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Cystatin cures visceral leishmaniasis by NF-κB-mediated proinflammatory response through co-ordination of TLR/MyD88 signaling with p105-Tpl2-ERK pathway

Susanta Kar[1], Anindita Ukil[2] and Pijush K. Das[1]

[1] Molecular Cell Biology Laboratory, Infectious Diseases and Immunology Division, Indian Institute of Chemical Biology, Kolkata, India
[2] Department of Biochemistry, Calcutta University, Kolkata, India

Cystatin could completely cure experimental visceral leishmaniasis by switching the differentiation of Th2 cells to Th1 type, as well as upregulating NO, and activation of NF-κB played a major role in these processes. Analysis of upstream signaling events revealed that TLR 2/4-mediated MyD88-dependent participation of IL-1R-activated kinase (IRAK)1, TNF receptor-associated factor (TRAF)6 and TGFβ-activated kinase (TAK)1 is essential to induce cystatin-mediated IκB kinase (IKK)/NF-κB activation in macrophages. Cystatin plus IFN-γ activated the IKK complex to induce phosphorylation-mediated degradation of p105, the physiological partner and inhibitor of the MEK kinase, tumor progression locus 2 (Tpl-2). Consequently, Tpl-2 was liberated from p105, thereby stimulating activation of the MEK/ERK MAPK cascade. Cystatin plus IFN-γ-induced IKK-β post-transcriptionally modified p65/RelA subunit of NF-κB by dual phosphorylation in infected phagocytic cells. IKK induced the phosphorylation of p65 directly on Ser-536 residue whereas phosphorylation on Ser 276 residue was by sequential activation of Tpl-2/MEK/ERK/MSK1. Collectively, the present study indicates that cystatin plus IFN-γ-induced MyD88 signaling may bifurcate at the level of IKK, leading to a divergent pathway regulating NF-κB activation by IκBα phosphorylation and by p65 transactivation through Tpl-2/MEK/ERK/MSK1.

Key words: Cystatin · Leishmaniasis · Macrophage · NF-κB · Tumor progression locus 2

Supporting Information available online

Introduction

Innate immunity coordinates the inflammatory response to pathogens and the essential role of TLR in this is widely recognized. Following infection, components of exogenous pathogens bind to TLR on host cell, triggering the activation of signaling pathways that stimulate the production of inflammatory mediators such as pro-inflammatory cytokines, IFN and chemokines. All these mediators are released into the circulation to elicit effective immune response against invading microorganisms. All TLR activate a common signaling pathway that culminates in the activation of transcription factor NF-κB as well as MAPK (ERK1/2, p38 and JNK) [1]. Following the recognition of pathogen-associated molecular pattern, all of the TLR except TLR3 recruit a signaling complex to the receptors that includes an adaptor protein, MyD88 and two protein kinases, termed

Correspondence: Dr. Pijush K. Das
e-mail: pijushdas@iicb.res.in

IL-1R-activated kinase (IRAK)1 and IRAK4 [2]. IRAK1 is then phosphorylated by IRAK4 as well as by autophosphorylation [3, 4], resulting in recruitment of TNF receptor-associated factor (TRAF)6 [5]. Phosphorylated IRAK1 and TRAF6 are thought to dissociate from the receptors [6], which is followed by TRAF6 autoubiquitination with lys-63-linked polyubiquitin chains [7]. TAB2 and TAB3, the regulatory subunits of the TGFβ-activated kinase (TAK) 1 complex, specifically interact with ubiquitinated TRAF6, which activates TAK1 [8]. Activated TAK1 is then thought to phosphorylate and activate downstream IκB kinase (IKK) complex as well as MAPK kinase 6 to activate NF-κB and MAPK signaling pathway. Activation of MAPK in macrophages requires another serine/threonine kinase, tumor progression locus 2 (Tpl-2) [9], also known as Cot/MAP3K8. Tpl-2 is critical for host defense and appropriate cytokine response against pathogens like *Toxoplasma gondii* and *Listeria monocytogenes* [10, 11]. It functions as a MAPK kinase kinase, which phosphorylates and activates MEK, the upstream activator of ERK1/2 kinase [12]. Tpl-2 is critically involved in linking TLR to TNF-α production through its activation of ERK1/2 and also stimulates MEK activation in presence of TNF-α and CD40 ligand, suggesting its important role in both innate and adaptive immune response [13]. In unstimulated macrophages, Tpl-2 is complexed with NF-κB1 (p105), an IκB family member, which blocks its MEK kinase activity [14, 15]. Stimulation of macrophages with LPS facilitates the release of Tpl-2 from p105 and thereby activates its kinase activity, which has been shown to occur as a consequence of p105 phosphorylation and proteolysis by the proteasome [14]. p105 phosphorylation is triggered by IKK complex, which is also critical for regulating the activation of NF-κB transcription factors through phosphorylation of IκB and p65/RelA subunit [15, 16].

Leishmaniases are spectrum of vector-borne parasitic diseases that are a major international public health problem, affecting the lives of millions of people worldwide with significant morbidity and mortality [17]. Gene knock-out studies in mice revealed an essential role of TLR signaling in the immune responses against *Leishmania* parasites [18]. For instance, MyD88-dependent pathway is required for the secretion of IL-1α by mouse peritoneal macrophages following infection with *L. major* [19] and MyD88-deficient mice developed progressive lesion with dominant Th2 response [20]. *In vivo* studies in mice revealed an important role for TLR4 in the control of *L. major* infection, possibly through the early induction of iNOS [21]. TLR9 is involved in NK-cell activation and pro-inflammatory cytokine synthesis in animal models of visceral (*L. infantum*) and cutaneous (*L. major and L. braziliensis*) leishmaniasis [22, 23], while *Leishmania* lypophosphoglycan, an abundant molecule on the surface of promastigotes, signals *via* TLR 2 [24]. Moreover, silencing of IRAK1, MyD88, TLR2 or TLR3 significantly attenuated the secretion of nitric oxide and TNF-α following infection of IFN-γ-primed macrophages by *L. donovani* promastigotes [25]. In addition, a recent study suggests that TLR2 and MyD88 appeared to have a critical role in both recognition as well as modulation of immune response against *Leishmania* parasites [26]. Our earlier work showed that cystatin, a natural cysteine protease inhibitor, in synergy with IFN-γ, could completely cure visceral leishmaniasis in BALB/c mice by inducing proinflammatory cytokine synthesis and NO generation [27]. Furthermore, this induction was found to be mediated by ERK 1/2/mitogen- and stress-activated protein kinase 1 (MSK1) leading to the activation of NF-κB-dependent gene expression [28]. In the present study, we sought to determine the detailed upstream signaling events involved in cystatin-induced activation of NF-κB, which is essential for its anti-leishmanial effector response.

Results

Activation of NF-κB is essential for cystatin plus IFN-γ-mediated anti-leishmanial response

Cystatin could completely cure experimental visceral leishmaniasis and this was shown to be associated with strong upregulation of Th1 cytokine and iNOS [27]. In the *in vitro* situation, a suboptimal dose of IFN-γ was required for cystatin-induced proinflammatory cytokine response and iNOS expression whereas IFN-γ was not a prerequisite *in vivo*. Because iNOS and many proinflammatory gene expressions are regulated by NF-κB, the effect of cystatin plus IFN-γ on NF-κB activation was assessed in infected macrophages using EMSA. DNA binding activity of NF-κB was found to be markedly enhanced in infected BM-derived macrophages (BMM) following cystatin plus IFN-γ treatment, being maximum at 4 h (3.8-fold) and still significant after 6 h (1.9-fold) (Fig. 1A). For NF-κB nuclear translocation, cytoplasmic IκBα must be phosphorylated, ubiquitinated and degraded. Because IκBα is phosphorylated by the IKK multiprotein complex, the effect of cystatin on the status of intrinsic cellular IKK activation was assessed. Accordingly, *L. donovani*-infected macrophages were treated with cystatin plus IFN-γ; IKKβ was immunoprecipitated and tested for the ability to phosphorylate GST-IκBα substrate. As shown in Fig. 1B, cystatin plus IFN-γ strongly induced IKKβ activity in infected BMM. In order to further ascertain the role of NF-κB in the modulation of disease progression, anti-leishmanial activity of cystatin plus IFN-γ was evaluated in the presence of BAY 11-7082 or BAY 11-7085, chemical compounds that block NF-κB expression by inhibiting IκB phosphorylation. In the *in vitro* situation of amastigote multiplication within macrophages, BAY-11-7082 (5 μM) could substantially reverse the parasite killing (78% reduction) by cystatin (Fig. 1C). In the *in vivo* situation of *L. donovani*-infected BALB/c mice, administration of cystatin (5 mg/kg/day) plus IFN-γ (5×10^5 U/kg/day) given for 4 consecutive days after 15 days of infection completely suppressed the parasite burden in the spleen cells of mice (Fig. 1D). However, a dose of 5 mg/kg/day of BAY-11-7085 given three times weekly for 4 wk starting at 15 days after infection greatly reduced cystatin-mediated protection (74% reduction in parasite killing). *In vivo* administration of BAY-11-7085 could also result in marked reduction of cystatin-mediated increase in TNF-α synthesis (77% reduction) (Fig. 1E) as well as iNOS expression (4.3-fold reduction) in infected mice (Fig. 1F). Taken together, these

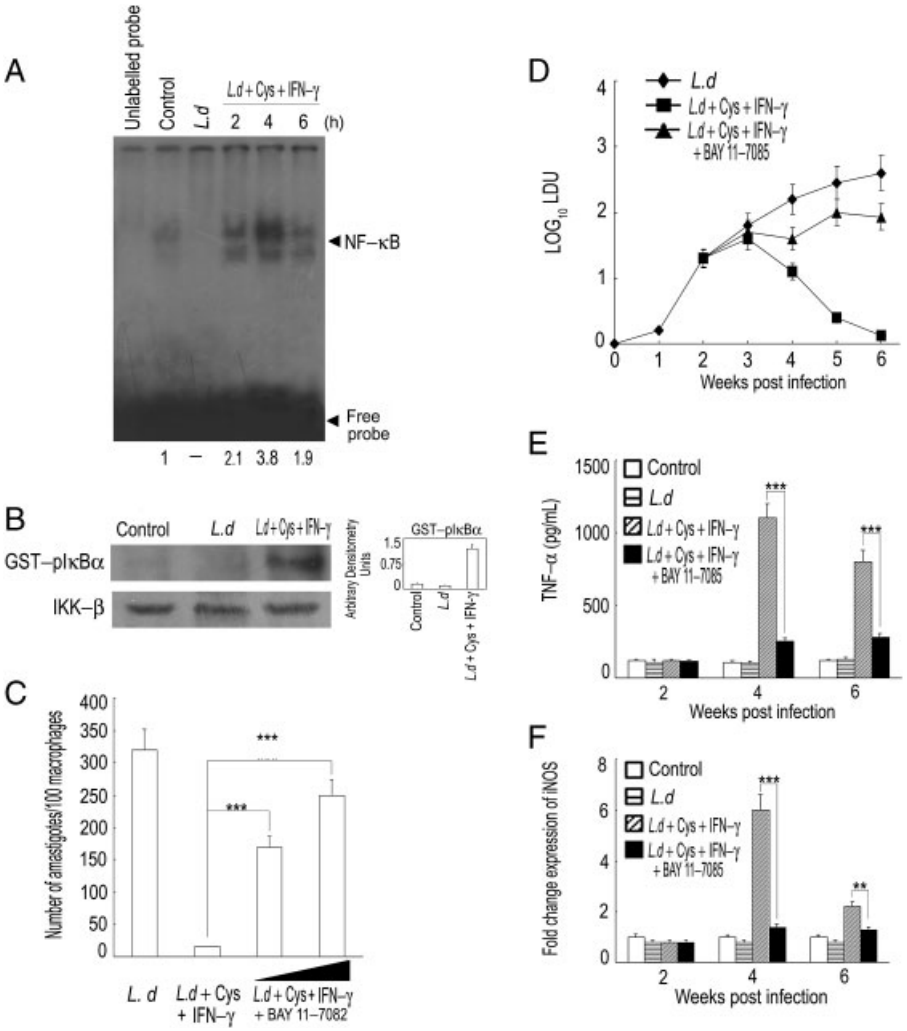

Figure 1. Involvement of NF-κB in cystatin plus IFN-γ-mediated anti-leishmanial response. (A) BMM were infected with *L. donovani* promastigotes (*L. d*) (cell:parasite ratio, 1:10) for 4 h. Noningested promastigotes were removed, and macrophages were cultured for another 20 h. *L. donovani*-infected (24 h) BMM were further treated with cystatin (0.5 μM) plus IFN-γ (100 U/mL) for 0–6 h and EMSA of NF-κB was performed with nuclear extract. Extracts were also run with cold competitor oligonucleotides. Bands were analyzed densitometrically, and fold changes are indicated at the bottom. (B) Whole-cell extracts from *L. donovani*-infected (24 h) and cystatin plus IFN-γ-treated (1 h) BMM were immunoprecipitated with Ab against IKKβ. IKKβ was assayed using GST-IκBα as substrate, and GST-phosphorylated IκBα was visualized by autoradiography. Relative amount of IKKβ in the whole-cell extracts was determined by Western blots. (C) Infected (24 h) and cystatin plus IFN-γ-treated (24 h) BMM were pretreated with BAY11-7085 (1 and 5 μM) for 1 h. Intracellular parasite number was determined by Giemsa staining. (D) Mice were challenged with 10^7 promastigotes, and after 15 days of infection, they were treated with cystatin plus IFN-γ daily for 4 consecutive days. Spleen parasite burdens were determined weekly after infection and were expressed as the mean of $\log_{10}$LDU. SD for six animals *per* group. BAY11-7085 (5 mg/kg/day) was used along with cystatin plus IFN-γ in a separate set of experiments. (E and F) Infected mice were treated either with cystatin plus IFN-γ or with cystatin plus IFN-γ plus BAY11-7085 as indicated in (D). ELISA was performed for the level of TNF-α (E) and real-time PCR analysis was performed for the expression of iNOS (F) in the splenocytes. The cultures were set in triplicate and the experiments were done a minimum of three times. Animal experiments were done with six animals *per* group and the results are representatives of four independent experiments. Data represent the mean+SD. $^{**}p<0.01$, $^{***}p<0.001$; Student's *t*-test.

results strongly suggest that the therapeutic effect of cystatin may be attributed to the activation of IKK-NF-κB pathway.

Cystatin plus IFN-γ-induced Tpl-2/MEK/ERK pathway

Apart from activation of NF-κB, IKK is also known to induce activation of Tpl-2, a serine-threonine kinase, which plays an important role in both innate and adaptive immune response [13, 14]. Since cystatin induced activation of IKK, we, therefore, examined whether cystatin plus IFN-γ could activate Tpl-2/MEK/ERK pathway. Treatment of infected BMM with cystatin plus IFN-γ (0−60 min) caused a marked increase in Tpl-2 kinase activity reaching a maximum at 60 min (Fig. 2A). The densitometric analysis in the right-hand panel of immunoprecipitated Tpl-2 (consisting of p58 and p52 isoforms) demonstrated the disappearance of p58 isoform at later time points (60 and 90 min) (Fig. 2A) suggesting its phosphorylation-mediated proteasomal degradation.

Proteasomal degradation of p58 isoform was further confirmed by the use of MG 132, a proteasomal inhibitor, which almost completely abrogated degradation of p58 as well as activation of Tpl-2 in cystatin plus IFN-γ-stimulated BMM (data not shown). This was further reflected in the activation of downstream kinases, MEK and ERK1/2. A marked phosphorylation of MEK and ERK1/2 were found in infected cells following cystatin plus IFN-γ treatment, which was significantly abrogated (2.3- and 2.2-fold for MEK and ERK1/2, respectively) in cells treated with Tpl-2-specific siRNA (Fig. 2B). To further determine whether cystatin plus IFN-γ induced the dissociation of Tpl-2 from p105, cell lysates were pre-cleared of p105 by immunodepletion and then subjected to Western blotting with anti-Tpl-2 antibody. As depicted in Fig. 2C, very little p105-free Tpl-2 was detected in infected cells. However, cystatin plus IFN-γ treatment induced the appearance of Tpl-2 in the p105-depleted lysate of infected BMM (Fig. 2C) suggesting a release of Tpl-2 from its inhibitor NF-κB p105. Moreover, the appearance of Tpl-2 in the p105-depleted lysate peaked at 60 min, the same time point at which the Tpl-2 MEK kinase activity was maximum. Tpl-2 activation is associated with p105 phosphorylation followed by its proteolysis. We found that treatment of infected BMM with cystatin plus IFN-γ induced phosphorylation of p105 on serine 927 followed by its rapid disappearance (Fig. 2D). The kinetics of p105 phosphorylation and degradation was correlated with Tpl-2 release from p105. To further address whether IKK is required for p105 phosphorylation as well as activation of Tpl-2, BMM were treated with the IKK inhibitor BAY-11-7082 prior to infection. Western blotting of cell lysates revealed that this inhibitor blocked cystatin plus IFN-γ-induced p105 phosphorylation and degradation (Fig. 2D). BAY-11-7082 also blocked cystatin plus IFN-γ-induced release of Tpl-2 from p105 (Fig. 2C) and Tpl-2 MEK kinase activity (Fig. 2F) in infected cells. Moreover, cystatin plus IFN-γ-mediated induction of MEK and ERK phosphorylation were also significantly reduced by BAY-11-7082 treatment (Fig. 2E). Collectively, these data suggest that IKK activity is required for cystatin plus IFN-γ-mediated activation of Tpl-2/MEK/ERK signaling pathway.

Cystatin plus IFN-γ induced dual phosphorylation of p65 by IKK

Phosphorylation of p65 at multiple residues has been implicated in p65 nuclear translocation and transcriptional activation of NF-κB [15, 16]. To investigate the possibility that IKK regulates NF-κB by a mechanism involving p65 phosphorylation, infected BMM were treated with cystatin plus IFN-γ for different time periods (0−3 h). Immunoblot analysis showed a strong induction of p65 phosphorylation at both Ser-536 and Ser-276 residues (Fig. 3A),

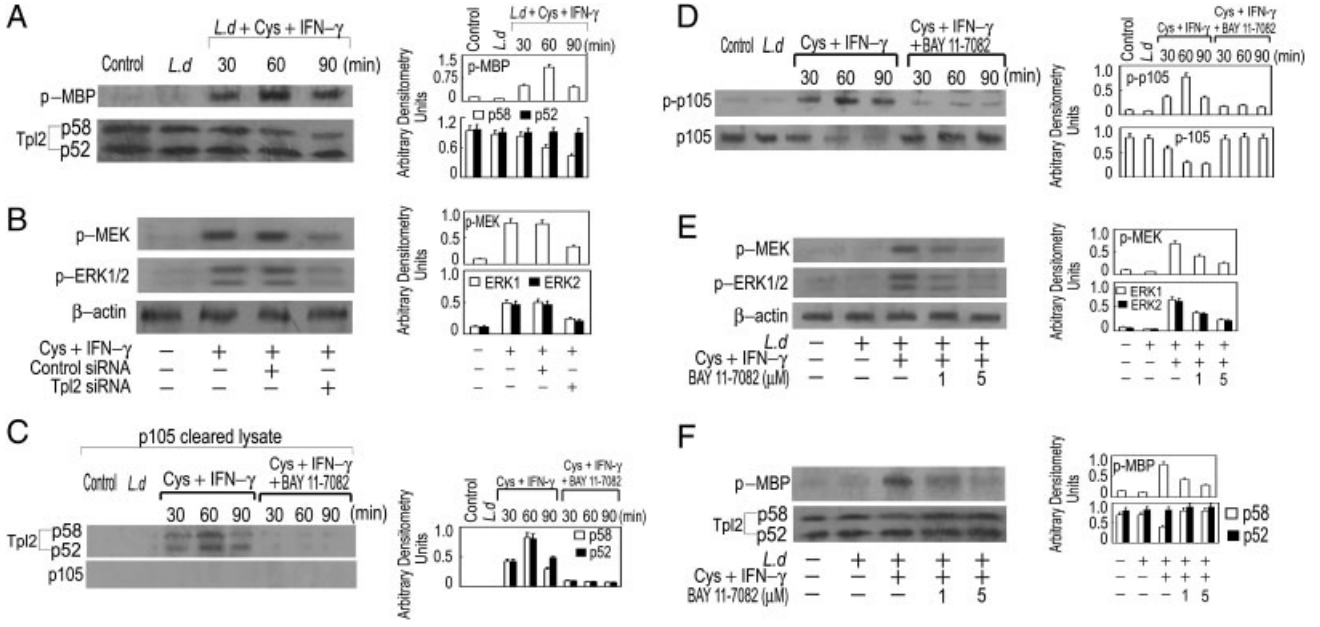

Figure 2. Effect of cystatin plus IFN-γ on Tpl-2/MEK/ERK pathway. (A) Infected BMM were treated with cystatin plus IFN-γ for the indicated times. Tpl-2 was immunoprecipitated and its MEK kinase activity was determined by coupled MEK/ERK kinase assay. Phosphorylated MBP substrate was visualized by autoradiography and the levels of immunoprecipitated Tpl-2 were determined by Western blotting to normalize the kinase assay. (B) RAW 264.7 cells were transfected (24 h) with either control or Tpl-2 siRNA, infected with *L. donovani* promastigotes (24 h) and further stimulated with cystatin plus IFN-γ for 4 h. The levels of p-MEK and p-ERK1/2 were measured by Western blotting. (C) *L. donovani*-infected BMM were treated with cystatin plus IFN-γ either alone or in combination with BAY11-7082 (5 μM) (pretreated for 1 h) for the indicated times. p105-cleared cell lysates were subjected to Western blotting for Tpl-2 and p105. (D) Infected BMM (24 h) were treated with cystatin plus IFN-γ and BAY11-7082 as indicated in (C). Levels of total and phosphorylated p105 were measured by immunobloting. (E) Infected BMM (24 h) were treated with cystatin plus IFN-γ either alone or in combination with BAY11-7082 (1 and 5 μM) (pretreated for 1 h) for 4 h. Levels of p-MEK and p-ERK1/2 were measured by Western blotting. (F) Infected BMM (24 h) were treated with cystatin plus IFN-γ either alone or in combination with BAY11-7082 (1 and 5 μM) (pretreated for 1 h) for 1 h. Tpl-2 was immunoprecipitated from total-cell lysates and then assayed for MEK kinase activity as indicated in (A). Densitometries are shown as bar graphs on the right-hand side of each panel. Results are expressed as mean+SD, $n = 3$. The results are representative of three separate experiments.

which was maximum at 2 h and almost completely abrogated in the presence of IKK inhibitor BAY-11-7082, suggesting the involvement of IKK in cystatin plus IFN-γ-induced dual phosphorylation of p65 (Fig. 3B). The decrease in p65 phosphorylation was not due to p65 degradation because the level of p65 remained constant (Fig. 3B). To further ascertain whether dual p65 phosphorylation was carried out by IKK directly or by some intermediate kinase, BMM were pretreated with apigenin, an inhibitor of ERK1/2 prior to cystatin plus IFN-γ stimulation. Interestingly, cell exposure to apigenin markedly reduced the phosphorylation of p65 (3.6-fold) at Ser-276 residue whereas the level of Ser-536 phosphorylation was unaltered (Fig. 3B). MSK1, a downstream target of ERK, phosphorylates multiple substrates including Ser-276 of p65. As depicted in Fig. 3C, phosphorylation of p65 at Ser-276 residue by cystatin was significantly abrogated (3.2-fold) in infected cells transfected with dominant-negative (dn) constructs of MSK1 with no alteration on p65 Ser-536 phosphorylation. Moreover, MSK1 activity could be significantly inhibited by apigenin (86%) and BAY11-7082 (91%) (Fig. 3D) suggesting IKK-ERK-mediated MSK1 activation, which in turn regulates Ser-276 phosphorylation of p65. Collectively, these results suggest that IKK induced the phosphorylation of p65 directly on Ser-536 residue and indirectly on Ser 276 residue by activation of ERK/MSK1 in cystatin plus IFN-γ-treated cells.

TAK1 is involved in cystatin plus IFN-γ-mediated NF-κB activation

The MAP3 kinase kinase kinase, TAK1 is known to mediate the pathway leading to IKKα/β phosphorylation resulting in classic NF-κB activation through IκB phosphorylation and degradation [8]. We, therefore, examined the possibility of TAK1 involvement in intermediate signaling events of cystatin plus IFN-γ-induced NF-κB activation. Kinetic analysis (0–60 min) following stimulation with cystatin plus IFN-γ revealed time-dependent phosphorylation of TAK1 in both normal and infected BMM with lesser induction and slower kinetics in infected cells (Fig. 4A). We further analyzed the role of cystatin-mediated TAK1 induction on both IKK activation and NF-κB-driven luciferase expression. Transient transfection of RAW 264.7 cells with dn constructs of TAK1 significantly reduced the amount of cystatin-induced phospho-IκBα and prevented IκBα degradation (Fig. 4B) suggesting a role of TAK1 in cystatin-mediated IKK activation. In addition, overexpression of dn-TAK1 markedly inhibited cystatin plus IFN-γ-induced IKKβ activity (Fig. 4C) as well as NF-κB-driven luciferase expression (2.5-fold reduction) (Fig. 4D). Cystatin-mediated anti-leishmanial activity was also significantly decreased in infected cells (68% reduction in parasite killing) transfected with dn TAK1 (Fig. 4E) implying the importance of TAK1 in cystatin-mediated activation of NF-κB pathway.

TLR/MyD88-dependent activation of NF-κB is essential for cystatin-mediated anti-leishmanial response

In the TLR/MyD88-signaling pathway, activated IRAK1 interacts with TRAF6, which in turn leads to the activation of TAK1-mediated NF-κB and MAPK [5, 6, 8] We, therefore, checked whether MyD88-dependent signaling is necessary for cystatin-induced activation of TAK1. To address this, RAW 264.7 cells

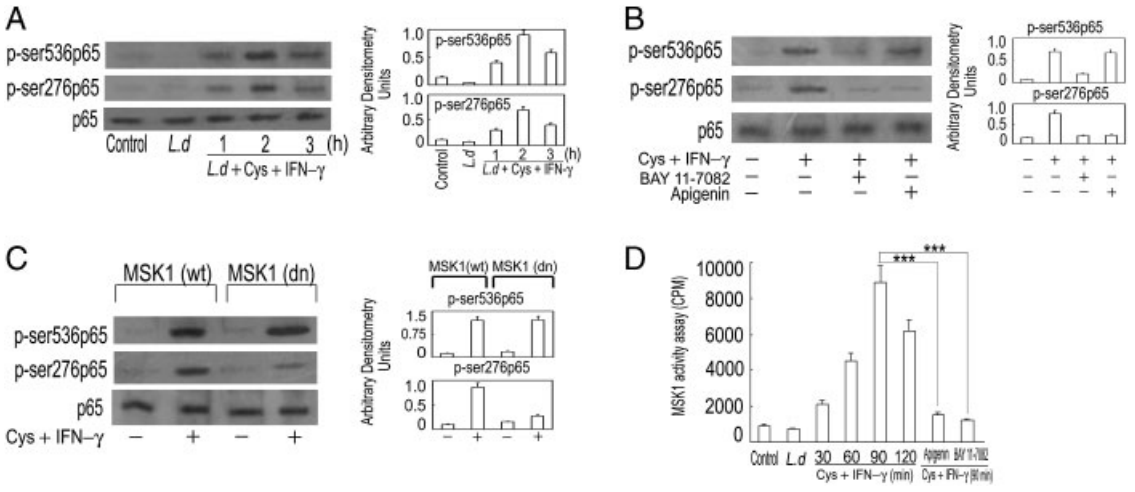

Figure 3. Role of cystatin plus IFN-γ on p65 phosphorylation. (A) *L. donovani* infected (24 h) BMM were treated with cystatin plus IFN-γ for the indicated times. Cells were lysed and levels of p-ser536p65, p-ser276p65 and p65 were determined by Western blotting. (B) Infected cells (24 h) were left untreated or pretreated with apigenin (40 μM) or BAY11-7082 (5 μM) for 1 h, followed by incubation with cystatin plus IFN-γ for 2 h. Levels of p-ser536p65, p-ser276p65 and p65 were determined by Western blotting. (C) Infected BMM (24 h) were transiently transfected with WT or dn-MSK1 expression plasmid. After 24 h of transfection, cells were stimulated with cystatin plus IFN-γ for 2 h. Levels of p-ser536p65, p-ser276p65 and p65 were determined by Western blotting. (D) Infected cells were incubated with cystatin plus IFN-γ for various time periods (0–120 min). In a separate set of experiment, infected cells were treated with apigenin (40 μM) or BAY11-7082 (5 μM) for 1 h before treatment with cystatin plus IFN-γ for 90 min. Cell lysates were then immunoprecipitated with anti-MSK1 Ab, and the immunoprecipitate was used to measure the phosphorylation of CREBTIDE as described in Materials and methods section ($n = 4$). Bands were analyzed densitometrically. Error bars represent mean + SD, $n = 3$. The data shown are representative of three independent experiments. ***$p<0.001$; Student's *t*-test.

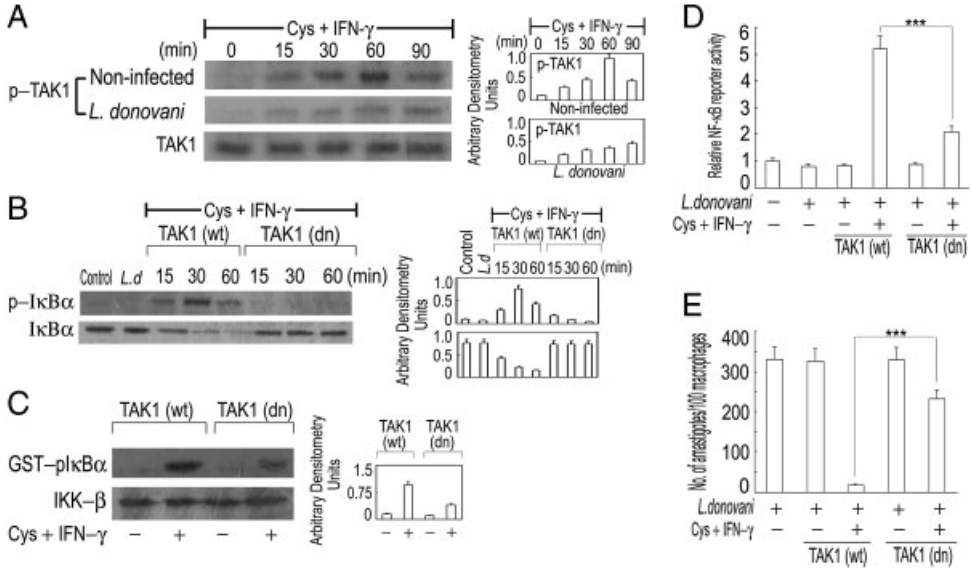

Figure 4. Role of TAK1 in cystatin plus IFN-γ induced NF-κB activation. (A) Both non-infected (upper row) and *L. donovani*-infected RAW 264.7 cells (lower row) were treated with cystatin plus IFN-γ for various time periods (0–90 min) and the levels of total and phosphorylated TAK1 were measured by Western blot analysis. (B) Infected cells (24 h) were transiently transfected with wt or dn-TAK1 expression plasmid (24 h) and stimulated with cystatin plus IFN-γ for the indicated times. Levels of total and phosphorylated IκBα were determined by Western blot analysis. (C) Infected macrophages were transfected with wt or dn-TAK1 expression plasmid for 24 h followed by stimulation with cystatin plus IFN-γ for 1 h. IKK activity was measured as described in the legend of Fig. 1B. (D) Infected macrophages (24 h) were transfected with pNF-κB-luciferase plasmid (1 μg) and 0.5 μg of pCMV-β-galactosidase together with wt or dn-TAK1 expression plasmid. After 24 h of transfection, cells were stimulated with cystatin plus IFN-γ for 24 h, lysed and processed for luciferase activity ($n = 4$). (E) Infected cells were transfected with either wt or dn-TAK1 constructs, followed by treatment with cystatin plus IFN-γ for 24 h. Intracellular parasite number was determined by Giemsa staining. Results are representative of four individual experiments. Data represent mean + SD, $n = 3$. ***$p<0.001$; Student's *t*-test.

were transiently transfected with dn constructs of MyD88, IRAK1 and TRAF6. As demonstrated in Fig. 5A, cystatin plus IFN-γ-induced phosphorylation of TAK1 was significantly diminished in infected macrophages overexpressing dn constructs of MyD88, IRAK1 and TRAF6 (3.2-, 3.3- and 2.9-fold, respectively) suggesting the involvement of MyD88-signaling in cystatin plus IFN-γ-mediated activation of TAK1. This was further reflected in cystatin-induced downstream NF-κB luciferase activity, which was substantially reduced in infected RAW 264.7 cells overexpressing dn-MyD88 (2.7-fold), dn-IRAK (2.5-fold) and dn-TRAF6 (2.6-fold) (Fig. 5B). Cystatin-induced inhibition of amastigote multiplication was also found to be markedly attenuated by overexpression of dn constructs of MyD88, IRAK1 and TRAF6 (73, 69 and 67% reduction in parasite killing, respectively) (Fig. 5C). These results suggest that anti-leishmanial effect of cystatin required upstream involvement of MyD88, IRAK and TRAF6. Since MyD88-dependent activation of NF-κB is necessary to induce anti-leishmanial effector response by cystatin, it might therefore induce the expression of TLR in infected macrophages. We studied the expression of various TLR in infected RAW 264.7 cells as well as BMM following cystatin plus IFN-γ treatment. As shown in Table 1, of all the TLR examined, cystatin plus IFN-γ led to a remarkable induction of TLR2 (3.8-fold) and TLR4 (3.5-fold) expression in infected macrophages at 12 h attaining maximal level at 24 h (8.3- and 8.7-fold for TLR2 and TLR4, respectively) and still significant after 36 h (2.9- and 2.6-fold for TLR2 and TLR4, respectively). The mRNA levels for remaining TLR did not change or were marginally increased. The expression patterns of all TLR observed in RAW 264.7 cells were similar to those observed in BMM (data not shown). To further assess the possible role of TLR2 and 4 in cystatin-mediated anti-leishmanial effector response, siRNA mediated knock-down system was used. As shown in Fig. 5D, transfection of RAW 264.7 cells with siRNA directed towards TLR 2 and TLR 4 significantly abrogated cystatin plus IFN-γ-mediated-NF-κB activation of luciferase expression (2.9- and 3.3-fold reduction for TLR2 and 4, respectively) as well as parasite suppressive effect (65 and 71% reduction in parasite killing for TLR2 and 4, respectively) (Fig. 5E). In order to check the presence of undetected contaminants in the parasite culture, macrophages (10^6 cells) were stimulated with culture supernatants obtained from 10^7 promastigotes grown at different time periods. mRNA expression profiling of iNOS and TNF-α by real-time PCR analysis after 24 h of incubation showed no alteration in culture supernatant-treated macrophages as compared with control macrophages (Fig. 5F). Since our cystatin preparation contains ~25 pg/mL LPS, we checked the iNOS and TNF-α profiling in macrophages treated with IFN-γ either alone or in combination with LPS. LPS (30 pg/mL) plus IFN-γ did not significantly induce iNOS and TNF-α expression (1.2- and 1.35-fold, respectively) compared with IFN-γ alone suggesting thereby that cystatin-mediated effect was not due to endotoxin contamination (Supporting Information Fig. 1). Collectively, these results suggest that TLR 2/4-MyD88-dependent activation of

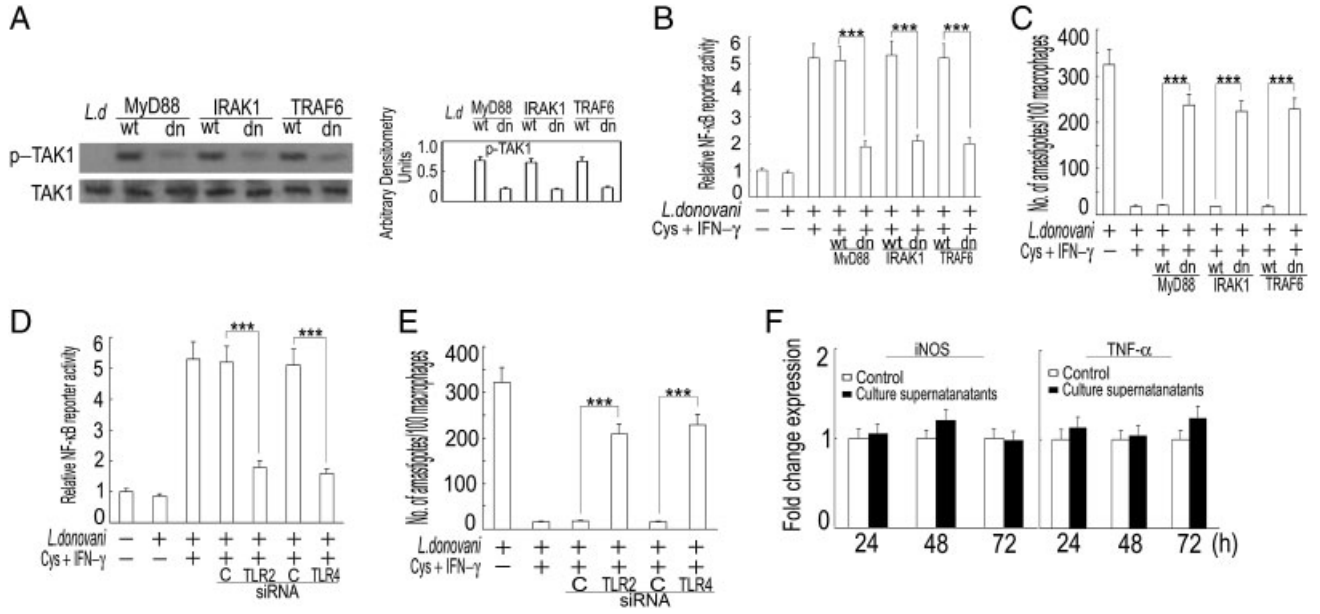

Figure 5. Effect of MyD88, IRAK1 and TRAF6 on cystatin plus IFN-γ-induced anti-leishmanial response. (A) *L. donovani*-infected RAW 264.7 cells were transiently transfected with wt or dn expression plasmids of MyD88, IRAK1 and TRAF6 for 24 h followed by stimulation with cystatin plus IFN-γ for 90 min. Levels of total and phosphorylated TAK1 were measured by Western blot analysis. (B) Infected cells were transiently transfected with various constructs along with pNF-κB-luciferase plasmid (1 μg) as indicated in (A), followed by stimulation with cystatin plus IFN-γ for 24 h, lysed and processed for luciferase activity ($n = 4$). (C) Infected cells were transiently transfected with various constructs as indicated in (A), followed by stimulation with cystatin plus IFN-γ for 24 h. Intracellular parasite number was determined by Giemsa staining. To determine the effect of TLR inhibition, macrophages were transfected (24 h) with TLR2 and TLR4 siRNA or control siRNA, infected and treated with cystatin plus IFN-γ as mentioned above. Cells were processed for luciferase activity (D) and intracellular parasite number was determined by Giemsa staining (E). (F) *L. donovani* promastigotes were grown for different time periods. BMM (10^6 cells) were stimulated with promastigote (10^7)-culture supernatants of each time point. iNOS and TNF-α mRNA expression were evaluated by real-time PCR analysis after 24 h of incubation. Experiments were done at least three times each and one set of representative data is shown. Error bars represent mean+SD, $n = 3$. ***$p<0.001$; Student's *t*-test.

Table 1. Real-time PCR for various TLR in cystatin plus IFN-γ-treated macrophages[a)]

Gene name	Assay ID	12 h	24 h	36 h
TLR 1	Mm00446095_m1	1.4±0.2	1.6±0.4	1.3±0.3
TLR 2	Mm00442346_m1	**3.8±0.4**	**8.3±1.1**	**2.9±0.4**
TLR 3	Mm00446577_g1	1.5±0.3	1.8±0.5	1.6±0.3
TLR 4	Mm00445273_m1	**3.5±0.6**	**8.7±1.2**	**2.6±0.5**
TLR 5	Mm00546288_s1	1.1±0.3	1.3±0.5	1.7±0.4
TLR 6	Mm02529782_s1	1.5±0.4	1.7±0.6	1.2±0.3
TLR 7	Mm00446590_m1	1.1±0.3	1.4±0.5	1.5±0.4
TLR 8	Mm01157262_m1	1.3±0.4	1.6±0.3	1.4±0.5
TLR 9	Mm00446193_m1	1.2±0.3	1.4±0.4	1.7±0.3
TLR 11	Mm01701924_s1	0.9±0.3	1.1±0.4	1.3±0.4

[a)] Values are fold change relative to infected samples.

NF-κB is essential for cystatin-induced anti-leishmanial effector response.

Restoration of lys-63-linked ubiquitination of TRAF6 by cystatin

We then examined the integrity of the protein–protein interactions between intracellular molecules of the TLR pathway in infected and cystatin plus IFN-γ-treated BMM. Because binding of TAK1–TAB2 complex to TRAF6 is a crucial step in the activation of TAK1 catalytic activity [6, 8], we analyzed the association of TAK1–TAB2 complex to TRAF6 by coimmunoprecipitation. Cell lysates from *L. donovani*-infected and cystatin plus IFN-γ-treated infected cells were subjected to immunoprecipitation using an anti-TAB2 antibody and immunocomplexes were analyzed by Western blotting with anti-TAK1 and anti-TRAF6 antibody (Fig. 6A). TAK1 was constitutively associated with TAB2 in both control and *L. donovani*-infected cells. However, TRAF6 did not immunoprecipitate with TAB2 obtained from infected cells. In contrast, cystatin plus IFN-γ treatment rapidly induced coimmunoprecipitation of TRAF6 with TAB2 from infected cells suggesting association of TRAF6 with TAK1–TAB2 complex and thereby activating TAK1-mediated IKK-NF-κB pathway. TLR signaling is thought to induce TRAF6 aggregation resulting in TRAF6 auto-ubiquitination at lys-63 residue, a required event for activation of TAK1 and downstream signal transduction [7, 8]. The role of TRAF6 ubiquitination is to provide an assembly scaffold for binding of downstream components to lys-63 ubiquitin. We observed that treatment of infected BMM with cystatin plus IFN-γ rapidly induced lys-63-linked polyubiquitination of TRAF6 (Fig. 6B), which is essential for its interaction and subsequent activation of TAK1.

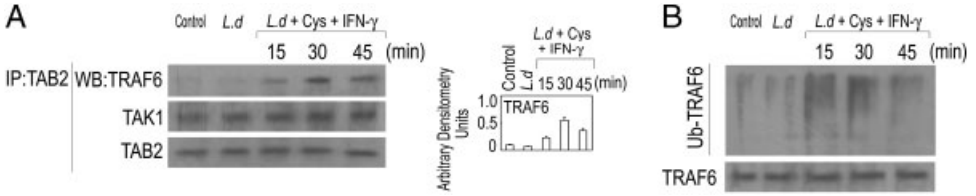

Figure 6. Cystatin plus IFN-γ-induced assembly of TRAF6–TAK–TAB complex. (A) *L. donovani*-infected (24 h) BMM were treated with cystatin plus IFN-γ for indicated times. The cells were lysed and subjected to immunoprecipitation and Western blot. IP: immunoprecipitation using the indicated antibody; WB: Western blot analysis using the indicated antibody. (B) Infected (24 h) BMM were treated with cystatin plus IFN-γ for the indicated times. The cell lysates were immunoprecipitated with Anti-TRAF6 antibody and were analyzed for polyubiquitination state by Western blot using a polyubiquitin-specific antibody. Bands were analyzed densitometrically and expressed as mean+SD, $n = 3$. Results are representative of three individual experiments.

Discussion

Parasites of the genus *Leishmania* rapidly alter several macrophage-signaling pathways in order to hijack the innate immune response, thereby favoring their invasion and survival within the host cell [29]. We previously elucidated the dual role of cystatin in suppressing the functional differentiation of Th2 type $CD4^+$ T cells, leading to the augmentation of Th1 response, and also upregulation of NO, resulting in the elimination of *Leishmania* parasites in both *in vitro* and *in vivo* experimental models of visceral leishmaniasis [27]. This was not related to cysteine protease inhibitory activity because saturation of cysteine protease inhibitory site by inactivated papain did not interfere with cystatin-induced NO release from activated macrophages [27]. Moreover, NO stimulatory site was different from cysteine protease inhibitory site and was identified to be present on the N-terminal 10-mer peptide sequence. This 10-mer peptide mimicked the effect of the whole cystatin molecule both in terms of NO upregulation and anti-leishmanial action [30]. We further showed that cystatin acts on the host cell signaling machinery resulting in the activation of MAPK pathway and transcription factor NF-κB. This, in turn, leads to the generation of NO and pro-inflammatory cytokines, which are responsible for the anti-leishmanial activity of cystatin [28]. The residual LPS content (<95 pg/mL) present in our culture system may not be significant in the present context as very negligible induction of iNOS and TNF-α mRNA expression was observed in case of LPS (100 pg/mL) plus IFN-γ (100 U/mL) treatment in *L. donovani*-infected macrophages (Supporting Information Fig. 2). In the present study we demonstrated that activation of NF-κB is absolutely essential for inducing proinflammatory gene expression and analysis of upstream signaling events revealed that TLR2/4 mediated MyD88-dependent participation of IRAK1-TRAF6-TAK1 is necessary to induce cystatin-mediated IKK/NF-κB activation in macrophages. In addition, cystatin activated the IKK complex to phosphorylate p105, triggering its proteolysis. Consequently, Tpl-2 is liberated from p105 thereby inducing activation of the MEK/ERK MAPK cascade. Moreover, cystatin plus IFN-γ-induced IKKβ post-transcriptionally modified p65/RelA subunit by dual phosphorylation directly on Ser-536 and indirectly on Ser-276 *via* Tpl-2/MEK/ERK/MSK1 sequence.

The importance of NF-κB in host defense against *Leishmania* infection has been clearly established by studies in mice deficient in different NF-κB family members [31]. NF-κB1 plays a key role in the adaptive immune response to *L. major* and is required for $CD4^+$ T-cell proliferation and development of Th1 response [32]. In contrast, NF-κB2 and c-Rel are involved in the innate responses to this intracellular pathogen [33, 34]. Moreover, macrophage from c-Rel-null mice showed a marked reduction in NO production and parasite killing compared with that from WT mice [35]. We have also demonstrated that cystatin might exert its curative effects on leishmaniasis through its ability to activate NF-κB by IKK-mediated IκB degradation resulting in *in vivo* induction of proinflammatory gene expression of TNF-α and iNOS. Moreover, cystatin plus IFN-γ induced the activation of Tpl-2, a serine-threonine kinase regulated by IKK. Release of Tpl-2 occurred as a consequence of IKK-mediated phosphorylation and subsequent proteolytic degradation of Tpl-2-associated p105. The IKK-dependent mechanism of Tpl-2 activation raises the question of the physiological advantage of linking cystatin activation of ERK and NF-κB *via* MSK1. It was demonstrated that induction of both TNF-α and COX-2 by LPS is blocked in Tpl-2-deficient BMM due to impairment in the activation of the ERK MAPK cascade [9, 36]. Transcriptional induction of both TNF-α and COX-2 by LPS involved co-ordinated activation of ERK and NF-κB [37, 38]. In the present study we observed that TNF-α synthesis and iNOS expression *in vivo* are under the control of NF-κB signaling cascade in cystatin-treated mice. Correlating the *in vitro* Tpl-2 activation data with the *in vivo* activation of NF-κB by IKK, it seems that ERK is activated simultaneously with NF-κB, facilitating induction of proinflammatory gene expression by cystatin. In addition, cystatin modulated IKK-induced phosphorylation of NF-κB p65 subunit at Ser-536 and Ser-276 residues. This is in accordance with a number of reports, which suggest that being a p65/RelA kinase, IKK post-translationally modifies the p65 subunit by phosphorylation, thereby promoting the transcriptional activation of NF-κB and the subsequent production of inflammatory cytokines [15, 16]. TLR/MyD88-dependent signaling is being implicated as essential for the immune responses against *Leishmania* parasites. A recent report showed

that MyD88 was important for dendritic cell recognition of *L. braziliensis* promastigotes *in vitro* and for the control of infection *in vivo* because of significant impairments in T-cell responses and protective immunity in MyD88$^{-/-}$ mice [26]. Moreover, *Leishmania* could interfere with TLR signaling by altering IRAK1's capacity to dissociate from the MyD88 complex [33]. Analysis of upstream signaling events in the present study revealed that inhibition of MyD88, IRAK1 and TRAF-6 strongly inhibited cystatin-induced NF-κB activation and parasite-suppressive effect in infected RAW 264.7 cells. In addition, cystatin-induced phosphorylation of TAK1 was substantially reduced in infected cells transfected with dn constructs of MyD88, IRAK1 and TRAF6. Since TAK1 is crucial for cystatin-induced activation of IKK/NF-κB pathway, these observations further strengthened the involvement of MyD88-dependent signaling in cystatin-mediated NF-κB activation. TRAF6 belongs to a member of E3 ubiquitin ligase family, and its auto-ubiquitination with lys-63 linked chains is necessary for TAK1 activation and ligand induced NF-κB activation [7]. Cystatin plus IFN-γ caused rapid induction of lys-63 ubiquitination of TRAF6 for facilitating its interaction with TAK1–TAB complex and thereby promoting activation of IKK-NF-κB pathway.

MyD88-dependent activation of NF-κB is primarily dictated by TLR and a number of *in vitro* and *in vivo* studies have already documented the importance of various TLR in host defense against different forms of leishmaniasis [20, 21, 25]. Control of *L. major* replication correlates with the early induction of iNOS in TLR4-competent mice, whereas increased parasite survival in host cells from TLR4 null mice is associated with higher activity of arginase, an enzyme essential for parasite growth [21]. Binding of *L. major* lipophosphoglycan to TLR2 enhances IFN-γ and TNF-α production and nuclear translocation of NF-κB in human NK cells [24]. Cystatin plus IFN-γ induced the expression of TLR2 and 4 in infected phagocytic cells and silencing of these receptors markedly attenuated the leishmanicidal activity of cystatin thereby suggesting the importance of downstream MyD88-dependent signaling in anti-leishmanial effector response.

In summary, the present study demonstrated that cystatin plus IFN-γ induced TLR/MyD88 signaling resulting in activation of IKK complex. IKK, in turn, induced p65 transactivation *via* Tpl-2/ERK pathway. TLR 2/4-induced IKK/NF-κB signaling in association with p105-Tpl-2-ERK pathway steers the course of cystatin-mediated host-protective immunological response in experimental visceral leishmaniasis.

Materials and methods

Reagents, Ab and constructs

Chicken cystatin (egg white) was obtained from Sigma (St. Louis, MO) and its purity is ≥95% (GE). All Ab were purchased form Cell Signaling Technology and Santa Cruz Biotechnology. BAY 11-7082 and BAY 11-7085 were obtained from Calbiochem. dn MyD88 (152–296) and IRAK1 (1–217) were kindly provided by Dr. M. Muzio, (Department of Immunology and Cell Biology, Mario Negri Institute, Milan, Italy) [39, 40]. WT and dominant-negative TRAF6 were gifts from Dr. J. Inoue (Institute of Medical Science, University of Tokyo,Tokyo, Japan) [41]. dn (K63W) TAK1 was a gift from K. Matsumoto (Nagoya University, Nagoya, Japan) [42]. pCMV5-Flag-WT MSK1 (MSK1-Wt), pCMV-Flag-MSK1-A195/N-terminal kinase dead (MSK1-Nd) were obtained from Dr D.R. Alessi (Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Scotland, UK) [43]. All other chemicals were purchased from Sigma-Aldrich unless otherwise mentioned. The pNF-κB-luciferase plasmid containing five copies of NF-κB consensus sequences (pNFκBLuc) were obtained from Stratagene. A pCMV-β-galactosidase reporter vector was purchased from Promega.

Parasites, cell culture and infection

The pathogenic strain of *L. donovani* promastigotes (MHOM/IN/1983/AG83) were maintained as described previously [27]. BMM were prepared, as described earlier [44], from the femurs and tibias of 6–8 wk old BALB/c mice. Splenocytes were cultured as described previously [27]. The murine macrophage cell line RAW 264.7 was cultured in RPMI 1640 containing 10% FCS and antibiotics as described previously [28]. *In vitro* infection experiments were carried out with macrophages using stationary phase promastigotes at a 10:1 parasite/cell ratio as described earlier [28]. LPS was quantified by a sensitive colorimetric LPS assay (QCL-1000, BioWhittaker, Walkersville, MD). LPS content of the reagents (cystatin and IFN-γ) was found to be less than 25 pg/mL. The concentration of LPS in media and FCS was less than 30 pg/mL. The parasite cultures and the final culture supernatants of cystatin plus IFN-γ-treated infected BMM and RAW 264.7 cells contained less than 95 pg LPS/mL.

In vivo priming and assessment of infection

Female BALB/c mice (20–25 g) were injected *via* the tail vein with 10^7 *L. donovani* promastigotes. Cystatin (5 mg/kg/day) in combination with suboptimal dose of IFN-γ (5×10^5 U/kg/day) were administered through tail vein on the 15th day post-infection for 4 consecutive days. Visceral infection was assessed by removing spleen from infected mice; multiple impression smears were prepared and stained with Giemsa. Spleen parasite burdens, expressed as Leishman–Donovan units (LDU), were calculated as the number of parasites *per* 1000 nucleated cells × organ weight (in grams) [27]. 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.

Real-time PCR

Total RNA was isolated from splenocytes, BMM or RAW 264.7 cells using the RNeasy Minikit (Qiagen) and quantitative real-time PCR were performed as described earlier [44] using TaqMan Fast Universal PCR Master Mix (Applied Biosystems). TaqMan probes for all TLR (Table 1) and iNOS were also purchased from Applied Biosystems. Relative quantitation was performed using the comparative $\Delta\Delta Ct$ method according to the manufacturer's instructions.

ELISA

The level of TNF-α in the single-cell suspension of spleen cells was measured as described previously using a sandwich ELISA Kit (Quantikine M; R&D systems) [44].

IKK assay

Cystatin plus IFN-γ-treated infected BMM were lysed in cold kinase assay lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM EDTA, 500 mM NaCl, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM Na_3VO_4, 10 mM pNPP, 1 mM benzamidine, 2 μM PMSF, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM DTT and 0.25% Nonidet P-40). IKK activity was measured as described earlier [45]. Briefly, the cell lysates (300 μg) were immunoprecipitated with anti-IKKβ Ab in immunoprecipitation buffer [45] followed by incubation with recombinant IκBα (4 μg) in kinase buffer [45] at 30°C for 1 h. The kinase reaction was stopped by addition of SDS-sample buffer. For visualization of phosphorylated IκBα, the reaction was subjected to SDS PAGE, dried and autoradiographed. Quantification of the radioactive bands was performed with a PhosphorImager. Western blotting for IKKβ was performed as a loading control by using anti-IKKβ antibody.

EMSA

Nuclear extracts from BMM were isolated and EMSA for NF-κB was performed as described earlier [45].

Tpl-2 kinase assay

To assay Tpl-2 MEK kinase activity, BMM (10^7 cells) were plated in 90-mm dishes (Nunc). After appropriate treatment, cells were washed with ice-cold PBS, lysed in 1 mL of kinase lysis buffer (Cell Signaling) and immunoprecipitated with agarose coupled anti-Tpl-2 antibody (Santa Cruz Biotechnology). After 4 h of incubation at 4°C, beads were washed four times with lysis buffer, twice with kinase buffer then resuspended in 25 μL of kinase buffer containing 1 μM ATP, 0.5 μg of GST-MEK1 (Upstate Biotechnology) and 1 μg of GST-ERK (Upstate Biotechnology). The beads were incubated on a shaker at 30°C for 30 min, pelleted and 4 μL of the supernatant was added to a reaction mix containing 10 μL of kinase buffer, 0.1 μM ATP, 50 μg of myelin basic protein (MBP) (Upstate Biotechnology), 10 μCi [γ-^{32}P] ATP and incubated at 30°C for 10 min. The assay was terminated by adding 2 × SDS sample buffer and labeled MBP was visualized by autoradiography. Tpl-2 bound to agarose beads was eluted with 0.2 M glycine (pH 2.4) and analyzed by immunoblotting to normalize the kinase assay.

Immunoprecipitation and Western blot

To analyze p105-free Tpl-2, lysates were first immunodepleted twice for 2 h with p105 antibody or control immunoglobulin coupled to protein A-Sepharose. Subsequently, these cleared lysates were immunoprecipitated with anti-Tpl-2 antibody, anti-p105 antibody or control IgG. Immunoblot was performed as described previously [28]. Densitometric analyses for all experiments were carried out using QUANTITY ONE software (Bio-Rad, Hercules, CA, USA). Band intensities for immunoblots were normalized to β-actin and expressed in arbitrary units.

MSK1 activity assay

MSK1 assay was performed as described previously [28]. *In vitro* kinase assays were carried out on MSK1 immunoprecipitates using the synthetic peptide EILSRRPSYRK (CREBTIDE) as substrate. Kinase activity is expressed in c.p.m. of [^{32}P] ATP incorporated in the substrate.

Transient transfection and NF-κB reporter assay

RAW 264.7 cells (2×10^6) were transfected with the appropriate constructs along with luciferase reporter plasmid and β-galactosidase reporter expression vector as described previously [28]. For promoter activation analysis, luciferase activity assays were performed in a luminometer, and the results were normalized for transfection efficiencies by assay of β-galactosidase activity. For siRNA transfection, cells were transfected with 1 μg of appropriate siRNA or control siRNA according to the manufacturer's instructions (Santa Cruz Biotechnology).

Statistical analysis

Data shown are representative of at least three independent experiments unless otherwise stated as n values given in the legend. Macrophage cultures were set in triplicate and the results are expressed as the mean ± SD. Statistical significances of differences among pair of data sets were determined by Student's t test with a p value <0.05 considered to be significant.

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Abbreviations: **BMM:** BM-derived macrophages · **IKK:** IκB kinase · **IRAK:** IL-1R-activated kinase · **LDU:** Leishman–Donovan units · **MBP:** myelin basic protein · **MSK1:** mitogen and stress-activated protein kinase 1 · **TAK:** TGFβ-activated kinase · **Tpl-2:** tumor progression locus 2 · **TRAF:** TNF receptor-associated factor

Full correspondence: Dr. Pijush K. Das, Molecular Cell Biology Laboratory, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Kolkata 700032, India
Fax: +91-33-2473-5197
e-mail: pijushdas@iicb.res.in

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