Results
Results

1. Profiling of MKs expression in *Leishmania major* infection

In *Leishmania major*, we have shown previously that CD40-induced p38MAPK activation and IL-12 expression are suppressed whereas ERK-1/2 activation and IL-10 expression are enhanced. In uninfected macrophages, a strong CD40 stimulation induces preferential phosphorylation of p38MAPK and proinflammatory cytokine IL-12 production whereas a weaker CD40 stimulation induces preferential phosphorylation of ERK-1/2 and anti-inflammatory cytokine IL-10 production (34). To find whether such a reciprocal responses are present in MAPKAPKs downstream of the p38 and ERK1/2 in particular and their role in anti-CD40 signaling these studies were carried out.

MK2 and RSK1/2 are specifically regulated by phosphorylation by p38 MAPK and ERK-1/2 kinase respectively (63) and *L.*major preferentially modulates these MAPK pathways (8), as phosphorylation of p38 and ERK ½ was found to be altered in *L. major* infection MK2 and RSK1/2 were chosen for further study.

We checked whether MAPKAPKs expression in uninfected and *Leishmania major* infected mouse macrophages is altered or not. Thus peritoneal macrophages were infected with *L. major* for 72 hours and followed by lysis and immunoblot for MAPKAPKs. It showed there was no change in MK2 and RSK1/2 expression in infected macrophages. Thus expression remains unaltered.

![Image of immunoblot showing MK2, RSK1, RSK2, and Actin expressions in uninfected (UIM) and infected (IM) macrophages.](image)

**Figure-1**: 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 immunoblot was done. Data show mean ± SEM. MK2, RSK1 and RSK2 expression after 72 hours of *L. major* infection.
2. CD40 regulates MAPKAPK activation

As CD40 reciprocally modulates the activation of p38MAPK and ERK1/2 and as MK2 and RSK ½ are MAPKAPKs specifically phosphorylated and activated by p38MAPK and ERK1/2, respectively, we examined whether CD40 regulates the phosphorylation of these MAPKAPKs. BALB/c derived thioglycolate-elicited macrophages were stimulated with the indicated doses of anti-CD40 antibody for given time points. The lower dose of α CD40 (1µg/ml), middle dose of α CD40(3 µg/ml), and highest dose of α CD40(6 µg/ml) increased time dependent phosphorylation of MK2 and RSK1/2. The strong and sustained activation of MK2 and RSK1/2 starts at 5th minute and lasts till 30th minute. So further stimulation were done at 15th minute.

![Figure -2: CD40 regulates phosphorylation of MK2 and RSK1/2.](image)

Uninfected macrophages were stimulated with 1µg/ml, 3µg/ml or 6µg/ml αCD40 antibody for the indicated time points- were lysed and subjected to western blot analysis of phosphorylated and total proteins of MK2 and RSK ½.

3. *L. major* infection regulates the anti CD40 mediated phosphorylation of MAPKAPKs

Role of MK2 and RSK1 and RSK2 were further investigated in *Leishmania major* infection. It showed that these MAPKAPKs are not reciprocally regulated similar to their upstream MAP kinase i.e. p38 and ERK1/2 in macrophages infected in vitro with *L. major* parasite. Mouse macrophage were stimulated with low, intermediate and high doses of anti CD40. It was observed that MK2 phosphorylation increases just like p38 and RSK1/2 phosphorylation decreases like ERK1/2 with increasing dose of anti CD40. Further the infected mouse macrophages were stimulated with increasing dose of antiCD40, it was observed that MK2 phosphorylation decreases in the infection. RSK activation involves phosphorylation of residues. Thus in infection, there was differential residual phosphorylation observed i.e. Thr359 / Ser363, directly phosphorylated by ERK1/2 was found to be more phosphorylated with increasing dose of anti CD40. While consequent residues like Ser380, Ser227 phosphorylation was
decreased in infected macrophages i.e. RSK activation was found to be decreased in infection.

![Image](image1.png)

**Fig:3** CD40 differentially regulates MK2 and RSK1/2 phosphorylation (a) Uninfected and *L. major* macrophages either untreated or stimulated with 1μg/ml, 3μg/ml or 6μg/ml αCD40 antibody for the 15 minutes were lysed and subjected to western blot analysis of pMK2 (Thr334) and MK2 total protein (b) pRSK (Thr359/ Ser363), pRSK(Ser380), and final activation residue pRSK (Ser227)and total RSK1 and RSK 2 is shown.

4. Studies of knocking down of MK2 and RSK s using siRNA

We next examine whether these MAPKAPKs regulate the CD40-induced p38MAPK and ERK-1/2 phosphorylation. Knockdown of MK2 and RSK1 and RSK2 by specific small interfering RNA (siRNA) suppressed the expression of MK2 and RSK 1 and RSK2. The control siRNA did not alter the expression of these MAPKAPKs(Figure 4.1B, Figure 4.2B and Figure 4.3B)

![Image](image2.png)

**Figure- 4.1 MK2 siRNA studies** Kinetics of MK2 knockdown. (A). 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) and (C).

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It was observed that reduced 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 (Figure 4.1C).

Figure-4.2: RSK 1 siRNA studies  Kinetics of RSK1 knockdown (A). Western blot analysis of lysates of P388D1 cells transfected with RSK 1 siRNA or control siRNA for 48h and left either untreated or anti-CD40 antibody (3μg/ml) treated for 15 min (B).

RSK1 inhibition reduced CD40-induced ERK-1/2 phosphorylation and p38 phosphorylation(Fig4.2). RSK2 inhibition increased CD40-induced ERK-1/2 phosphorylation and decreased p38 phosphorylation. This suggest RSK2 is involved in negative feedback mechanism of ERK1/2 phosphorylton(Fig4.3).

Figure-4.3: RSK 2 siRNA studies Kinetics of RSK2 knockdown (A).Western blot analysis of lysates of P388D1 cells transfected with RSK 1 siRNA or control siRNA for 48h and left either untreated or anti-CD40 antibody (3μg/ml) treated for 15 min (B).
5. Effect of inhibitor of p38- SB203580 and inhibitor of ERK1/2 – PD098059 on anti CD40 mediated MAPKAP kinase signalling

The previous observation indicated that SB203580 when pretreated to macrophages and then stimulated with anti CD40 leads to reduced anti -CD40 induced activation of p38 but augmented anti –CD40 induced ERK1/2 (34). Also in accordance with earlier reports when ERK1/2 inhibitor –PD 098059 when pretreated to macrophages and then stimulated with antiCD40 leads decrease in anti-CD40 induced ERK1/2 phosphorylation and increase in anti –CD40 induced p38 phosphorylation(34).

Thus peritoneal macrophages were treated with increasing doses of SB203580- p38 inhibitor along with antiCD40 stimulation , it was found that p38 and MK2 phosphorylation decreases but ERK1/2 phosphorylation increases. Similarly the phosphorylation was found to be increased in residues of RSKs like Thr359 /Ser363 , Ser380 and Ser227.

![Figure 5.1 : Effect of p38 inhibitor on MKs.](image)

Uninfected macrophages were treated with p38 inhibitor SB203580 for 90minutes with increasing doses followed by stimulation with αCD40-3 µg/ml for 15 minutes and the cell were lysed and immunoblot analysis of phospho p38 and MK2 and total p38 , MK2 was done(a). While phospho and total ERK and various phosphorylation residues of RSK and total RSK is shown in(b).

When macrophages were treated with increasing dose of PD098059 –ERK inhibitor along with antiCD40 phosphorylation of the ERK1/2 was reduced . Similarly Thr 359/
Ser 363 phosphorylation of RSK1/2 was decreased. While in further serially activated residues of RSK1/2 like Ser 380/ Ser 227 there was no differences in phosphorylation whereas it was found that p38 phosphorylation increases but MK2 phosphorylation was found to be decreased.

![Image](image_url)

**Figure 5.2 : Effect of ERK inhibitor on MKs.** Uninfected macrophages were treated with ERK inhibitor PD098059 for 90 minutes with increasing doses followed by stimulation with αCD40-3 µg/ml for 15 minutes and the cells were lysed and immunoblot analysis of phospho p38 and MK2 and total p38, MK2 was done(a). While phospho and total ERK and various phosphorylation residues of RSK and total RSK is shown in(b).

### 6. Effect of MK2 inhibitor on anti CD40 mediated p38 and ERK1/2 MAP kinase and IL10-IL12 cytokine synthesis.

Because both p38 and ERK1/2 inhibition resulted in decreased CD40 induced MK2 phosphorylation, we further tested effect of MK2a inhibitor - a pharmacological inhibitor (131).

Mouse macrophages were treated with various doses of MK2 inhibitor along with anti CD40 stimulation till the inhibition of phosphorylation was observed in MK2, analysed by western blot. Then macrophages were treated for 90 minutes at these increasing doses, followed by 15 minutes of αCD40 stimulation. Cells were then lysed and subjected for western blot analysis of phospho and total proteins of MK2, p38 and ERK1/2. We found that MK2a inhibitor which inhibits phosphorylation of MK2, inhibits CD40 induced phosphorylation of p38 and ERK1/2.
We further confirmed effect of MK2a inhibitor by treating the macrophages with similar doses along with anti CD40 stimulation and checking IL10, IL12 and TNF-α expression by RT PCR. As MK2 plays key role in TNF-α synthesis, MK2 inhibition showed decrease in expression of TNF-α. Also MK2 inhibition showed decrease in expression of IL10 and IL12 which confirmed our findings of its effect on decrease in phosphorylation of p38 and ERK1/2.

![Figure 6](image)

**Figure 6**: (a) MK2 inhibition decreases phosphorylation of both p38 and ERK. Uninfected macrophages were treated with MK2a inhibitor for 90 minutes with increasing doses followed by stimulation with αCD40-3 µg/ml for 15 minutes and the cell were lysed and immunoblot analysis of phospho and total protein of MK2, p38 and ERK was done. (b) MK2 inhibition decreases the expression of IL12, IL10 and TNFα. RT-PCR analysis of IL12, IL10 and TNFα in uninfected macrophages treated with increasing doses of MK2a inhibitor for 90 minutes followed by stimulation with αCD40-3 µg/ml for 8 hours.

7. Studies of phosphorylation of various proteins and effect on IL-10/IL12 synthesis in uninfected and *L. major* infected macrophages treated with MK2a inhibitor and αCD40 stimulation.

Uninfected and (72 hours) infected macrophages were pre treated with MK2a inhibitor at 75 µM for 90 minutes followed by stimulation of anti CD40 -3µg/ml for 15 minutes. Cells were lysed and subjected for western blot analysis of phospho and total forms of various proteins like p38, MK2 –substrate of p38, Hsp27- substrate of MK2. Also
phospho and total forms of ERK½, RSK1/2 and Stat3- Substrate of ERK1/2 were analysed (Figure 7.1).

Phosphorylation of Hsp 27 and MK2 was decreased in *L. major* infected macrophages like the decrease in the phosphorylation of their upstream kinase p38 in MK2 inhibitor treated and anti CD40 stimulated macrophage. Also almost complete phosphorylation of anti CD40 induced ERK1/2 was abolished in macrophages treated with MK2 inhibitor. Stat 3, being involved in the synthesis of IL10, its phosphorylation was also studied and was found to be decreased just like ERK1/2 phosphorylation with treatment of inhibitor and anti CD40 stimulation.

**Figure 7.1 : Effect of MK2 inhibitor on various proteins in uninfected and *L. major* infected mouse macrophages.** Uninfected and (72 hours) *L. major* infected macrophages were treated with MK2a inhibitor (75µM) for 90 minutes followed by stimulation with αCD40-3 µg/ml for 15 minutes and the cell were lysed and immunoblot analysis of phospho and total protein of HSP27, MK2, p38 and ERK, stat 3 was done.
We next examined the effect of treatment of MK2 inhibitor and anti CD40 stimulation, on IL10- IL12 cytokine synthesis in uninfected and *L. major* infected macrophages. The supernatant collected after 90 minutes of inhibitor treatment and αCD40 stimulation for 48 hours. Decrease in synthesis of both IL10 and IL12 was seen in macrophages treated with inhibitor and anti CD40 treatment(Figure 7.2).

![Graphs](image)

**Figure 7.2**: MK2 inhibitor decreases both IL10 and IL12 cytokine levels. Uninfected macrophages were treated with MK2a inhibitor (75µM) for 90 minutes followed by stimulation with αCD40-3 µg/ml for 48 hours and the supernatants were harvested and IL10(a)-IL12(b) cytokine levels were analyzed by ELISA.

8. *Invitro Studies of effect of MK2 inhibitor on parasite load*

Because reduced phosphorylation of ERK1/2 and decreased IL10 cytokine synthesis in macrophages are hallmarks for anti leishmanial effects (34), 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 (Figure 8.1) Parasite burden was assessed by Giemsa staining. Distribution curve analysis of number of infected macrophages and number of amastigotes in macrophages was plotted (Figure 8.2).
Figure -8.1 : Parasite burden assessed by Giemsa Staining

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Figure-8.2: Distribution curve analysis (A) and statistical analysis (B) of number of infected macrophages and number of amastigotes in macrophages

9.MK2 inhibitor protects the BALB/c mice from *Leishmania major* infection

Because MK2 inhibitor, a pharmacological inhibitor of MK2 suppressed CD40-induced expression of anti-inflammatory cytokine and inhibited in vitro parasite
multiplication, we tested whether it can suppress *Leishmania major* infection in susceptible BALB/c mice. *Leishmania major* infected BALB/c mice were treated with MK2 inhibitor intra-peritoneally for five days. Disease progression was assessed by footpad thickness and parasite burden for five weeks. Treatment of BALB/c mice with MK2 inhibitor resulted in significantly reduced footpad thickness (Figure 9A) and parasitic burden (Fig.9B). Our data thus indicates that MK2 inhibitor indeed has antileishmanial potential.

**MK2 inhibitor enhances anti-leishmanial influence of CD40 in susceptible BALB/c mice**

Previous in vitro as well as in vivo results demonstrated that MK2 inhibitor, has antileishmanial functions. It inhibited CD40-induced p38MAPK phosphorylation and IL-12 expression also it inhibits ERK-1/2 phosphorylation, IL-10 expression and invtro parasite burden.

We next 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, those treated with MK2 inhibitor alone or in combination with anti-CD40 antibody had less footpad thickness (Fig-9A), parasite burden (Fig- 9B) and lymph node weight (Fig- 9C ) and lymph node cells from the infected mice treated with MK2 inhibitor alone or with anti-CD40 together produced significantly higher IFNγ (Fig-9F) and less IL-4 (Fig -9G) 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.

We further investigated the requirement of CD40 for mounting MK2 inhibitor dependent anti-leishmanial immune response in CD40-deficient mice. It was observed that host-protective effect of MK2 inhibitor was completely lost in CD40-deficient mice. CD40-deficient mice treated with MK2 inhibitor did not show any differences in the footpad thickness ( Fig-9D ) and parasite load ( Fig-9E ) as compared to the control untreated mice. All together, these data suggest an important role for MK2 inhibitor in the function of CD40 in protection against *L. major* infection.
Figure -9 : MK2 inhibitor protects the Balb/c from L. major infection. a) Weekly footpad thickness b)Lymph node weight c)Parasite load in BALB/c (CD40+/+) mice .BALB/c mice were infected with L. major (2x 10^6) subcutaneously in the hind foot pad; 2 days later, they were treated with MK2 inhibitor (40μg/mouse; i.p.) for 5days, some BALB/c mice were injected intra-peritoneally with anti-CD40 antibody (50μg/mouse) for 3 alternate days beginning 3 days after infection. Footpad thickness (A) assessed in mice weekly, lymph node weight (B) and parasite load (C) 5 weeks after infection. MK2 inhibitor plus anti-CD40 antibody treatment significantly reduced footpad thickness (p=0.003) and parasite load (p=0.004). Footpad thickness (D) and parasite load (E) in CD40 deficient (CD40−/−) mice infected with L. major and treated with MK2 inhibitor. (F and G) Lymph node cells were stimulated with anti-CD3 and anti-CD28 antibody for 48h and IFNγ and IL-4 production was assessed by ELISA. Data show mean values ± SEM.
10. Effect of lentivirus expressed MK2 shRNA on CD40-induced MAPK phosphorylation and effector functions

Previous results demonstrated that MK2 siRNA inhibited CD40-induced ERK-1/2 phosphorylation and also inhibits p38MAPK phosphorylation in P388D1 macrophage cell line. To specifically inhibit MK2 expression in peritoneal macrophages, lentivirus expressed MK2 shRNA was tested. It was observed that lentivirus expressed MK2 shRNA but not control shRNA inhibited MK2 expression in peritoneal macrophages (Figure – 10.1).

Inhibition of MK2 by MK2a inhibitor resulted in less parasite burden in the *Leishmania major* infected BALB/c mice however; a pharmacological inhibitor has always some non-specific effects. To rule out that possibility a more specific approach with lentivirus expressed MK2 shRNA was tested to check whether it can also recapitulate the same effect of MK2 inhibitor in *L. major* infected BALB/c mice. Indeed, the Lv-MK2 shRNA but not the control shRNA significantly enhanced the CD40-induced anti-leishmanial effect in BALB/c mice. It was observed that mice treated with Lv-MK2 shRNA alone or together with anti-CD40 had significantly less footpad thickness (Fig. 10.2A) and parasite burden (Fig.10.2B) compared to mice either untreated or treated with control-Lv. The effect of such treatments was not observed in CD40-deficient mice.

Administration of Lv-MK2 shRNA in *L. major* infected CD40-deficient mice did not show any differences in the footpad thickness (Fig.10.2D) and only marginal differences were observed in parasite burden (Fig.10.2E) suggesting the CD40 requirement for the anti-leishmanial effects exerted by the MK-2 shRNA.
Figure 10.2: Lv-MK-2 shRNA protects susceptible BALB/c mice from L. major infection. Mice were infected with L. major; 2 days later, they were injected with lentivirus expressing MK2 shRNA or control shRNA some BALB/c mice were injected intra-peritoneally with anti-CD40 antibody (50μg/mouse) for 3 alternate days beginning 3 days after infection. Footpad thickness (A) assessed in mice weekly, lymph node weight (B) and parasite load (C) 5 weeks after infection. MK2 shRNA plus anti-CD40 antibody treatment significantly reduced footpad thickness (p = 0.006 ) and parasite load (p=0.003). Footpad thickness (D) and parasite load (E) in CD40 deficient (CD40 -/-) mice infected with L. major and treated with MK2 shRNA (F and G) Lymph node cells were stimulated with anti-CD3 and anti-CD28 antibody for 48h and IFNγ and IL-4 production was assessed by ELISA. Data show mean values ± SEM.

11. Effect of RSK inhibitor – SL0101 on anti CD40 mediated p38 and ERK1/2 MAP kinase and IL10- IL12 cytokine synthesis.

Because both p38 and ERK1/2 inhibition resulted in decreased CD40 induced MK2 phosphorylation, we further tested effect of RSK inhibitor - a pharmacological inhibitor. Mouse macrophages were treated with various doses of RSK inhibitor-SL0101 along with anti CD40 stimulation till the inhibition of phosphorylation was observed in RSK, analysed by western blot. Then macrophages were treated for 90 minutes at these increasing doses, followed by 15 minutes of αCD40 stimulation .Cells were then lysed.
and subjected for western blot analysis of phospho and total proteins of RSK 1/2, p38 and ERK1/2. We found that RSK inhibitor which inhibits phosphorylation of RSK, inhibits CD40 induced phosphorylation of p38 and augments CD40 induced phosphorylation of ERK1/2.

We further confirmed effect of RSK inhibitor by treating the macrophages with similar doses along with anti CD40 stimulation and checking IL10, IL12 and TNF α expression by RT PCR. RSK inhibition showed increase in expression of IL10 and decrease in expression of IL12 and TNF α which confirmed our findings of its effect on decrease in phosphorylation of p38 and ERK1/2.

- **Figure 11**: (a) RSK inhibition decreases phosphorylation of p38 and augments phosphorylation of ERK1/2. Uninfected macrophages were treated with SL0101-RSK inhibitor for 90 minutes with increasing doses followed by stimulation with αCD40-3 µg/ml for 15 minutes and the cell were lysed and immunoblot analysis of phospho and total protein of RSK ½, p38 and ERK was done. (b) RSK inhibitor augments IL10 expression and decreases IL12 and TNF α production. RT-PCR analysis of IL12, IL10 and TNF α in uninfected macrophages treated with increasing doses of SL0101-RSK inhibitor for 90 minutes followed by stimulation with αCD40-3 µg/ml for 8 hours.
12. Studies of Phosphorylation of various proteins and IL10/IL12 synthesis in uninfected and *L. major* infected macrophages treated with RSK inhibitor- SL0101 and αCD40 stimulation.

Uninfected and (72 hours) infected macrophages were pre treated with RSK inhibitor SL0101 at 75 µM for 90 minutes followed by stimulation of anti CD40 -3µg/ml for 15 minutes. Cells were lysed and subjected for western blot analysis of phospho and total forms of p38 and ERK (Figure12.1).

There was increase in phosphorylation in ERK1/2 and decrease phosphorylation of p38 in *L.major* infected macrophages in SL0101 treated and anti CD40 stimulated macrophages.

![Image](image.png)

**Figure 12.1: Effect of RSK inhibitor on p38 and ERK1/2 MAPKinase in uninfected and *L.major* infected mouse macrophages.** Uninfected and (72 hours ) *L major* infected macrophages were treated with RSK inhibitor –SL0101(50µM ) for 90minutes followed by stimulation with αCD40-3 μg/ml for 15 minutes and the cell were lysed and immunoblot analysis of phospho and total protein of p38 and ERK was done.

We next examined the effect of treatment of RSK inhibitor-SL0101 and anti CD40 stimulation, on IL10- IL12 cytokine synthesis (Fig-12.2) in uninfected and *L. major* infected macrophages. The supernatant collected after 90 minutes of inhibitor treatment and αCD40 stimulation for 48 hours. Decrease in synthesis of IL12 cytokine was seen
in macrophages treated with inhibitor and anti CD40 treatment. But IL10 ELISA was not conclusive in repeated experiment.

![Graph a)](image1.png)

![Graph b)](image2.png)

**Figure 12.2:** RSK inhibitor decreases IL12 cytokine synthesis. Uninfected macrophages were treated with RSK inhibitor (50µM) for 90 minutes followed by stimulation with αCD40-3 µg/ml for 48 hours and the supernatants were harvested and IL10-IL12 (a and b) cytokine levels were analysed by ELISA.

13. In vitro Studies of effect of RSK inhibitor on parasite load

![Image 1](image3.png)

![Image 2](image4.png)

**Figure 13.1** Parasite burden assessed by Giemsa Staining
Results

Because there was increase in phosphorylation of ERK1/2 and in IL10 cytokine synthesis and decrease in phosphorylation of p38 and IL12 in macrophages treated with the SL0101, showing anti-inflammatory role of RSK, we tested if RSK inhibitor can prevent parasite multiplication in vitro. We observed that co-treatment of RSK inhibitor and anti-CD40 antibody resulted in parasites load equal to control. Parasite burden was assessed by Giemsa staining (Figure 13.1). Distribution curve analysis of number of infected macrophages and number of amastigotes in macrophages was plotted (Figure 13.2).

14. RSK inhibitor enhances anti-inflammatory but not anti-leishmanial influence of CD40 in susceptible BALB/c mice

Previous in vitro result demonstrated that RSK inhibitor, has anti-inflammatory functions like decrease in p38 phosphorylation and IL12 synthesis and increase in ERK1/2 phosphorylation and IL10 synthesis. Also with the treatment of inhibitor persistence of parasites in macrophages were observed.

We next investigated the role of CD40 in disease progression of RSK inhibitor on the course L. major infection in susceptible BALB/c mice. Compared to the untreated control mice, those treated with RSK inhibitor in combination with anti-CD40 antibody
had less footpad thickness (Fig. 14A), parasite burden (Fig. 14B) lymph node weight (Fig. 14C) and lymph node cells from the infected mice treated with RSK inhibitor alone or with anti-CD40 together produced significantly lower IFNγ (Fig. 14D) and higher IL-4 (Fig. 14E) compared to the cells from untreated infected mice. It suggests that RSK inhibitor augments anti-inflammatory functions but in vivo results indicated it does not have pro-leishmanial function of CD40 in susceptible BALB/c mice from Leishmania major infection.

**Figure 14:** RSK inhibitor enhances anti-inflammatory but not anti-leishmanial influence of CD40 in susceptible BALB/c mice

a) Weekly footpad thickness
b) Lymph node weight
c) Parasite load in BALB/c (CD40+/+) mice. BALB/c mice were infected with L. major (2x10⁶) subcutaneously in the hind foot pad; 2 days later, they were treated with RSK (10μM/mouse; i.p.) for 5 days, some BALB/c mice were injected intra-peritoneally with anti-CD40 antibody (50μg/mouse) for 3 alternate days beginning 3 days after infection. Footpad thickness (A) assessed in mice weekly, lymph node weight (B) and parasite load (C) 5 weeks after infection. RSK inhibitor plus anti-CD40 antibody treatment significantly reduced footpad thickness (p = 0.002) and but parasite load is not reduced significantly (p = 0.33). (D and E) Lymph node cells were stimulated with anti-CD3 and anti-CD28 antibody for 48 h and IFNγ and IL-4 production was assessed by ELISA. Data show mean values ± SEM.
Taken together all these data revealed a novel parasite employed immune evasion strategy whereby the parasite exploits host intracellular MAPKAPKs in order to establish infection.