), spleen (○)). In one group of cured mice, NMMA (50 mM) was given in drinking water 1 wk after reinfection for 2 wk (liver (□), spleen (■)). Results are from three experiments and indicate the mean ± SD for five to seven mice at each time point. Time-course pattern of the mRNA expression of IFN-γ, IL-12 and TNF-α in the splenocytes of infected (G) and cystatin (5 mg/kg/day)-treated mice (H) ($n = 3$). iNOS expression by RT-PCR (I) and Western blot (J) in splenocytes of *L. donovani*-infected mice treated with cystatin (5 mg/kg/day) along with various anticytokine mAb and control mouse IgG (200 μg/mouse) ($n = 3$). β-actin was used as internal control. Band intensities quantified by densitometry are shown as bar graphs on the right-hand side of each panel.

Involvement of MAPK pathways in cystatin plus IFN-γ-mediated NO generation

In order to identify the signal transduction mechanisms underlying this cellular response, we examined the induction of MAPK, which are known to play an important role in iNOS and proinflammatory cytokine regulation in response to various stimuli. Activation of all three MAPK was examined by their phosphorylation in RAW 264.7 macrophages stimulated with cystatin (0.5 μM) plus IFN-γ (100 U/mL) for 4 h. Marked induction was observed in case of phosphorylation of ERK1/2 with very little induction for p38 and no induction for p46 and p54 JNK (Fig. 2A). Preincubation of cells for 1 h before cystatin stimulation with specific ERK1/2 inhibitors, PD98059 and apigenin resulted in dose-dependent reduction of iNOS mRNA and protein expression with a maximum reduction of ~80% at the highest concentration (40 μM) of inhibitors (Fig. 2B). Kinetic analysis (0–8 h) following stimulation with cystatin plus IFN-γ revealed time-dependent phosphorylation of ERK1/2 in both normal and infected macrophages with lesser induction and slower kinetics in infected cells (Fig. 2C). The maximum induction of phosphorylation of ERK1/2 was observed at 4 h in normal macrophages and at 8 h in infected macrophages. These results suggest that the ERK1/2 pathway is activated in response to cystatin plus IFN-γ and is functionally relevant to the modulation of iNOS induction in macrophages. Consistent with these observations, BM-derived macrophages (BMM) also displayed enhanced activation of ERK (Fig. 2D) and reduced expression of iNOS at both mRNA and protein levels (Fig. 2E) in the presence of specific ERK inhibitors.

Role of NF-κB in iNOS modulation by cystatin

A luciferase reporter assay was used to determine the effect of cystatin on the NF-κB-dependent iNOS gene expression. The macrophages were transiently transfected with a plasmid containing five copies of the NF-κB-binding sites, and the luciferase activities were measured. Cystatin plus IFN-γ significantly increased the NF-κB-dependent luciferase activity in a dose-dependent manner, which is consistent with the production of NO (Fig. 3A). In order to further examine the putative mechanism, the effect of cystatin on the activation of NF-κB was monitored using EMSA. In the presence of both cystatin and IFN-γ, a much higher NF-κB DNA-binding activity was detected than when they were added separately (Fig. 3B), being maximal

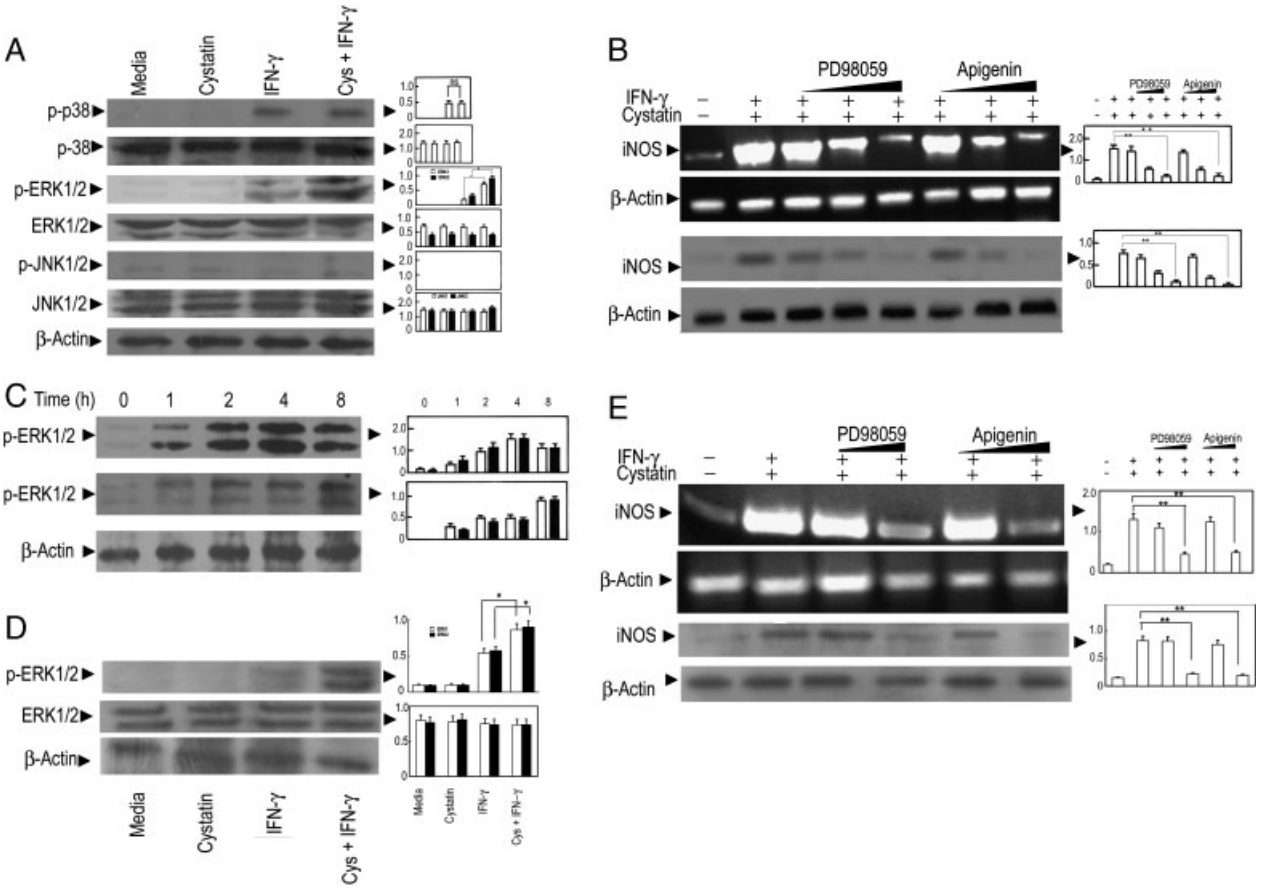

Figure 2. Effect of cystatin on the regulation of iNOS by MAPK activation. (A) RAW 264.7 cells were treated either with cystatin (0.5 μM), IFN-γ (100 U/mL) or both for 4 h. The expression and phosphorylation of MAPK were detected by Western blotting ($n = 4$). (B) Cells were treated (1 h) with either PD98059 (1, 15 and 30 μM) or apigenin (1, 20 and 40 μM) before stimulation with cystatin plus IFN-γ (24 h), and their effects on iNOS mRNA (upper panels) and protein (lower panels) expression were evaluated by RT-PCR and Western blotting, respectively ($n = 3$). (C) Kinetics of ERK1/2 activation. Both normal (upper panel) and *L. donovani*-infected (lower panel) macrophages were treated with cystatin plus IFN-γ for 0–8 h and the levels of phosphorylated ERK1/2 were measured by Western blotting ($n = 3$). (D) Western blot analysis of phospho-ERK1/2 in cystatin plus IFN-γ-treated BMMΦ ($n = 3$). (E) iNOS expression of cystatin plus IFN-γ-treated BMMΦat mRNA (upper panel)) and protein (lower panel)) level in the presence of either PD98059 (1 and 30 μM) or apigenin (1 and 40 μM) ($n = 3$). β-actin was used as an internal control. Densitometries are shown as bar graphs on the right-hand side of each panel. Open bars denote p38, ERK1 and JNK1 whereas closed bars denote ERK2 and JNK2. Densitometric evaluations are mean ± SD. $^{*}p<0.01$ *versus* IFN-γ; $^{**}p<0.001$ *versus* Cystatin plus IFN-γ.

at 4 h and still significant after 8 h (Fig. 3C). Since selective blockage of the ERK1/2 pathway inhibited cystatin-induced iNOS expression, we wanted to determine whether NF-κB activation was also under the control of this signaling cascade. Cell exposure to either apigenin or PD98059 resulted in significant decrease of cystatin plus IFN-γ-inducible NF-κB DNA-binding activity (Fig. 3D). Further, cell pretreatment with BAY11-7082, a chemical compound that blocks NF-κB expression by inhibiting IκB phosphorylation, before cystatin stimulation, resulted in a concentration-dependent reduction of iNOS expression at both mRNA and protein levels (Fig. 3F and G). All these results indicate that ERK1/2-mediated NF-κB activation appears to be necessary for the induction of macrophage iNOS expression in response to cystatin. This observation was further validated in BMM, where cystatin plus IFN-γ-induced NF-κB activation was significantly reduced by specific ERK inhibitors (Fig. 3E).

Activation of mitogen- and stress-activated protein kinase 1 and induction of p65 phosphorylation

The MAPK control several cellular processes through the phosphorylation of an array of substrates including nuclear mitogen- and stress-activated protein kinase 1 (MSK1), which in turn regulates transcription by phosphorylating specific residues on multiple substrates including Ser^{276} on p65/RelA resulting in transcriptional activation of NF-κB. Since MSK1 is also a downstream target of ERK, we explored the possibility of the involvement of MSK1 in cystatin-induced and ERK1/2-directed activation of NF-κB. MSK1 activity was determined in cystatin-treated macrophages by immunoprecipitating it from cell lysates and assaying the phosphorylation of specific peptide substrate [20]. Cystatin activated MSK1 in a time-dependent manner with optimal activity at 90 min after treatment (Fig. 4A). MSK1 activity could be significantly inhibited ($p<0.001$; $n = 3$) by

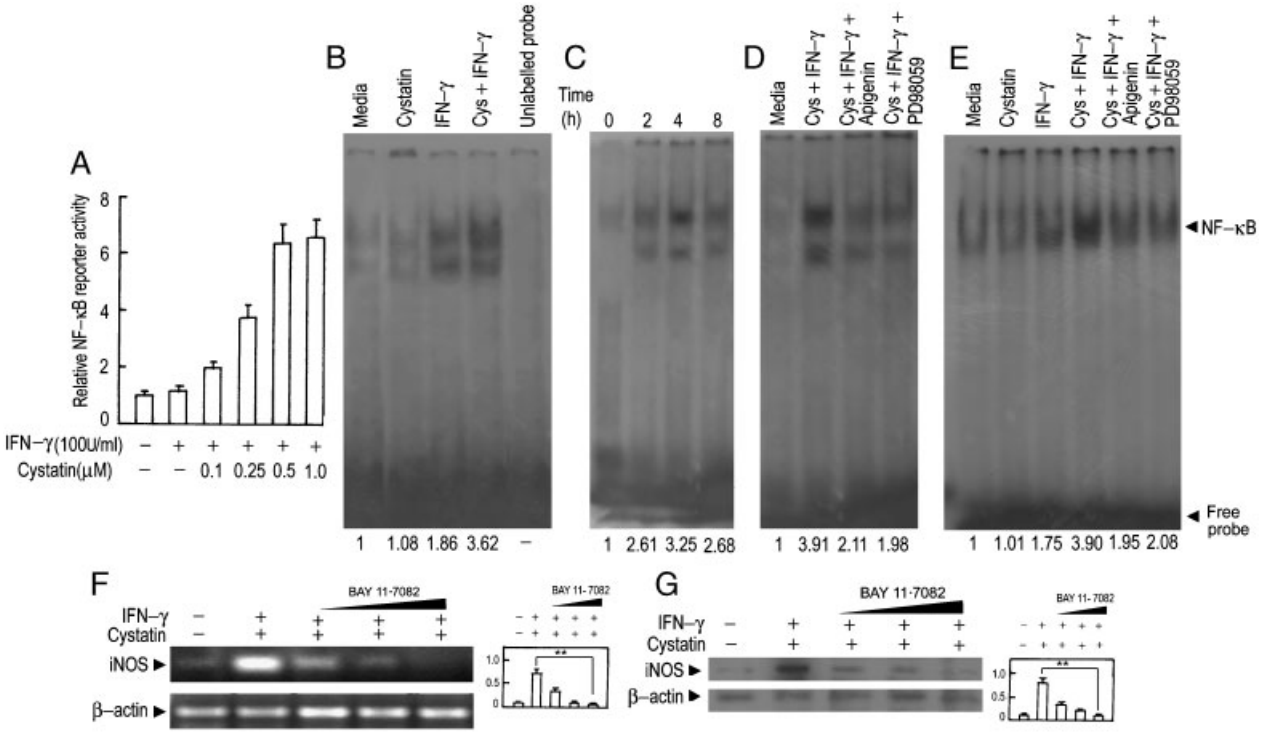

Figure 3. Involvement of NF-κB in cystatin-dependent iNOS expression. (A) RAW 264.7 cells were transiently transfected using Lipofectamine reagent with 1 μg NF-κB luciferase reporter vector along with 0.5 μg pCMV-β-gal. After 24 h of transfection, cells were stimulated with cystatin (0.01–1 μM) and IFN-γ (100 U/mL) for 12 h. Cells were lysed and processed for luciferase activity (mean ± SD, $n = 4$). (B) EMSA of NF-κB complexes in macrophage nuclei after treatment with cystatin (0.5 μM) or IFN-γ (100 U/mL) or both for 4 h. Extracts were also run with cold competitor oligonucleotides. (C) EMSA of NF-κB was performed with nuclear extracts from cystatin plus IFN-γ-treated cells for 0–8 h. EMSA of NF-κB in RAW cells (D) and BMMΦ (E) pretreated (1 h) with either apigenin (40 μM) or PD98059 (30 μM) and then stimulated with cystatin plus IFN-γ for 4 h. Bands were analyzed densitometrically, and fold changes are indicated at the bottom. Cells were treated (1 h) with BAY 11-7082 (0.55 μM) before stimulation with cystatin plus IFN-γ (24 h) and iNOS mRNA (F) and protein (G) expressions were evaluated by RT-PCR and Western blotting, respectively ($n = 3$). Densitometric evaluations are mean ± SD. **$p<0.001$ *versus* Cystatin plus IFN-γ.

apigenin (40 μM) suggesting a role of ERK in MSK1 activation (Fig. 4B). We further analyzed the role of cystatin-mediated MSK1 activation on both NF-κB-driven luciferase expression and p65 phosphorylation. Cystatin treatment resulted in increased luciferase expression and p65 phosphorylation, which was abrogated in cells transfected with dominant-negative (dn) MSK1 (Fig. 4C and D). Moreover, cells transfected with dnMSK1 produced significantly lower amounts of iNOS at both mRNA and protein level ($p<0.01$; $n = 3$) in response to cystatin than transfected controls (Fig. 4E and F). Collectively, these results indicate that cystatin-induced ERK1/2 has a major role in controlling NO production through its activation of MSK1, the direct activator of NF-κB.

Requirement of JAK/STAT signaling pathway

Since a suboptimal dose of IFN-γ is required for cystatin to induce NO production, to elucidate whether the synergistic effect of IFN-γ depends on STAT-1α and IRF-1, nuclear extracts from RAW 264.7 cells treated with either cystatin, IFN-γ or both were subjected to EMSA using the specific binding elements for each transcription factor. DNA-binding activity of both STAT-1α and IRF-1 following 2 h stimulation were much higher in cystatin plus IFN-γ-treated cells than in cells treated with either component (Fig. 5A and B). Because binding of IFN-γ to its receptors results in activation of the receptor-associated kinases JAK-1 and JAK-2, we analyzed the involvement of the JAK/STAT signaling pathway (Fig. 5C and D). Our results demonstrated increased JAK-1 and JAK-2 tyrosine phosphorylation in RAW 264.7 cells after treatment with cystatin plus IFN-γ. When JAK-mediated phosphorylation of STAT-1α was assessed in Ser^{727} and Tyr^{701} residues by immunobloting, significantly increased phosphorylation ($p<0.01$; $n = 3$) was observed at Tyr^{701} residue whereas Ser^{727} phosphorylation was unaltered in cystatin plus IFN-γ-treated cells compared with IFN-γ-treated cells (Fig. 5E). When we assessed the effects of JAK inhibitor, AG490 and IRF-1 inhibitor, genistein on iNOS expression by macrophages stimulated with cystatin plus IFN-γ, both the inhibitors reduced iNOS expression in a dose-dependent manner (Fig. 5F–I). However, at the maximal inhibitor concentration (50 μM) of AG490 and genistein, the inhibition obtained was 50 and 40%, respectively, which was much less than that obtained by ERK1/2 and NF-κB inhibitors, apigenin and BAY11-7082. Further, the expression of iNOS was significantly suppressed ($p<0.01$; $n = 3$) at both RNA and protein levels by transient transfection of an IRF-1-specific

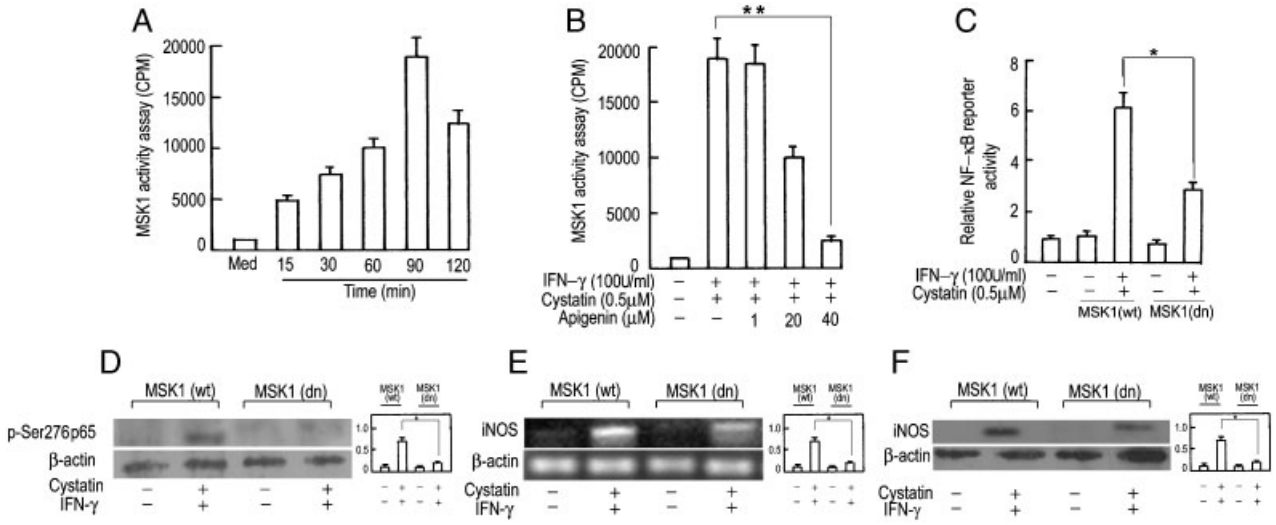

Figure 4. Role of MSK1 in cystatin-dependent iNOS expression. (A) RAW 264.7 cells were incubated with cystatin (0.5 μM) plus IFN-γ (100 U/mL) for 0–120 min. Cell lysates were immunoprecipitated with anti-MSK1 antibody, and the immunoprecipitate was subjected to MSK1 assay. (B) Cystatin plus IFN-γ-stimulated (4 h) cells were pretreated (1 h) with apigenin and MSK1 activity was assayed in the cell lysate. (C) Cells were transiently transfected with pNF-κB-luciferase plasmid and 0.5 μg pCMV-β-gal together with 1 μg of WT or dn-MSK1 expression plasmid. After 24 h of transfection, cells were stimulated with cystatin (0.5 μM) and IFN-γ (100 U/mL) for 12 h, lysed and processed for luciferase activity. (D) Cells were transfected with WT or dn-MSK1 constructs, treated with cystatin plus IFN-γ for 2 h, lysed and immunoblotted with anti-phospho Ser^{276} p65 antibody ($n = 4$). Cells were also evaluated for iNOS mRNA (E) and protein (F) expression by RT-PCR and Western blot, respectively ($n = 3$). In this case cystatin plus IFN-γ treatment was given for 24 h. Statistical evaluations show mean ± SD. *$p<0.01$; **$p<0.001$ *versus* Cystatin plus IFN-γ.

siRNA oligonucleotide into macrophages (Fig. 5J and K). Nitrite levels were also reduced in IRF-1 siRNA-treated cells (Fig. 5M). The efficacy of siRNA on IRF-1 expression was assessed by Western blotting. IRF-1 was significantly reduced in cells expressing IRF-1-specific siRNA compared with cells expressing control siRNA (Fig 5L). These results suggest that cystatin plus IFN-γ-mediated macrophage NO modulation, at least in part, depends on the activation of JAK/STAT pathway.

Effect of cystatin plus IFN-γ on *in vivo* activation of NF-κB

We wanted to determine the involvement of NF-κB in cystatin plus IFN-γ-mediated iNOS expression *in vivo* at post-infective stages. To this end, a dose of either 5 mg/kg/day of cystatin or 5×10^5 U/kg/day of IFN-γ or both were given for 4 consecutive days 15 days after infection and we examined the relative abundance, subcellular distribution and functional activity of NF-κB in nuclear extracts of splenocytes isolated 3 days after final treatment. Binding to NF-κB-specific oligonucleotides (as assessed by EMSA) showed a 1.52-fold increase in cystatin plus IFN-γ-treated infected cells compared with infected cells treated with either component (Fig. 6A). The specificity of these interactions was confirmed by the ability of antisera against p50 and p65 subunits of NF-κB to retard the mobility ("super-shift") of these binding species (Fig. 6B). The levels of both nuclear p65 and p50 subunits were four- to fivefold higher in infected cystatin plus IFN-γ-treated mice compared with either uninfected or infected animals; similarly, the infected treated mice also had relatively less cytoplasmic p65 and p50. To ascertain the effects of cystatin plus IFN-γ on the phosphorylation and subsequent degradation of IκBα, splenocyte lysates from infected animals were subjected to Western blot analysis using antiphospho IκBα antibody. Treatment of infected animals with cystatin plus IFN-γ markedly induced the phosphorylated IκBα signal whereas very little IκBα phosphorylation was observed in normal or untreated infected cells (Fig. 7B). Because IκBα is phosphorylated by the IκB Kinase (IKK) multiprotein complex, the effect of cystatin on the status of intrinsic cellular IKK activation was determined. Accordingly, the IKK activity in splenocytes was measured directly by immunocomplex kinase assay and it was significantly higher ($p<0.001$; $n = 3$) in treated mice than in untreated and infected mice (Fig. 7C). These results show that the increase in IKK activity and the decrease of IκBα level trigger the translocation and activation of NF-κB in infected mice treated with cystatin plus IFN-γ.

Discussion

In the present study we have demonstrated that chicken cystatin, a natural CP inhibitor, acts as an immunomodulator in activating macrophages for the release of NO, which play a central role in curing visceral leishmaniasis in the experimental animal model. The synergistic role of cystatin plus IFN-γ on intracellular parasite killing relies on the induction of NOS pathway and was almost completely abrogated in the presence of NMMA, a specific NOS inhibitor. Although our *in vitro* studies demonstrated the inability of cystatin alone to induce NO synthesis in macrophages, our

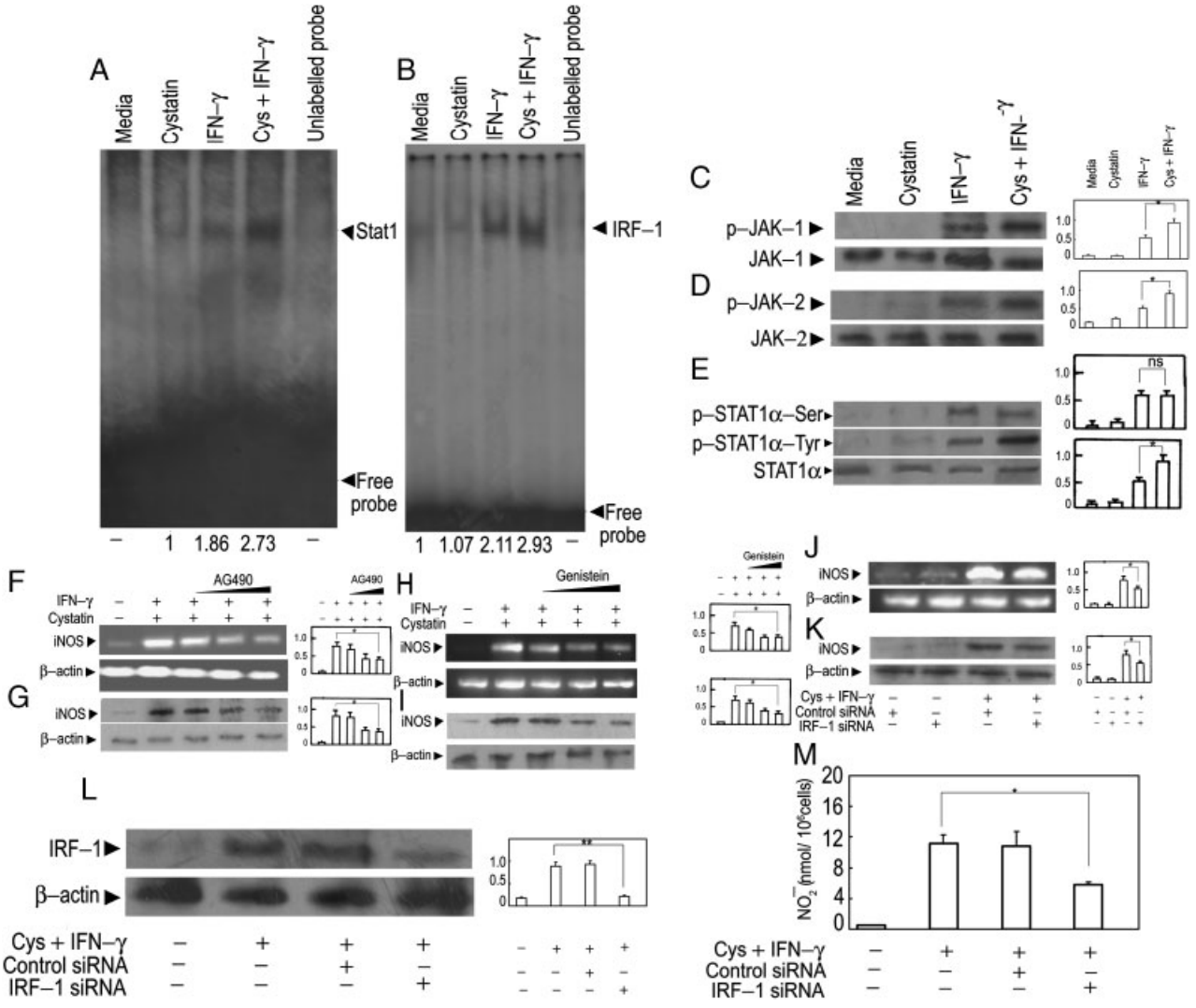

Figure 5. Effect of cystatin plus IFN-γ on JAK-2/STAT1α activation. EMSA of Stat1α (A) or IRF-1 (B) in macrophage nuclear extracts after treatment (2 h) with cystatin (0.5 μM) or IFN-γ (100 U/mL) or both. Extracts were run with cold competitor oligonucleotides for specific binding. Protein lysates from cystatin- or IFN-γ- or cystatin plus IFN-γ-stimulated macrophages were subjected to Western blotting. JAK-1 and JAK-2 phosphorylation patterns were determined with phosphor-JAK-1 (C) and phosphor-JAK-2 antibodies (D) whereas STAT1α phosphorylation patterns were determined with phospho-STAT1α-Ser and phospho-STAT1α-Tyr antibodies ($n = 3$) (E). Equal loading was verified using an anti-STAT1α antibody. Cells were treated (1 h) with 1, 25 and 50 μM of AG 490 or genistein before stimulation (24 h) with cystatin plus IFN-γ and iNOS mRNA (F and H) and protein (G and I) expressions were determined using RT-PCR and Western blotting, respectively ($n = 3$). For specific inhibition of IRF-1, macrophages were transfected (24 h) with IRF-1 siRNA or control siRNA, stimulated (24 h) with cystatin and IFN-γ, lysed and processed for RT-PCR (J) and immunoblot analysis (K) ($n = 4$). (L) The specificity of IRF-1 siRNA was determined in whole extracts from macrophages expressing either IRF-1 targeting or control siRNA by Western blotting using specific antibody against IRF-1 ($n = 3$). (M) Nitrite levels in control and IRF-1 siRNA treated cells. Statistical evaluations show mean ± SD. *p<0.01 *versus* IFN-γ (C-E); *p<0.01; **p<0.001 *versus* Cystatin plus IFN-γ (F–L).

in vivo studies argue against this. Thus, cystatin given *in vivo* induced NO synthesis in peritoneal macrophages, along with an enhancement of inhibition of parasite growth. This suggests that NO generation by macrophages may be an indirect effect of cystatin activation requiring the cooperation of macrophages and other cells of the immune system. The requirement of IFN-γ in the *in vitro* situation is suggestive of its compensatory role for the effector molecules of immune system *in vivo*. Cystatin-induced generation of NO depends on ERK, which in turn activates NF-κB-dependent gene expression through the participation of MSK1. Further, the present study provides the first *in vivo* evidence that cystatin may exert its therapeutic effect on visceral leishmaniasis by activating NF-κB pathway in spleen at all levels in the activation cascade as far back as IKK activity. The current data indicating that cystatin plus IFN-γ-enhanced NF-κB activity is under the control of the ERK1/2 pathway correlate with a number of studies showing direct correlation between ERK1/2 activation and upregulation of both NF-κB activity and NO production [21–23]. On the other hand, infection with *L. amazonensis* amastigotes inhibited ERK phosphorylation in response to LPS [13], whereas infection with *L. donovani* amastigotes resulted in inactivation of ERK1/2, which was

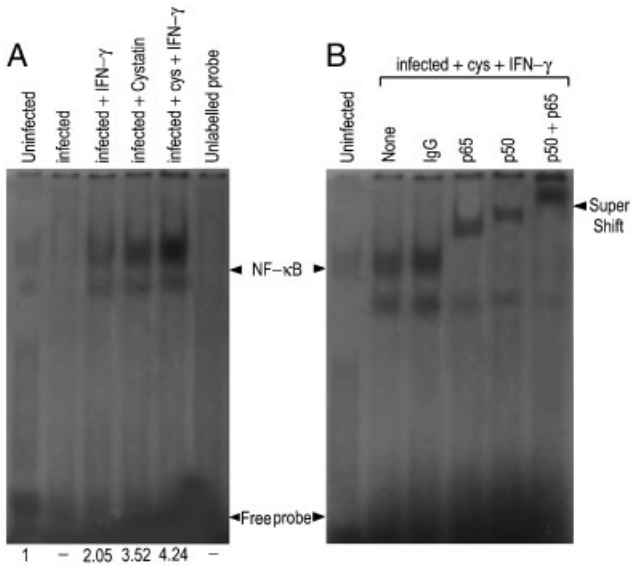

Figure 6. Effect of cystatin plus IFN-γ on *in vivo* activation of NF-κB. *L. donovani*-infected mice were treated with cystatin (5 mg/kg/day), IFN-γ (5×10^5 U/kg/day) or both for 4 consecutive days starting at day 15 after infection. Splenocytes were isolated 3 days after last treatment. (A) EMSA of NF-κB complexes were performed in splenocyte nuclear extract. Cold competitor oligonucleotides were used for specific binding. Bands were analyzed densitometrically, and fold changes are indicated. (B) Splenocyte nuclear proteins were super shifted using antibodies against the indicated NF-κB subunits. Normal rabbit IgG was used as control.

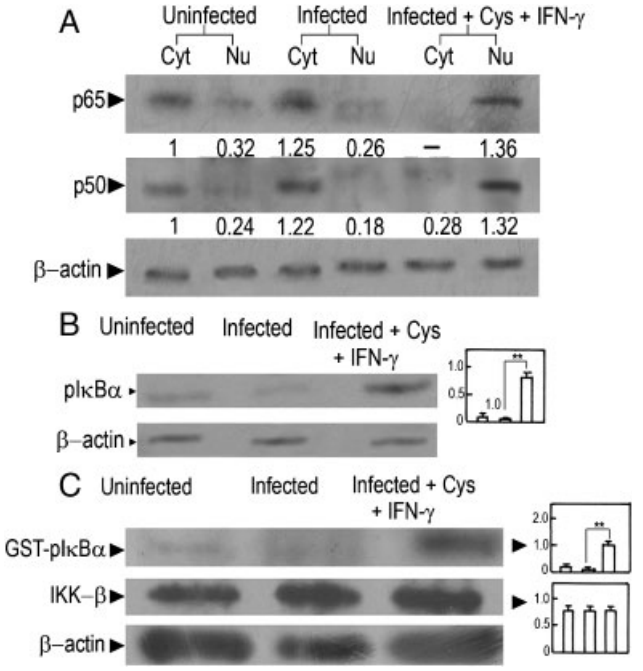

Figure 7. Nuclear translocation of NF-κB subunits and cystatin-induced activation of IKK. (A and B) Following treatment of *L. donovani*-infected mice with cystatin plus IFN-γ as described in Fig. 6, splenocytes were isolated for the preparation of cytoplasmic and nuclear protein fractions for Western blot analyses. Bands reacting with antibodies against p65, p50 (A) and phosphorylated IκBα (B) were visualized ($n = 3$). The blots were analyzed densitometrically, and the values were normalized to β-actin. (C) Splenocyte cell lysates were immunoprecipitated with anti-IKKβ antibody, IKK was assayed using GST-IκBα as substrate, and GST-phosphorylated IκBα was visualized by autoradiography ($n = 3$). A relative amount of IKK-β in the whole cell extracts was determined by Western blots. Densitometric evaluations show mean ± SD. $^{**}p<0.001$ *versus* infected.

accompanied by the inhibition of transcription factors Elk-1 and c-fos expression [23]. In a more recent study, ceramide-mediated inactivation of ERK1/2 and resulting inhibition of AP-1 and NF-κB have been proposed to explain the absence of NO generation by *L. donovani*-infected cells [24]. All these studies suggest that compounds having the ability to augment signaling pathways leading to macrophage activation might demonstrate protective action against *Leishmania* infection.

Post-translational modifications achieved by both phosphorylation and acetylation of p65 subunit play particularly important roles in the activation of NF-κB in addition to the activation of classical pathway. MSK1, a downstream kinase of both ERK1/2 and p38, can phosphorylate p65 subunit at Ser^{276} residue and acts as a post-translational modifier that integrates the upstream MAPK signaling cascades with NF-κB transactivation [25]. Our results demonstrate a significant role of ERK in the generation of NO by the activation of NF-κB in an MSK1-dependent fashion. Transient overexpression of dn-MSK1 substantially inhibited cystatin-induced p65 phosphorylation at serine^{276} residue as well as NF-κB-driven luciferase promoter activity suggesting that MSK1 is the major kinase responsible for phospho-Ser^{276} RelA formation. Although p65 serine phosphorylation has also been shown to be mediated by cAMP-dependent protein kinase at least in the case of LPS treatment [26], in the present study complete abrogation of this phosphorylation by dn-MSK1 emphasizes the predominant role of MSK1. However, as far as cystatin-induced iNOS transcriptional activity is concerned, silencing of MSK1 could only partially inhibit the process. This observation corroborates the concept that MSK1-mediated post-translational modification of RelA works in parallel with the classical IKK pathway leading to the full extent of NF-κB activation. In addition to NF-κB activation, cystatin along with IFN-γ also induced phosphorylation and nuclear translocation of STAT-1α and IRF-1, which possibly contributed to the induction of cystatin-induced iNOS expression. It may be worthwhile to mention that subthreshold concentrations of IFN-γ, which did not activate macrophages, were shown to sensitize macrophages for subsequent IFN-γ stimulation resulting in increased STAT-1α activation and increased IFN-γ-dependent gene activation [27]. Maximal STAT-1α activation has been found to require phosphorylation at both Tyr^{701} and Ser^{727} residues [28]. Our results demonstrated increased phosphorylation at Tyr^{701} but unaltered phosphorylation status of Ser^{727} residues by cystatin treatment. This is not surprising as phosphorylation of STAT-1α in the carboxy terminal Ser^{727} is known to be mediated by p38, which was not induced by cystatin activation.

Targeted gene deletion studies established that NF-κB family members play complex regulatory roles in controlling innate and adaptive immune response in leishmaniasis [29, 30]. It was reported that in mouse macrophages exposed to *L. donovani* the

NF-κB signaling pathway is not triggered [12, 24], which leads to silencing of any effector host molecules that prevent the parasite progeny from developing. More recently, it was documented that *L. mexicana* amastigotes disrupt the NF-κB signaling pathway and this is at least partly responsible for parasite survival in its mammalian host [31]. All these findings suggest that agents that lead to activation of NF-κB pathway might prove attractive candidates to control infection with *Leishmania*. In the present study, in addition to the *in vitro* studies, we have focused on the signaling mechanism of NF-κB activation in the *in vivo* situation in relation to the curative effect of cystatin in experimental visceral leishmaniasis. The results provide the first *in vivo* evidence that cystatin may exert its beneficial effects on leishmaniasis mostly through its ability to activate NF-κB by inducing phosphorylation and subsequent degradation of IκBα as studied in the spleen of both normal and infected animals. This is consistent with our observation that cystatin alone can induce NO generation as well as antileishmanial activity in the *in vivo* situation. Our *in vivo* studies further suggest that IKK was stimulated in cells that were activated in presence of cystatin through a mechanism that most likely involves the upstream signaling of MAPK pathways as shown extensively by our *in vitro* studies. It may be mentioned that in Hs294T cells, ERK regulation of NF-κB activation has been shown to involve increased IκB phosphorylation with concomitant elevation in the NF-κB DNA-binding activity [32].

Collectively, the findings in the present study suggest that the sequential activation of MEK-, ERK-, MSK1- and NF-κB-dependent signaling events play pivotal roles in cystatin plus IFN-γ-inducible macrophage NO generation and that STAT-1α is also involved in the regulation of iNOS gene expression. Overall, a better knowledge of the transductional mechanism by which cystatin triggers macrophage microbicidal functions, such as production of NO, could permit the development of immunomodulators useful not only for nonhealing leishmaniasis but also for other chronic infectious diseases.

Materials and methods

Reagents and constructs

All antibodies were from Santa Cruz Biotechnology and Cell Signaling Technology. All other chemicals were from Sigma unless otherwise indicated. WT and dn (D195A) MSK1 in pCMV-FLAG were obtained from Dr. Dario Alessi, University of Dundee, UK.

Cell culture and infections

L. donovani promastigotes (MHOM/IN/1983/AG83) were grown as described previously [33]. The murine macrophage cell line RAW 264.7 was maintained at 37°C/5% CO_2 in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). BMM and BM-derived splenocytes were cultured as described earlier [17, 34] For *in vitro* infection experiments, thioglycollate-elicited murine macrophages on glass cover slips (18 mm^2; 5×10^5 macrophages/cover slip) in 0.5 mL of RPMI/10% FCS were infected with stationary phase promastigotes at a ratio of 10 parasites/macrophage [17]. Infection was allowed to proceed for 4 h, unphagocytized parasites were removed by washing with medium and cells were resuspended in RPMI 1640/10% FCS with or without cystatin and IFN-γ, along with each component added alone, for 24 h at 37°C. Cells were fixed in methanol and stained with Giemsa stain for determination of intracellular parasite numbers. The number of amastigotes in 100 macrophages in drug-treated and control cultures were determined. Percentages of suppression of *Leishmania* in drug-treated cultures were calculated on the basis of considering the number of amastigotes in untreated cultures as 100%. Nitrite formation in macrophage culture supernatants was detected by the Griess reaction as previously described [17]. For *in vivo* experiments, female BALB/c mice were injected *via* the tail vein with 10^7 *L. donovani* promastigotes. For reinfection experiments, promastigotes was injected 45 days after the first infection. Cystatin (0.1–10 mg/kg/day), either alone or in combination with IFN-γ (5×10^5 U/kg/day) was administered on the 15th day post infection for 4 consecutive days. Infection was assessed by removing liver and spleen from 45 day infected mice and parasite burdens were determined from Giemsa stained impression smears. Data are presented as LDU [35]. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23 revised 1996) and with the approval of the Institutional Animal Care and Use Committee.

RT-PCR

RT-PCR was performed for iNOS as described previously [33].

EMSA

Nuclear extracts from either the splenocytes or RAW 264.7 cells were isolated as described earlier [33]. For the preparation of radiolabeled probes representing standard consensus sequences of various transcription factors, the following oligonucleotides were used: IRF-1: 5′-GGA AGC GAA AAT GAA ATT GAC T-3′; STAT-1: 5′-AAG TAC TTT CAG TTT CAT ATT ACT CTA-3′ and NF-κB: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′. As a control, a 100-fold molar excess of unlabelled competitor oligonucleotide was added. For NF-κB supershift assay, the nuclear extracts from splenocytes were incubated with anti-p65 or anti-p50 polyclonal antibody for 30 min at 25°C and analyzed by EMSA in the presence of all components of the binding reaction described

earlier [33]. As control, the nuclear extracts were also treated with normal rabbit IgG.

IKK assay

Subcellular protein fractions were prepared from freshly isolated spleen in cold kinase assay lysis buffer (Cell signaling Technology). The IKK activity was measured as described earlier with slight modification [36]. The sample was resolved by SDS-PAGE, dried and autoradiographed. To determine the total amount of IKK-β, 30 μg of the whole cell extract protein was subjected to SDS-PAGE and analyzed by Western blotting.

Immunoblot analysis

Equal amount of protein (30 μg) from each sample was resolved by 10% SDS-PAGE and then transferred to nitrocellulose membrane (Millipore). The membranes were blocked with 5% BSA in wash buffer (TBS/0.1% Tween 20) for 1 h at room temperature and probed with primary antibody overnight at dilution recommended by the suppliers. Membranes were washed three times with wash buffer and then incubated with HRP-conjugated secondary antibody and detected by ECL detection system (Amersham Biosciences) according to the manufacturer's instructions.

MSK1 activity assay

MSK1 assay was performed as described previously [20]. Immunoprecipitated MSK1 pellets were incubated at 30°C for 30 min in 50 μL of kinase buffer containing 30 μM substrate peptide: EILSRRPSYRK (CREBTIDE) and 0.1 mM (γ^{32}P) ATP (200 000 cpm/pmol). Reactions were stopped by placing the tubes on ice. After centrifugation (30 s, 4°C, 10 000 × *g*), 30 μL of supernatant was spotted on p81 phosphocellulose paper, washed thrice with 0.75 M orthophosphoric acid and incorporation of radiolabeled phosphates in CREBTIDE was determined.

Transient transfection and NF-κB reporter assay

Transfections were carried out in 2×10^6 cells with the appropriate constructs in serum-free medium using Lipofectamine (Invitrogen) according to the manufacturer's instructions. For NF-κB luciferase activity, cells were harvested using reporter lysis buffer (Promega) and luciferase activity was then assessed *via* luminometry. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector. For siRNA transfection, cells were transfected with 1 μg of IRF-1 siRNA or control siRNA according to the manufacturer's instructions (Santa Cruz Biotechnology).

Statistical analysis

Experiments were repeated three times unless otherwise stated, and probability (p) was calculated using Student's t-test. $p<0.05$ were considered statistically significant.

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Abbreviations: **BMM:** BM-derived macrophages · **CP:** cysteine protease · **dn:** dominant-negative · **IKK:** IκB kinase · **IRF-1:** IFN regulatory factor-1 · **NMMA:** N^G-monomethyl-L-arginine

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