RESULTS
Results

1. Collection of peritoneal exudates and macrophages purity

My most of the study was on peritoneal macrophages, so first of all we checked the purity of the peritoneal macrophages. In order to this, firstly peritoneal exudates were harvested from 3% thioglycolate injected BALB/c mice. Cells were cultured overnight for adherence of macrophages and were washed to remove the non adherent cell types. To check the purity of macrophage population, the FACS staining was performed for CD11b (Mac-1), an exclusive marker of macrophages. The results showed that macrophages were found to be around 97% pure (Figure-1).

![Figure 1](image)

I. Expression profile of all known Toll Like Receptors (TLRs) in *Leishmania* infected mouse peritoneal macrophages

Toll like receptors are known for their ability to recognize pathogen associated molecular patterns and initiate immune response against them. Despite the presence of these receptors we often get infection suggesting that pathogens have the strategy to modulate the function of these receptors which helps them to escape from this host defense machinery. Pathogens can modulate the function of a receptor by either modulating its expression or by skewing its signaling towards their survival. In this section we have compared the expression profile of all known TLRs in uninfected and *Leishmania* infected macrophages.
2. Expression profile of TLRs in uninfected and (5ASKH) *Leishmania major* infected macrophages

Thioglycolate-elicited macrophages from BALB/c mice, a susceptible host, were infected with *Leishmania major* promastigotes for 72 hours. Cells were lysed and RNA and protein were isolated for RT-PCR and Western blot. *Leishmania* infection alters the expression of TLR1, TLR4, TLR9, TLR11 and TLR12 in macrophages (Fig-2.a), among the altered TLRs, TLR1, TLR11 and TLR12 were up regulated and TLR4 and TLR9 were down regulated in macrophages upon *Leishmania* infection. Western blot and FACS recapitulate the expression of altered TLRs at protein level (Fig-2.b and 2.c). Peritoneal macrophages from Naïve and *Leishmania* infected mice were isolated and lysed for RNA and protein. RT-PCR data (Fig-2.d) and western blot data (Fig-2.e) showed similar TLR alteration as the in vitro infection data. RNA was also isolated from foot pad tissue of the naïve and infected mice and it showed the similar alteration of TLR expression (Fig-2.f). Above data suggested that *Leishmania*, a protozoan parasite differentially regulates toll-like receptor (TLR) expression in macrophages involving up regulation of TLR1, TLR11 and TLR12 whereas down regulation of TLR4 and TLR9.

![Fig-2.a](image)

*Fig-2.a* Macrophages from BALB/c mice were infected with *Leishmania major* promastigotes at a 1:10 ratio for 72 hours. The uninfected (UIM) and infected (IM)
macrophages were lysed to extract RNA for reverse transcription-polymerase chain reaction (RT-PCR) for specific TLR (a, left panel; the densitometry data from the described three experiments are plotted (right panel) as mean±SD). The horizontal arrows signify no alteration in TLR expression upon *Leishmania* infection whereas the downward and upward arrows signify reduced or increased expressions of the respective TLRs.

**Fig-2.b** Western blot was performed from proteins of the 72hr. uninfected (UIM) and infected (IM) macrophages by probing with TLR-specific antibodies. TLR1 and TLR11 were up regulated and TLR4 and TLR9 were down regulated at protein level upon *L. major* (5ASKH) infection.

**Fig-2.c** Uninfected (UIM) and 72hr. infected (IM) macrophages were stained for TLR1,
TLR4, TLR9 and TLR11 with their respective antibodies. Samples were acquired in FACS vantage™ flow cytometer. Expression of TLR1 and TLR11 were found up regulated and TLR4 and TLR9 were found down regulated upon L. major (5ASKH) infection.

2.d

![Western Blot Image](image)

**Fig-2.d and Fig-2.e** NPMØ and IPMØ are the peritoneal macrophages from uninfected and infected mice, respectively showing TLR1, TLR11 up regulation and TLR4, TLR9 down regulation at RNA level (Left panel), densitometry of RT-PCR data plotted (right panel). Western blot with proteins of NPMØ and IPMØ for TLR1, TLR4, TLR9 and TLR11 showing similar TLR up regulation and down regulation at protein level (Left panel), densitometry of RT-PCR data plotted (right panel).

2.e

![Western Blot Image](image)

2.f

![Western Blot Image](image)
**Fig-2.f** RNA was prepared from the foot pad tissue of the naïve and *Leishmania* infected mice and RT-PCR was done for TLR1, TLR4, TLR9 and TLR11. Infected foot pad tissue shows TLR1, TLR11 up regulation and TLR4, TLR9 down regulation (Left panel), densitometry of RT-PCR data plotted (right panel).

**II-Relation of virulence of *Leishmania* with the modulation in expression of Toll Like Receptors (TLRs)**

Virulence of a pathogen is their ability to infect the host or cause damage to the host, pathogens possess certain molecules which are required for their virulence and they are termed as virulence factors. In this section, we have shown the relation of the virulence of *Leishmania* parasite with their ability to modulate the expression of TLRs.

3. **Relation of the virulence of *Leishmania* with its ability to modulate TLRs expression**

As virulence of a pathogen is required for causing disease, we checked whether above alteration of TLRs is related with the virulence of *Leishmania* or not? To relate the virulence of the parasite with its ability to modulate TLR expression, peritoneal macrophages were infected with two virulent strain (5ASKH (LP), LV39) and two avirulent strain (5ASKH (HP), L119) of *Leishmania* for 72hr. Virulence of the strains were checked with their ability to induce high foot pad swelling, parasite load in poppliteal lymph node and ability to induce high IL-10 in BALB/c mice (Fig-3.a). Cells were lysed and RNA and protein were isolated for RT-PCR and Western blot. Virulent strain of *Leishmania* alters the expression of TLR1, TLR4, TLR9, TLR11 but avirulent strain of *Leishmania* failed to do such alteration in macrophages (Fig-3.b). Western blot recapitulate the expression of altered TLRs at protein level (Fig-3.c), suggesting that virulence of *Leishmania* is required for modulating the expression of TLRs in macrophages.
BALB/c mice were infected with $2 \times 10^6$ parasite of each strain (5ASKH (LP), LV39 and 5ASKH (LP), L119) in foot pad. Foot pad thickness readings were taken every week. After completion of five weeks of infection mice were sacrificed and popliteal lymph nodes were taken out for seeing parasite load and measuring cytokine. Infection with 5ASKH (LP), LV39 strain leads to very high foot pad swelling, parasite load and T-cell mediated IL-10 production as compared to 5ASKH (HP), L119.
3.b and 3.c BALB/c-derived macrophages were infected with two virulent (5ASKH (LP), LV39) and two avirulent (5ASKH (HP), L119) *Leishmania* strains at a 1:10 ratio for 72 hours, followed by RT-PCR (Left panel) (b) and Western blots (Left panel) (c) for TLR1, TLR4, TLR9 and TLR11 expressions. Densitometry of RT-PCR and Western blot data plotted (right panel) of Figure (a) and (b).

4. Role of Lipophosphoglycan (LPG) a virulence factor of Leishmania and ligand for TLR2 in modulating TLRs expression

As virulent strain (5ASKH (LP) and LV39) were only capable of modulating TLRs expression not the avirulent strain (5ASKH (HP) and L119), as virulence is a result of presence of virulence factors, so we checked whether these strains differ in their ability to express LPG a virulence factor of *Leishmania* or not? Different strains of *Leishmania* were stained with anti LPG antibody and their FACS analysis revealed that virulent strain express very high level of LPG in comparison to avirulent strain (Fig-
4.a). Further we checked that if the presence of LPG is the main factor which helps in modulating the expression of TLRs then these changes should happen with the purified LPG also. Macrophages were stimulated with purified LPG and RNA isolated from cells for RT-PCR. Stimulation of macrophages with LPG also results in modulation in expression of TLR1, TLR4, TLR9 and TLR11 (Fig-4.b). LPG is responsible for modulating the expression of TLRs was further confirmed by the inability of *Leishmania* to cause alteration of TLRs in macrophages when their LPG was blocked by anti LPG antibody (Fig-4.c). As LPG binds to TLR2, blocking TLR2 also inhibits the LPG mediated alteration of TLRs expression (Fig-4.d). Blocking of TLR2 and LPG also inhibits the parasite burden in macrophages (Fig-4.e). The above data suggest that modulation of expression of TLR is mediated by LPG of *Leishmania* upon interaction with TLR2. In vivo experiment involving BALB/c mice recapitulate the in vitro data as administration of TLR2 blocking antibody before and after parasite injection reduces the foot pad swelling as well as parasite load in mice (Fig-4.f) and also inhibits *Leishmania* induced modulation of expression of TLR1, TLR4, TLR9 and TLR11 in peritoneal macrophages from infected mice (Fig-4.g).

**Fig-4.a** Different strains of Leishmania were fixed with methanol followed by staining with LPG specific antibody and samples were acquired in FACS vantage \textsuperscript{TM} flowcytometer. Virulent strains (5ASKH (LP) and LV39) expresses very high level of LPG as compared to avirulent strain (5ASKH (LP) and L119).
**Fig-4.b** Peritoneal macrophages were stimulated with purified LPG of *Leishmania* with dose 10µg/ml for 24hr. Cells were lysed to isolate RNA followed by RT-PCR of TLR1, TLR4, TLR9 and TLR11 with specific primers. Purified LPG induces up regulation of TLR1, TLR11 and down regulation of TLR4, TLR9 (right side). Densitometry of RT-PCR data plotted (left side).

**Fig-4.c** Peritoneal macrophages were infected with 5ASKH (LP), 5ASKH (LP) pre-incubated with blocking anti LPG and 5ASKH (LP) pre-incubated with isotype antibody. Blocking of LPG inhibits the ability of virulent 5ASKH (LP) to induce alteration in expression of TLR1, TLR4, TLR9 and TLR11 (RT-PCR), densitometry of RT-PCR data plotted along with the RT-PCR data.
4.d Peritoneal macrophages were pre-incubated with TLR2 blocking and isotype antibody followed by infection with 5ASKH (LP), for 72hr. Blocking of TLR2 inhibits the ability of virulent 5ASKH (LP) to induce alteration in expression of TLR1, TLR4, TLR9 and TLR11 (left panel), densitometry of RT-PCR data plotted (right panel).

4.e Macrophages and Leishmania (5ASKH (LP)) were pre-incubated with anti TLR2 and anti LPG antibody to block TLR2 and LPG followed by infection of macrophages with parasites in 1:10 ratio. After 72 hr. of infection macrophages were fixed and Geimsa stained. Amastigotes were counted in macrophages under microscope. Blocking of LPG and TLR2 results in less parasite burden in macrophages after 72 hours of infection.
Fig-4.f and Fig-4.g Anti TLR2 antibody (2.5µg/foot pad) was injected in the foot pad of BALB/c mice prior or after parasite infection. Foot pad thickness readings were taken every week. After completion of five weeks of infection mice were sacrificed and popliteal lymph nodes were taken out for seeing parasite load from each group of animals. Blocking of TLR2 reduces footpad swelling and parasite burden in mice (4.f). Peritoneal macrophages were isolated from the above group of animals and lysed to get RNA. RT-PCR data shows up regulation in TLR1, TLR11 and down-regulation of TLR4 and TLR9 (4.g).
III-TLR2 and cytokine mediated regulation of Toll Like Receptors (TLRs) expression

As LPG binds to TLR2 of the host cell and LPG also induces the modulation of TLRs, so in this section we have tried to show that how TLR2 signaling and also cytokines helps in modulating the expression of TLR1, TLR4, TLR9 and TLR11.

5. TLR2 mediated trans regulation of TLR1, TLR4, TLR9 and TLR11

From the previous data we come to know that LPG a virulence factor of *Leishmania* induces the changes in TLRs expression via TLR2. We thought if it is the result of TLR2 activation then any TLR2 ligand like LPG may induce similar changes in TLR expression. Peptidoglycan (PGN) a commercially available TLR2 ligand was used to stimulate the macrophages and cells were lysed to isolate RNA and protein for RT-PCR and western blot respectively. PGN induce similar changes in expression of TLR1, TLR4, TLR9 and TLR11 like purified LPG and infection with virulent *Leishmania* (Fig-5.a and 5.b). Furthermore use of TLR2 siRNA reconfirmed the role of TLR2 in modulating the expression of TLRs as PGN stimulation in P388D1 macrophage cell line transfected with TLR2 siRNA does not result in alteration of TLRs expression (Fig-5.c).
Fig-5.a and Fig-5.b Macrophages were treated with PGN (16µg/ml) for 24 hours, followed by RT-PCR and western blot for TLR1, TLR4, TLR9 and TLR11. RT-PCR data (a) and Western blot data (b) shows PGN induced up regulation in TLR1, TLR11 and down-regulation of TLR4 and TLR9 (left panel). Densitometry of RT-PCR and western blot data plotted (right panel of (a) and (b) respectively).

Fig-5.c P388D1 cell line were transfected with TLR2 siRNA followed by stimulation with PGN (16µg/ml) for 24hr. RT-PCR data (left panel) shows inhibition of PGN induced modulation of expression of TLR1, TLR4, TLR9 and TLR11. Densitometry of RT-PCR data plotted (right panel).
6. Modulation of expression of TLR1, TLR4, TLR9 and TLR11 expression through TLR2 involves MyD88 pathway

As previous literature suggests that upon activation, TLR2 elicit MyD88 pathway, we checked whether the alteration in expression of TLR1, TLR4, TLR9 and TLR11 is dependent on MyD88 pathway or not? Use of MyD88 siRNA inhibits the PGN induced up-regulation of TLR1, TLR11 and restores the level of TLR9. There was not much restoration in the level of TLR4 (Fig-6.a), suggesting that TLR2 mediated modulation of TLR4 expression is somehow independent of MyD88 pathway. Cell permeable MyD88 peptide inhibitor reconfirmed the siRNA data (Fig-6.b).

**Fig-6.a and Fig-6.b** P388D1 macrophage cell line were transfected with MyD88 siRNA followed by stimulation with PGN. Presence of MyD88 siRNA inhibits the PGN induced modulation of TLR1, TLR9 and TLR11 (6.a left panel). Similarly, macrophages pre-incubated with MyD88 peptide inhibitor does not show PGN induced modulation of expression of TLR1, TLR9 and TLR11 (6.b left panel). Expression of
TLR4 was not much affected in both the cases. Densitometry or RT-PCR data of both the experiment plotted (right panel of 6.a and 6.b respectively).

7. **Induction of IL-10 modulate the TLR expression**

From the above data we come to know that LPG and PGN when binds to TLR2 results in Myd88 dependent and independent modulation of TLR1, TLR9, TLR11 and TLR4 respectively. We further try to elucidate the exact mechanism of this regulation of TLRs expression by checking whether these changes are the consequence of any cytokine induced as a result of *Leishmania* infection or TLR2 ligation. As *Leishmania* infection augments IL-10 to establish infection, so we checked the induction of IL-10 by virulent and avirulent strains of *Leishmania* and also by purified LPG and PGN. Virulent strain, purified LPG and PGN induces high IL-10 in comparison to avirulent strain of *Leishmania* (Fig-7.a, 7.b and 7.c). Whether IL-10 is responsible for these TLR alterations or not was checked by adding different doses of rIL-10 in culture of naïve macrophages and also by neutralizing IL-10 in culture of macrophages infected with virulent *Leishmania* or stimulated with Peptidoglycan (PGN). Addition of increasing doses of rIL-10 increased the up-regulation of TLR1, TLR11 and down-regulation of TLR9 but expression of TLR4 was not much affected (Fig-7.d). Neutralization of IL-10 in culture of infected macrophages or macrophages stimulated with Peptidoglycan (PGN) restored the level of TLR1, TLR11 and TLR9 like the uninfected macrophages and unstimulated macrophages but failed to restore the level of expression of TLR4(Fig-7.e, 7.f).
Fig-7.a, 7.b and 7.c Macrophages infected with virulent strain induces high IL-10 as compared to avirulent strain at both RNA and protein level (7.a). LPS and PGN stimulation of macrophages leads to IL-10 production at both RNA and protein level (7.b and 7.c).

Fig-7.d Different doses of recombinant IL-10 were added in culture of naïve macrophages for 6hr, followed by RNA isolation for RT-PCR. Addition of rIL-10 induces up-regulation of TLR1, TLR11 and down regulation of TLR9. TLR4 expression remains unaffected.
IL-10 was neutralized with anti IL-10 antibody (10µg/ml) in culture of infected macrophages and PGN stimulated macrophages. After 72 hr. of infection and 24hr of PGN stimulation cells were lysed to isolate RNA for RT-PCR. Neutralization of IL-10 inhibits *Leishmania* and PGN induced up-regulation of TLR1, TLR11 and down regulation of TLR9. TLR4 expression remains unaffected.

8. Induction of TGF-β regulate the TLR4 expression

As alteration of TLR4 expression was independent of IL-10, so we checked whether induction of TGF-β which is another cytokine which helps in establishing *Leishmania* infection has any regulatory effect on expression of TLR4. Macrophages infected with virulent strains of *Leishmania* induce high TGF-β as compared to avirulent and also purified LPG and PGN stimulation results in induction of TGF-β (Fig-8.a, 8.b, and 8.c). Addition of increasing dose of rTGF-β down-regulate expression of TLR4 (Fig-8.d). Neutralization of TGF-β in culture of infected macrophages or macrophages stimulated with Peptidoglycan (PGN) restores the level of TLR4 like the uninfected macrophages and unstimulated macrophages (Fig-8.e and Fig-8.f).
**Fig-8.a, 8.b and 8.c** Macrophages infected with virulent strain induces high TGF-β as compared to avirulent strain at both RNA and protein level (8.a). LPS and PGN stimulation of macrophages leads to TGF-β production at both RNA and protein level (8.b and 8.c).

**Fig-8.d** Different doses of recombinant TGF-β were added in culture of naïve macrophages for 6hr, followed by RNA isolation for RT-PCR. Addition of rTGF-β induces down-regulation of TLR4 expression. Densitometry graph plotted below RT-PCR data.

**Fig-8.e and 8.f** TGF-β was neutralized with anti TGF-β antibody (10µg/ml) in culture of infected macrophages and PGN stimulated macrophages. After 72 hr. of infection and 24hr of PGN stimulation cells were lysed to isolate RNA for RT-PCR. Neutralization of TGF-β restores the normal level of TLR4 expression.
IV. Effector functions of TLR and its role in disease progression and disease amelioration

Leishmania a pathogen, modulate the expression of some TLRs and some TLRs expression remains unchanged. Among the modulated TLRs TLR1, TLR11 and TLR12 get up-regulated whereas TLR4 and TLR11 get down-regulated. In this section, we have shown how the Leishmania induced up-regulated TLRs helps in their survival and also the early treatment with the ligands of some down-regulated and unchanged TLRs helps the host in disease amelioration.

9. Pam3CSK4 and Profilin induces high IL-10 and low IL-12 whereas LPS and CpG induces less IL-10 and high IL-12

After elucidating the mechanism of alteration of TLR expression we studied the effector functions of the altered TLRs to reveal the purpose of *Leishmania* parasite behind these alterations. As in *Leishmania* infection TLR1, TLR11 get up-regulated and TLR4, TLR9 get down-regulated, so we checked whether up-regulated TLRs have effector response which helps in parasite survival or disease progression and those down-regulated have any anti-leishmanial effect. We found that Pam3CSK4 a ligand for TLR1 and Profilin a ligand for TLR11 induces high IL-10, which is helpful for disease establishment and progression, whereas LPS a ligand for TLR4 and unmethylated CpG a ligand for TLR9 induces high IL-12 which is required for host to elicit anti-leishmanial immune response (Fig-9.a and 9.b). So, *Leishmania* target TLR4 and TLR9 to down-regulate their expression. Different doses of CpG induce less IL-12 in infected macrophages as compared to the naïve macrophages (Fig-9.c and 9.d).
Fig-9.a and Fig-9.b Naïve peritoneal macrophages were stimulated with Pam3CSK4 (5ng/ml), LPS (1.5ng/ml), CpG (0.25µM) and Profilin (500ng/ml) for 24 hr and 6hr for ELISA and RT-PCR. Cells were lysed to isolate RNA for RT-PCR and supernatant were collected for IL-10 and IL-12 cytokine ELISA. Pam3CSK4, Profilin induces high IL-10 and IL-12 at RNA level (9.a), whereas LPS, CpG induces high IL-12. Cytokine ELISA for IL-10 and IL-12 shows similar results like RT-PCR (9.b).

Fig-9.c and Fig-9.d Uninfected and infected macrophages were stimulated with different doses of CpG for RT-PCR and ELISA. Supernatant was collected for ELISA and cells were lysed to isolate RNA for RT-PCR. Different dosage of CpG induces high IL-12 in naïve macrophages and less IL-12 in infected macrophages at RNA level (9.c) as well as at protein level (9.d).
10. Pam3CSK4 and Profilin decreases the expression of TLR9 and also inhibits CpG induced IL-12 production

We have found that signaling through TLR1 and TLR11 which are up-regulated in case of Leishmania infection can also inhibit the expression and function of TLR9 in naïve macrophages suggesting an existence of reciprocal relation between these TLRs. Different doses of Pam3CSK4 and Profilin decreases the expression of TLR9 (Fig-10.a), also different doses of Pam3CSK4 and Profilin inhibits the CpG induced IL-12 production in naïve macrophages (Fig-10.b).

**Fig-10.a** Naïve peritoneal macrophages were stimulated with different dosage of Pam3CSK4 and Profilin for 6hr. Cells were lysed and RNA were isolated for RT-PCR. Increasing dose of Pam3CSK4 and Profilin decreases TLR9 expression.

**Fig-10.b** Naïve peritoneal macrophages were stimulated with CpG along with different dosage of Pam3CSK4 and Profilin for 24hr. Supernatant was collected for cytokine ELISA. Increasing dose of Pam3CSK4 and Profilin decreases CpG induced IL-12 production.
11. Pam3CSK4 a TLR1 ligand and Profilin a TLR11 ligand promote parasite burden and disease progression

As previously we have shown that TLR1 and TLR11 upon activation with their respective ligand induces IL-10, which is an important cytokine required for parasite survival and disease progression, so we checked whether different doses of Pam3CSK4 and Profilin enhance parasite burden in infected macrophages? Our observation suggests that TLR1 and TLR11 ligand increase the parasite burden or no. of amastigotes in macrophages with increase in the dosage of the ligands (Fig 11.a). In continuation to this observation we performed invivo experiment with BALB/c mice in which we injected different dosage of Leishmania parasite like $2 \times 10^4$, $2 \times 10^5$, and $2 \times 10^6$. We injected TLR1 ligand Pam3CSK4 for five days started from 2nd day of the infection in $2 \times 10^4$ parasites injected mice. After five weeks mice were sacrificed and we found that injection of Pam3CSK4 promoted the parasite burden around 100 fold (Fig-11.b). PGN promoted the disease but not as significant as Pam3CSK4 in $2 \times 10^4$ parasite injected mice.

11.a

![Graph](attachment:image)

**Fig-11.a** Macrophages were infected with *Leishmania* (5ASKH (LP)) and after 24hr of infection they were treated with different doses of Pam3CSK4 and Profilin for 48hr. After 72 hr. of infection macrophages were fixed and Geimsa stained. Amastigotes were counted in macrophages under microscope. Increasing dose of Pam3CSK4 and Profilin increases the parasite burden in macrophages.
Fig-11.b BALB/c mice were infected with the indicated inoculums of 5ASKH (LP) or with the lowest dose of the parasite along with either Pam3CSK4 or PGN (both at 10μg/mouse; s.c. in the infected foot-pad). The course of infection was measured by footpad thickness (*, p<0.01 for comparison between 2x10⁴ parasite and 2x10⁴+PGN; **, p<0.001 for comparison between 2x10⁴ parasite and 2x10⁴+P3C); the parasite load in these mice also showed an exacerbated infection by Pam3CSK4 (f; p<0.01). The error bars represent mean±SD.

12. Pam3CSK4 a TLR1 ligand promote parasite burden and disease progression in BALB/c mice and it can be inhibited by neutralizing IL-10 or by administration of rIL-12

In the above section as we have shown that Pam3CSK4 increase the parasite burden in mice and also previously we have shown that Pam3CSK4 induces high IL-10, so we thought if this disease progression is due to their high IL-10 and less IL-12 inducing property then upon IL-10 neutralization or administration of rIL-12 may inhibit this disease progression and parasite burden. We have demonstrated that in vivo neutralization of IL-10 and administration of rIL-12 inhibits parasite burden and disease progression in infected BALB/c mice (Fig-12.a).
Balb/c mice were infected with the 2x10^6 parasite of 5ASKH (LP) or with the lowest dose of the parasite along with or without Pam3CSK4 (10µg/mouse); s.c. in the infected foot-pad. Pam3CSK4 injected mice were also injected with rIL-12 (20ng/mouse) and αIL-10 (10µg/mouse) separately. The course of infection was measured by footpad thickness for comparison between 2x10^4 parasite and 2x10^4+P3C, comparison between 2x10^4+P3C and 2x10^4+P3C+rIL-12, comparison between 2x10^4+P3C and 2x10^4+P3C+αIL-10); the parasite load in these mice also showed an exacerbated infection by Pam3CSK4 but effects of Pam3CSK4 were neutralized by injecting rIL-12 and αIL-10 along. The error bars represent mean±SD.

**Fig-12.a** BALB/c mice were infected with the 2x10^6 parasite of 5ASKH (LP) or with the lowest dose of the parasite along with or without Pam3CSK4 (10µg/mouse); s.c. in the infected foot-pad. Pam3CSK4 injected mice were also injected with rIL-12 (20ng/mouse) and αIL-10 (10µg/mouse) separately. The course of infection was measured by footpad thickness for comparison between 2x10^4 parasite and 2x10^4+P3C, comparison between 2x10^4+P3C and 2x10^4+P3C+rIL-12, comparison between 2x10^4+P3C and 2x10^4+P3C+αIL-10); the parasite load in these mice also showed an exacerbated infection by Pam3CSK4 but effects of Pam3CSK4 were neutralized by injecting rIL-12 and αIL-10 along. The error bars represent mean±SD.

**13. CpG therapy at early time point reduces parasite burden but it becomes ineffective at later time point of infection in Leishmania infected BALB/c mice**

CpG a ligand for TLR9 has anti-leishmanial effect as it induces IL-12 mediated Th1 response, whereas Leishmania as an evasion strategy decreases the TLR9 expression, so we checked the effect of CpG therapy in the Leishmania infected BALB/c mice. Mice were infected with 2x10^6 parasites and followed by CpG treatment started from 2nd day of infection. After five weeks mice were sacrificed and parasite load were checked. Mice treated with CpG were found to have less footpad swelling and less parasite load in comparison to untreated mice (Fig13.a). In continuation, we have checked whether this anti-leishmanial effect of CpG works even at later time point of infection? As we have observed that Leishmania infection decreases the TLR9
expression. Mice were infected with the $2 \times 10^6$ parasites and treatment began with CpG at different time point of infection started from 2\textsuperscript{nd} day, 1\textsuperscript{st} week, 2\textsuperscript{nd} week, 3\textsuperscript{rd} week and 4\textsuperscript{th} week. We have found that early treatment only shows anti-leishmanial effect of CpG and this effect goes down as much we delay the treatment. This may be due to the induction of less IL-12 by CpG as we have shown that *Leishmania* infection down regulate the TLR9 expression in *in vitro* as well as in vivo. To confirm this, we injected rIL-12 along with the CpG in infected mice after 2\textsuperscript{nd} week of infection and we have found that anti-leishmanial effect was more in mice as compared to the alone CpG injected group (Fig-13.b).

![Graph](image1.png)

**Fig-13.a** BALB/c mice were infected with the $2 \times 10^6$ parasite of 5ASKH (LP) with or without unmethylated CpG (10μg/mouse; s.c. in the infected foot-pad). The anti-leishmanial effect of CpG was measured by footpad thickness, by comparison between $2 \times 10^6$ parasites and $2 \times 10^6$+CpG injected mice; Lymph node weight in treated mice was found less as compared to the untreated mice, parasite load in these mice also showed regression of infection by CpG. The error bars represent mean±SD.
Fig-13.b BALB/c mice were infected with the 2x10^6 parasite of 5ASKH (LP) with or without unmethylated CpG (10μg/mouse; s.c. in the infected foot-pad) with different time point of infection. Group of mice to which treatment started after 2nd week were injected CpG with or without rIL-12 (20ng/mouse). The anti-leishmanial effect of CpG and rIL-12 at early and later time point of infection was measured by footpad thickness. The anti-leishmanial effect of CpG decreased in later time point of infection results in less reduction in parasite load in these mice as compared to earlier time point. Injecting rIL-12 compensated the decrease in anti-leishmanial effect of CpG in mice subjected to CpG treatment after 2nd week. The error bars represent mean±SD.

14. Imiquimod and Loxoribine reduces parasite burden in Leishmania infected BALB/c mice

In the profiling data, we come to know that among the total twelve TLRs expressed in mice five of them get modulated at expression level and rest were unaltered or same at expression level. So, we thought whether we can utilize these receptors effector function for the disease amelioration in Leishmania infected mice. TLR7 which remains unaltered in case of Leishmania infection were activated with their ligands like Imiquimod and Loxoribine. We have found that Imiquimod and Loxoribine injection lowers the footpad swelling as well as parasite load in Leishmania infected
mice as compared to the control untreated mice (Fig-14.a).

Fig-14.a BALB/c mice were infected with the $2 \times 10^6$ parasite of 5ASKH (LP). Treatment of infected mice were started with TLR7 ligand like Imiquimod and Loxoribine ($10 \mu g/mouse$ each, s.c. in the infected foot-pad). The anti-leishmanial effect of Imiquimod and Loxoribine were measured by footpad thickness, by comparison between $2 \times 10^6$ parasites and $2 \times 10^6$+Imiquimod injected mice, comparison between $2 \times 10^6$ parasites and $2 \times 10^6$+Loxoribine injected mice; Lymph node weight in treated mice were found less as compared to the untreated mice, parasite load in these mice also showed regression of infection by Imiquimod and Loxoribine at different dilutions (A, B and C). The error bars represent mean±SD.