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Inhibition of CD40-Induced MAPK Activated Protein Kinase 2 (MK2) Reduces *Leishmania major* Infection

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Abstract: CD40, a macrophage expressed costimulatory molecule induces counteractive immune responses to *Leishmania major* infection through p38 MAPK and ERK1/2. CD40- induced IL10 or IL12 plays crucial role in promotion or protection for *L. major* infection, respectively. Because MK2 and RSK-1/2 are MAP Kinase activated protein kinases downstream of p38 and ERK1/2, respectively, we tested the role of CD40 in the regulation of these MAPKAPKs in *L. major* infection. In *Leishmania* infected macrophages we found phosphorylation and activation of MK2 like p38 was reduced and thus there was reduction in synthesis of IL12 and IL10. MK2a inhibitor of MK2 enhanced the CD40 induced anti leishmanial functions and significantly protected susceptible BALB/c mice from *L. major* infection. To the best of our knowledge the present study illustrates for the first time that CD40 regulates MAPKAPKs phosphorylation and activation while *Leishmania* targets MK2 in order to manipulate CD40 towards ERK1/2 dependent IL10 production. The findings reveal a novel parasite – devised immune strategy and an effective target to redirect CD40 – regulated immune response.

Keywords: *Leishmania*, MAPKAPK2, CD40, Macrophages, Phosphorylation

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Introduction

CD40 is a macrophage expressed costimulatory molecule that interacts with the T cell expressed CD40 L and signals through the ERK1/2 and p38 MAPK to differentially regulate IL10 and IL12 production, respectively (Mathur *et al.*, 2006; Rub *et al.*, 2009). IL10 and IL12 are two cytokines responsible for disease promotion or prevention, respectively in *Leishmania major* infection. CD40 induced p38 MAPK pathway activation leads to

IL12 production whereas ERK1/2 activation is associated with IL10 production (Mathur *et al.*, 2004, 2006). In *L. major* infection p38 MAPK phosphorylation is decreased whereas ERK1/2 phosphorylation enhanced (Awasthi *et al.*, 2003; Mathur *et al.*, 2004). MAPKs catalyse the phosphorylation and activation of several protein kinases, termed MAPK- activated protein kinases (MKs). The MK family comprises the $\approx$ 90 kDa

ribosomal S6 kinase (RSKs), the mitogen and stress-activated kinase (MSKs), the MAPK interacting kinases (MNKs), MAPK-activated protein kinases 2 and 3 (MK2 and 3, also termed MAPKAP-K2 and -3) and MAPK-activated protein kinase 5 (MK5, also termed MAPKAP-K5). MK family comprises 11 members that are activated by mitogens through the ERK1/2 kinase cascade (RSK1, RSK2, RSK3, RSK4 and MNK2), stress stimuli through the p38 kinase cascade (MK2, MK3 and possibly MK5), or both (MSK1, MSK2 and MNK1) (Philippe *et al.*, 2004). As RSKs are exclusively phosphorylated through the ERK1/2, and MK2 is exclusively phosphorylated through the p38, we focussed our studies on MK2 and RSKs. Among these MKs, MK2 is most characterized member of the family. MK2 plays direct role in synthesis of proinflammatory cytokine such as TNF, IL6 and iNOS2 (Kotlyarov *et al.*, 1999, 2002). However, it is involved in production of anti-inflammatory cytokine IL-10 (Christian *et al.*, 2011). MK2-deficient mice shows increased susceptibility to *Listeria monocytogenes* infection (Lehner *et al.*, 2002). In contrast RSK1/2 is less characterized and its role in immune response remains unknown. RSK is very specific substrate of ERK1/2 (Philippe *et al.*, 2004). Thus, these reports imply that MK2 and RSKs are though activated by their upstream MAP kinase i.e. p38 and ERK1/2, their selective roles in immune response remains unknown.

In this study, we show that MK2 is differentially activated in *L. major* infected macrophages. CD40 stimulation of macrophages induces the phosphorylation and activation of these two MAPKAPs. CD40 induced p38 MAPK and ERK1/2 phosphorylation were significantly reduced by knockdown of MK2 using siRNA and also by MK2 inhibition by MK2a inhibitor, suggesting MK2 is activated mainly by p38, it is also activated by ERK1/2 to some extent. This resulted in significant decrease in IL10 synthesis leading to less parasite burden *in vitro*. Administration of MK2 inhibitor along with α CD40 resulted in decreased *L. major* infection in a

susceptible BALB/c mouse strain. Our data demonstrate for the first time the role of CD40 induced MAPKAPs activation in *L. major* infection. We also show how the *Leishmania* devises a novel MK2 targeted immune evasion strategy.

Materials and Methods

Reagents:

MK2 Inhibitor (MK2a) was purchased from Calbiochem (San Diego, CA). Abs specific to MK2, p-p38, p38, p-ERK1/2, ERK1/2, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ELISA Abs (IL-12, IL-10, IFN- γ , and IL-4) were from BD Biosciences (San Diego, CA).

Mice, parasites, and infection:

BALB/c mice and CD40-deficient mice (BALB/c background) were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were subsequently bred in the experimental animal facility at the National Centre for Cell Science, Pune, Maharashtra, India in Thoren caging units (Thoren Caging System, Hazleton, PA). *L. major* (strain MHOM/Su73/5ASKH) was maintained *in vitro* in RPMI 1640 medium (Life Technologies-BRL, Grand Island, NY), supplemented with 10% FCS (Life Technologies-BRL), and the virulence was maintained by passage through BALB/c mice. These organisms and animals were cared for in accordance with the Guidelines approved by the appropriate Ethical Committee at the NCCS, Pune where the experiments were carried out. Stationary-phase *L. major* promastigotes (2×10^6) were used to infect BALB/c mice subcutaneously in the hind footpad and were treated with MK2 inhibitor (i.p. 4 μ g /mouse) for 5 d beginning 2 d postinfection. Some mice were treated with anti-CD40 (i.p. 50 μ g/mouse; clone 3/23, a gift from Dr. G. Klaus, London, U.K.) for 3 days alternatively beginning day 4 postinfection. Disease severity was assessed by measurement of footpad swelling using a digital micrometer (Mitutoyo Japan) and parasite burden.

Macrophage culture and L. major infection of macrophages:

Thioglycolate-elicited BALB/c-derived peritoneal macrophages were cultured in RPMI 1640 medium, supplemented with 10% FCS. Macrophages were pretreated with different doses of MK2a inhibitor for 1 h, followed by infection with *L. major* promastigotes at a ratio of 1:10 for 6 h (Mathur *et al.*, 2004; Awasthi *et al.*, 2003). Extracellular parasites were washed and macrophages were cultured with or without anti-CD40 Ab or anti-IL-10 neutralizing Ab (10 µg/ml) for 60 h and Giemsa stained, followed by counting the number of amastigotes per 100 macrophages. The percentage infectivity in macrophages untreated and *L. major*-infected was 67–75%.

RT-PCR:

RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) as described previously (Mathur *et al.*, 2004; Awasthi *et al.*, 2003). RT-PCR for IL-10, IL-12p40, and GAPDH was performed using the specific primers (Awasthi *et al.*, 2003; Mathur *et al.*, 2004; Rub *et al.*, 2009) GAPDH Forward, 5'-GAGCCAAACGGGTCATCATC-3'; Reverse, 5'-CCTGCTTCACCACCTTCTTG -3'; IL-10 Forward, 5'-CTGCTATGCTGCCTGCTCTT-3'; Reverse, 5'-CTCTTCACCTGCTCCA CTGC-3'; IL-12p40 Forward, 5'-CACGCCTGAAGAAGATGACA-3; Reverse, 5'-GACAGAGACGCCATTCCACA-3.

Western blotting:

Cells were washed twice with chilled PBS and lysed in cell lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM EDTA, protease inhibitor mixture (Roche Applied Science, Mannheim, Germany), and phosphates inhibitor mixture (Pierce, Rockford, IL)]. Protein was quantified by a BCA kit (Pierce) and an equal amount of protein was run on SDS-PAGE. Resolved proteins were blotted to polyvinylidene difluoride (Millipore, Bedford, MA) and then blocked with

5% nonfat dried milk in TBST (25 mM Tris [pH 7.6], 137 mM NaCl, and 0.2% Tween 20). Membranes were incubated with primary Ab at 4 C overnight, washed with TBST, and incubated with HRP-conjugated secondary Ab. Immunoreactive bands were visualized with the Luminol reagent, purchased from Santa Cruz Biotechnology.

Small interfering RNA transfection:

The P388D1 macrophage cell line was used to carry out transient transfection studies. MK 2, control small interfering RNA (siRNA), transfection medium, and transfection reagent were procured from Santa Cruz Biotechnology. siRNA transfection was carried out according to the manufacturer's protocol. Briefly, 5×10⁵ cells were plated in 6-well plates 24 h before transfection. siRNA duplex (60 pmol) was used for transfection and cells were transfected in serum-free media. After 6 h, complete medium (1 ml, RPMI 1640 plus 10% FCS) was added; 12 h later, this medium was replaced with standard culture media. After 36 h, cells were stimulated with anti-CD40 and harvested for Western blot or quantitative RT-PCR analysis.

Cytokine ELISA:

Macrophages, either unstimulated or stimulated with CD40 (anti-CD40 Ab; 3 µg/ml), or lymph node cells stimulated with anti-CD3 (0.5 µg/ml) and anti-CD28 (2 µg/ml) Abs for 48 h and culture supernatants were assayed for IL-10/IL-12 or IL-4/IFN-γ, respectively, using OptEIA kits (BD Biosciences) as described previously (Rub *et al.*, 2009).

Statistical analysis:

The significance of difference between the means was calculated by using Student *t* test. The minimum number of mice used for *in vivo* experiments was five per group. The *in vitro* cultures were always set in triplicates. The results were plotted as mean ± SEM.

Results

MKs phosphorylation but not expression is altered in Leishmania major infection:

As change in expression of MAPKAPKs (MKs) can in principle be an indicator of their possible involvement in *L. major* infection, we checked the expression of MK2 in 72 h *L. major* infected macrophages by western blotting. It was seen that expression of MK2 remains unaltered (Fig. 1A). Next we studied whether CD40 regulates the phosphorylation of these MAPKAPKs. Also role of MK2 was further investigated in *Leishmania major* infection. BALB/c derived thioglycolate-elicited macrophage were stimulated with low, intermediate and high doses of anti CD40. It was observed that MK2 phosphorylation increases just like p38 with increasing dose of anti CD40. Further the infected mouse macrophages were stimulated with increasing dose of anti CD40. It was observed that MK2 phosphorylation decreases in the infection (Fig. 1B).

Studies of knockdown of MKs using siRNA:

We further examined whether these MAPKAPKs regulate the CD40-induced p38MAPK and ERK-1/2 phosphorylation. Knockdown of MK2 by specific small interfering RNA (siRNA) suppressed the expression of MK2. The control siRNA did not alter the expression of these MAPKAPKs. It was observed that decrease in MK2 expression by its siRNA resulted in reduced CD40-induced p38MAPK phosphorylation and reduced ERK-1/2 phosphorylation suggesting MK2 is phosphorylated and regulated by both i.e. p38 and ERK1/2 *in vitro* (Fig. 2A).

MK2 inhibition decreases CD40 induced phosphorylation of both p38 and ERK1/2:

Because both p38 and ERK1/2 inhibition resulted in decreased CD40 induced MK2 phosphorylation, we further tested effect of MK2a inhibitor - a pharmacological inhibitor. It was observed that MK2a inhibitor which inhibits phosphorylation of MK2, inhibits CD40 induced phosphorylation of p38 and ERK1/2 (Fig. 2B).

Fig. 1: (A) Profiling of MAPKAPKs expression in *L. major* infection. Peritoneal macrophages isolated from BALB/c mice and were infected with *L. major* at 1:10, MΦ: parasite ratio. After 72h of infection the macrophages were lysed and immunoblots were done. Data show mean $\pm$ SEM. MK2 expression after 72h of *L. major* infection. (B) CD40 differentially regulates MK2 phosphorylation in *L. major* infection: Uninfected and *L. major* macrophages either untreated or stimulated with 1µg/ml, 3µg/ml or 6µg/ml α CD40 antibody for the 15 min were lysed and subjected to western blot analysis of pMK2 (Thr334) and MK2 total protein is shown.

Studies of phosphorylation of p38 and ERK1/2 MAP kinase in uninfected and L. major infected macrophages treated with MK2a inhibitor along with α CD40 stimulation:

Uninfected and infected (72 h) macrophages were pre-treated with MK2a inhibitor at 75 µM for 90 min followed by stimulation of anti CD40- 3µg/ml for 15 min. Cells were lysed and subjected for western blot analysis. Phosphorylation of MK2 was found to be decreased in *L. major* infected macrophages similar to the decrease in the phosphorylation of their upstream kinase p38. Also almost complete phosphorylation of anti CD40 induced ERK1/2 was abolished in macrophages treated with MK2 inhibitor (Fig. 3).

MK2 inhibitor along with α CD40 reduces parasite load in vitro:

Because reduced phosphorylation of ERK1/2 and decreased IL10 cytokine synthesis in

macrophages are hallmarks for anti-leishmanial effects, we tested if MK2 inhibitor can prevent parasite multiplication *in vitro*. We observed that co-treatment of MK2 inhibitor anti-CD40 antibody resulted in decrease in parasites load (Fig. 4A). Parasite burden was assessed by Giemsa staining.(Fig. 4B).

Fig. 2: (A) MK2 siRNA studies : Western blot analysis of lysates of P388D1 cells transfected with MK2 siRNA or control siRNA for 48h and left either untreated or anti-CD40 antibody (3μg/ml) treated for 15 min. (B)MK2 inhibition decreases phosphorylation of both p38 and ERK. Uninfected macrophages were treated with MK2a inhibitor for 90min with increasing doses followed by stimulation with αCD40-3 μg/ml for 15 min and the cell were lysed and immunoblot analysis of phospho and total protein of MK2, p38 and ERK was done.

MK2 inhibitor protects the BALB/c mice from Leishmania major infection:

We investigated the role of CD40 in regulating the protective functions of MK2 inhibitor on the course *L. major* infection in susceptible BALB/c mice. Compared to the untreated control mice (Fig. 5A), those treated with MK2 inhibitor alone or in combination with anti-CD40 antibody had less footpad thickness (Figs. 5B, 6A) and parasite

burden (Fig. 6B). Lymph node cells from the infected mice treated with MK2 inhibitor alone or with anti-CD40 together produced significantly higher IFNγ (Fig. 6C) and less IL-4 (Fig. 6D) compared to the cells from untreated infected mice. It suggests that MK2 inhibitor augments anti-leishmanial functions of CD40 and thereby, it protects susceptible BALB/c mice from *Leishmania major* infection. However, the host protective effect of MK2 inhibitor was abrogated in CD40 deficient mice (Fig. 7) suggesting important role of MK2 in the function of CD40 in protection against *L. major* infection.

Discussion

We have demonstrated for the first time to our knowledge, how an intracellular parasite *L. major* for evading host immune response, exploits intracellular MAPKAPKs (MKs) particularly MK2. Although MAPKAPKs are main substrates and executioners of functions of MAP kinase (Philippe *et al.*, 2004), to our knowledge their role in CD40 signalling has never been addressed. We have previously proposed CD40 signalling proceeds by phosphorylation of MK2 downstream of p38 and RSK1/2 downstream of ERK1/2 as function of strength of CD40 signalling (Mathur *et al.*, 2004, 2006; Rub *et al.*, 2009). The current set of data indicates *L. major* preferentially skews CD40 signalling, towards MK2 and thus leading to more IL10 synthesis and increases chances of *Leishmania* survival intracellularly, revealing novel parasite employed immune evasion strategy.

In this report, we first studied whether CD40 regulate various MAPKAPKs downstream of MAPKs. It was observed that anti CD40 stimulation of BALB/c mouse macrophages in three different doses resulted in MAPKAPKs activation. CD40 cross linking with αCD40, augments p38 phosphorylation in macrophages at higher dose and inhibits ERK1/2 activation. While at lower dose of αCD40 stimulation in macrophages ERK1/2 phosphorylation is higher and p38 phosphorylation is down regulated

Fig. 3: Effect of MK2 inhibitor on MK2, p38 and ERK1/2 MAP kinase in uninfected and *L. major* infected mouse macrophages. Uninfected and (72 h) *L. major* infected macrophages were treated with MK2a inhibitor (75μM) for 90 min followed by stimulation with αCD40-3 μg/ml for 15 min and the cell were lysed and immunoblot analysis of phospho and total protein of MK2, p38 and ERK was done (Data of 2 experiments is shown).

Fig. 4: MK2 inhibitor along with αCD40 reduces parasite load in vitro. (A) Parasite burden assessed by Giemsa staining. (B) 5x10⁴ macrophages were pre treated with MK2 inhibitor for 1 h and then infected with *Leishmania major* promastigotes at a ratio of 1:10 for 6 h. The extracellular parasites were washed and macrophages were cultured with or without αCD40 (3 μg/ml) for 60 h. Cells were then washed, fixed with methanol and stained with Giemsa. The parasite load was counted microscopically and expressed as number of amastigotes per100 macrophages. Data show mean values ± SEM.

Fig. 5: (A) Foot pad thickness in Control BALB/c mice with *L. major* infection and (B) BALB/c mice with *L. major* infection treated with MK2 inhibitor and Anti CD40.

Fig. 6 (A-D): MK2 inhibitor protects the Balb/c from *L. major* infection: BALB/c mice were infected with *L. major* (2×10^6) subcutaneously in the hind foot pad; 2 days later, they were treated with MK2 inhibitor ($40 \mu\text{g}/\text{mouse}$; i.p.) for 5 days, some BALB/c mice were injected intraperitoneally with anti-CD40 antibody ($50 \mu\text{g}/\text{mouse}$) for 3 alternate days beginning 3 days after infection. Footpad thickness (A) assessed in mice weekly and parasite load (B) 5 weeks after infection. MK2 inhibitor plus anti-CD40 antibody treatment significantly reduced footpad thickness ($p=0.003$) and parasite load ($p=0.001$). Lymph node cells were stimulated with anti-CD3 and anti-CD28 antibody for 48h and IFN γ and IL-4 production was assessed by ELISA. IFN γ cytokine levels was significantly increased ($p=0.008$) and IL-4 cytokine levels was significantly reduced ($p=0.009$) in lymph node cells obtained from mice treated with MK2 inhibitor along with anti-CD40 as compared to cells from control (C) and (D). Data shows mean values $\pm$ SEM.

Fig. 7: Host protective effect of MK2 inhibitor was abrogated in CD40 deficient mice . BALB/c (CD40 Knock out) mice were infected with *L. major* (2×10^6) subcutaneously in the hind foot pad; 2 days later, they were treated with MK2 inhibitor ($40 \mu\text{g}/\text{mouse}$; i.p.) for 5 days. Footpad thickness (A) assessed in mice weekly and parasite load (B) 5 weeks after infection. No significant difference was observed.

(Mathur *et al.*, 2004). The increase in MK2 phosphorylation was observed similar to its upstream p38 kinase at higher dose of αCD40 . *Leishmania major* protozoan parasite skews CD40 signaling in macrophage towards ERK1/2 dependent IL10 production and inhibits anti-parasitic IL12 production (Mathur *et al.*, 2006). So we further checked the activation of MAPKAPKs in *Leishmania* infected macrophages and we found phosphorylation and activation of MK2 like as its upstream kinase p38 was reduced.

Inhibition of MK2 phosphorylation using MK2 inhibitor showed decrease in phosphorylation of both p38 and ERK1/2 and also decrease in synthesis of IL12 and IL10. MK2 silencing using MK2siRNA gave similar results. The treatment of MK2 inhibitor to macrophages along with anti CD40, leads to decrease in parasite load *in vitro*. Thus inhibition of MK2 phosphorylation using MK2 inhibitor showed inhibition in phosphorylation of p38 and ERK1/2. MK2 has direct role in IL10 synthesis as use of MK2 inhibitor almost nullifies the phosphorylation of ERK1/2 in infected macrophages decreasing IL10 synthesis to great extent and thus doesn't allow *Leishmania major* to survive in the macrophages. This was further studied by using MK2 inhibitor in BALB/c infected with *L. major* along with administration of αCD40 . Disease progression was

observed for 6 weeks. It was observed that there is significant decrease in footpad thickness and parasite load in mice treated with MK2 inhibitor and αCD40 together. Thus *Leishmania* targets MK2 in order to manipulate CD40 towards ERK1/2-dependent IL10 production. MK2-deficient mice shows increased susceptibility to *Listeria monocytogenes* infection (Lehner *et al.*, 2002). The role of MK2 in synthesis of proinflammatory cytokine such as TNF, IL6, iNOS2 is well documented (Kotlyarov *et al.*, 1999; Kotlyarov *et al.*, 2002). However, it is involved in production of anti-inflammatory cytokine IL-10 (Christian *et al.*, 2011). But contradictory we observed, knock down of MK2 by siRNA, MK2a inhibitor treatment decreased CD40 induced IL10 production. Previous reports suggested that LPS induced IL10 production is mediated through prominently by p38 MAPK and to lesser extent by ERK1/2 or JNK (Chi *et al.*, 2006).

This difference can be explained by the facts that CD40 induced IL10 is mediated through ERK1/2 activation and also that CD40 signalling reciprocally regulates IL10 and IL12 production (Mathur *et al.*, 2004, 2006; Rub *et al.*, 2009).

Conclusion

In this study, we show that MK2 silencing decreases ERK1/2 phosphorylation, which results

in decreased IL10 expression. Thus MK2 is related to IL10 via ERK1/2 activation. We conclude that CD40 stimulation activates MAPKAPKs. MK2 inhibition and MK2 silencing enhances CD40 induced anti-leishmanial effect.

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