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# STAT4 is critical for immunity but not for antileishmanial activity of antimonials in experimental visceral leishmaniasis

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## Summary

We and others have previously shown that IL-12 is indispensable for immunity and is required for optimal antiparasitic activity of antimonials in experimental visceral leishmaniasis caused by *Leishmania donovani*. In this study we investigated the role of STAT4 in immunity against *L. donovani* using STAT4 knockout mice and also determined the effect of STAT4 deficiency in response to antimonial therapy. Upon infection with *L. donovani*, *stat4*$^{-/-}$ BALB/c and C57BL/6 mice showed enhanced susceptibility to *Leishmania* during late time points of infection which was associated with a marked reduction in Th1 responses and hepatic immunopathology. Interestingly, these defects in Th1 responses in *stat4*$^{-/-}$ did not impair the antimonial chemotherapy as both *stat4*$^{-/-}$ and WT mice showed comparable levels of parasite clearance from the liver and spleen. These findings highlight the role of STAT4 in immunity to *L. donovani* infection and also provide evidence that STAT4 is dispensable for antimonial based chemotherapy.

### Keywords

*Leishmania donovani*; IL-12; IFN-γ; STAT4 chemotherapy and sodium stibogluconate

## Introduction

Visceral leishmaniasis (VL) affects millions of people worldwide and is a major health concern. This fatal disease is caused by the intracellular protozoan parasite, *Leishmania donovani*, which infects host macrophages. In experimental VL, *L. donovani* infection targets the hosts' spleen and liver resulting in the development of organ specific immunity [1]. Strain dependent resistance profiles of mice to *L. donovani* have been linked to the ability of the host to mount a strong Th1 immune response with high amounts of IFN-γ early in infection. As such, while C57BL/6 mice clear *L. donovani* rapidly within the first 30 days of infection, BALB/c mice have a delayed recovery and develop chronic infection [2].

IL-12 is a critical Th1 cytokine associated with the host protection in VL [3] which stimulates the production of IFN-γ from NK cells [4, 5], $CD4^+$ T cells [6, 7] and $CD8^+$ T cells [8]. IL-12 deficient C57BL6 [9] and BALB/c [10] mice are highly susceptible to *L.*

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*donovani* infection exhibiting high hepatic parasite burdens. IL-12 exerts its biological activity through STAT4 dependent and STAT4 independent pathways [11]. However, it is not known whether STAT4 pathway is required for resistance to VL. Furthermore, we and others have previously shown that IFN-γ, which signals via STAT1, promotes Th1 responses and mediates immunity against *L. donovani*, but *stat1*$^{-/-}$ mice are highly resistant to *L. donovani* and develop less liver immunopathology despite impaired Th1 immunity [12]. These findings indicate that a cytokine and its signaling mediators could play distinct roles in determining the outcome of *L. donovani* infection and that STAT4 may or may not be required for resistance against VL.

Host immune responses are also crucial for determining the outcome of antimonial therapy during VL as it is less effective in immunocompromised individuals and SCID mice [13]. Studies have shown that sodium stibogluconate (SSG) based therapy are also less effective in mice deficient in IL-10 [14], IL-4 [15], IFN-γ [16] and IL-12 [17]. STAT4 is involved in IL-12 signaling, which could subsequently regulate the production of other cytokines, but the role of STAT4 in antimonial therapy during experimental VL has not been investigated.

In this study, we investigate the role of STAT4 in immunity against *L. donovani* using *stat4* deficient C57BL/6 and BALB/c mice. We also examine the effect of *stat4* deficiency during antimonial based chemotherapy of VL.

## Results

### STAT4 is indispensable for protection against VL caused by *Leishmania donovani*

To determine the role of STAT4 in immunity against VL, we examined the course of *L. donovani* infection in *stat4*$^{-/-}$ mice of BALB/c and C57BL/6 backgrounds and compared these to their WT counterparts following intravenous infection with $2 \times 10^7$ *L. donovani* amastigotes. At day 20 post-infection, WT and *stat4*$^{-/-}$ mice (BALB/c or C57BL/6 background) showed similar levels of parasite loads in their spleens and livers (Figures 1A-D). At day 40 post-infection, both *stat4*$^{-/-}$ BALB/c and C57BL/6 mice had significantly higher parasite loads in their livers compared to WT BALB/c and C57BL/6 mice respectively, but not in spleens (Figures 1A-1D). At day 60 post infection, both *stat4*$^{-/-}$ BALB/c and C57BL/6 mice had more elevated parasite loads in their livers and spleens compared to WT (Figures 1A-D). These data demonstrate that the absence of STAT4 results in the failure of both C57BL/6 and BALB/c mice to control *L. donovani* infection in spleens and livers.

### STAT4 is critical for mounting an efficient Th1 response during *Leishmania donovani* infection

IL-12, a critical Th1 cytokine which induces IFN-γ, can function via a STAT4 dependent or independent pathway [18]. To determine whether *stat4*$^{-/-}$ mice infected with *L. donovani* were able to mount a Th1 response, we measured IL-12 and IFN-γ production from splenocytes of WT and *stat4*$^{-/-}$ mice following *in vitro* stimulation with *L. donovani* antigen (LdAg). At day 40, IFN-γ levels were impaired in *stat4*$^{-/-}$ BALB/c mice and at day 60 the production of IFN-γ was impaired in both *stat4*$^{-/-}$ BALB/c and C57BL/6 mice compared to WT counterparts (Figures 2A and 2C). IFN-γ producing $CD4^+$ T cell populations were also significantly reduced in spleens of *L. donovani* infected *stat4*$^{-/-}$ mice compared to WT mice (Figures 2M-O). Although IL-12 production from both *stat4*$^{-/-}$ and WT BABL/c or C57BL/6 mice were comparable at day 40 (Figures 2B and 2D), it was impaired in *stat4*$^{-/-}$ BALB/c and C57BL/6 mice at day 60 compared to their respective WT counterparts (Figures 2B and 2D). Real time PCR analysis of splenic RNA from WT and *stat4*$^{-/-}$ BALB/c and C57BL/6 mice showed similar Th1 cytokine profiles (Figures 2E-H).

These findings show that STAT4 is required for mounting efficient Th1 immune responses in *L. donovani* infected BALB/c and C57BL/6 mice.

We also determined the role of STAT4 in the induction of the inflammatory chemokines CCL2 and CXCL10. Both CCL2 and CXCL10 are required for the recruitment of Th1 cells to sites of inflammation [19-21] and activation of host immune response pathways against *Leishmania* [22-24]. Although there were no significant differences in CXCL10 and CCL2 mRNA expression in WT and *stat4*$^{-/-}$ BALB/c mice, there was a significant decrease in both CXCL10 and CCL2 mRNA expression in *stat4*$^{-/-}$ C57BL/6 mice compared to WT at day 60 post-infection (Figures 2I-L).

## *Stat4* deficiency does not increase the production of IL-4 and IL-10 during *L. donovani* infection

Previous studies using *stat4*$^{-/-}$ mice have shown that the absence of STAT4 enhances Th2 development [25]. We therefore analyzed the production of the Th2 cytokine IL-4, as well as the anti-inflammatory cytokine IL-10 in *L. donovani* infected *stat4*$^{-/-}$ BALB/c and C57BL/6 mice. At day 40 post infection, *stat4*$^{-/-}$ BALB/c mice showed significantly low levels of IL-10 compared to WT BALB/c mice (Figure 3A), while *stat4*$^{-/-}$ C57BL/6 mice showed slightly increased IL-10 levels compared to WT C57BL/6 mice although not statistically significant (Figure 3C). Furthermore, at day 60 post-infection there was no significant difference in the levels of IL-10 and IL-4 in *stat4*$^{-/-}$ and WT BALB/c mice while *stat4*$^{-/-}$ C57BL/6 mice showed a reduction in the production of IL-10 and IL-4 at day 60 post-infection compared to its WT counterpart (Figures 3A-D). We obtained similar results using quantitative PCR of splenic mRNA (Figures 3E-H). Taken together our results show that STAT4 is not involved in suppressing Th2 cytokine production during VL.

## *Stat4*$^{-/-}$ mice have delayed formation of granulomas in the liver

The hallmark of *L. donovani* infection is the formation of granulomas, comprised of macrophages and T cells, aimed at controlling the spread of the parasite [26]. We therefore analyzed granuloma formation in *stat4*$^{-/-}$ mice and determined its correlation with hepatic parasite burdens during experimental VL. We observed an inefficient and delayed granulomatous response in *stat4*$^{-/-}$ BALB/c and C57BL/6 mice compared to WT counterparts. At day 40, while WT BALB/c and C57BL/6 mice exhibited well-formed granulomas, *stat4*$^{-/-}$ mice did not show foci of inflammation (Figures 4C and D). Further, at day 60 hepatic granulomas in *stat4*$^{-/-}$ mice were still ill-formed (Figures 4A-D). This inability to mount an efficient granulomatous response correlated with increased hepatic parasite burdens in *stat4*$^{-/-}$ mice infected with *L. donovani* (Figures 1B and D).

## STAT4 is not required for sodium stibogluconate based chemotherapy of *Leishmania donovani* infected mice

Previous studies have shown that IL-12 is important for mediating antimonial therapy of *L. donovani* infected mice [27]. To determine whether STAT4 is required for the anti-parasitic activity of SSG based chemotherapy, we evaluated the parasite loads in the spleen and liver of *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice treated with SSG. BALB/c mice were chosen for this treatment study because they present a more susceptible phenotype to *L. donovani* infection. *L. donovani* infected WT mice treated with 500mg per kg body weight of SSG showed about 85% and 94% clearance of parasites from the spleen and the liver respectively in comparison to PBS treated WT infected controls (Figures 5A, B and 6A). Interestingly, *stat4*$^{-/-}$ infected mice showed similar rates of parasite clearance (about 69% and 82% clearance in the spleen and the liver respectively) compared to PBS treated *stat4*$^{-/-}$ infected mice (Figures 5A, B and 6A).

To expand our understanding of the role STAT4 plays in SSG based chemotherapy of *L. donovani* infected mice, we treated WT and *stat4*$^{-/-}$ BALB/c mice at either a later time point when STAT4 deficiency significantly affected parasite infection levels, or at suboptimal doses which would require a greater immune response involvement. *L. donovani* infected mice treated with 500mg per kg body weight of SSG at day 40 post infection resulted in a comparable reduction in parasite burdens between *stat4*$^{-/-}$ and WT infected mice (Figure 6B). Further, suppression of parasite burdens was similar to *stat4*$^{-/-}$ infected mice treated with SSG at day 14 (Figure 6A and B). Treatment of *L. donovani* infected BALB/c mice with the suboptimal dose of 50mg per kg SSG [28, 29] at day 14 post infection resulted in about 35% mean suppression of parasite burdens in the liver, while minimal effects were observed in splenic parasite burdens of SSG treated WT mice (Figure 6C). As in previous SSG treatment experiments, suppression of parasite burdens in *stat4*$^{-/-}$ mice was similar to that observed in WT mice (Figure (6C). These results suggest that STAT4 plays a negligible role in SSG based chemotherapy of *L. donovani* infected mice.

We also analyzed host cytokine responses of splenocytes restimulated with LdAg *in vitro* following SSG treatment of *L. donovani* infected WT and *stat4*$^{-/-}$ mice. In WT mice, although IFN-γ levels were lower in SSG treated mice compared to PBS treated controls (Figure 5C), IL-10 and IL-4 levels were also significantly lower in the SSG treated group compared to PBS treated controls (Figures 5D and E). More significantly, the ratio of IFN-γ to IL-10 was higher in SSG treated WT mice compared to PBS treated WT controls (Figure 5F), suggesting the existence of a protective balance between cytokines necessary for the eradication of the parasite. Similar to WT mice, *stat4*$^{-/-}$ mice displayed slightly lower levels of IL-10 and IL-4 (Figures 5D and E) and an increased ratio of IFN-γ to IL-10 in SSG treated *stat4*$^{-/-}$ mice infected with *L. donovani* compared to the PBS treated control group (Figure 5F). Taken together, these results suggest that STAT4 is dispensable for SSG based chemotherapy of *L. donovani* infected mice.

## Discussion

This study clearly establishes a role for STAT4 in mediating protection against experimental VL caused by *L. donovani*. We and others have previously shown that IL-12 is essential for protective immunity during *L. donovani* infection [3, 9]. Although IL-12 can function independently of STAT4 [18], it is evident from this study that STAT4 independent pathways do not result in resistance to *L. donovani* infection in mice. This is true for both BALB/c and C57BL/6 mouse backgrounds which are genetically and immunologically distinct and display dissimilar phenotypes in response to VL. While C57BL/6 WT mice have elevated levels of IFN-γ during the initial acute phase of *L. donovani* infection leading to early recovery, BALB/c mice have a delayed IFN-γ response resulting in chronic infection [2]. It is noteworthy that during early time points of infection (day 20), STAT4 does not appear to contribute to immunity in both BALB/c and C57BL/6 mice. However, at later time points (day 40 and day 60), STAT4 deficiency led to a progressive increase in parasite loads in the spleens and livers of both BALB/c and C57BL6 mice. A similar trend was observed in previous studies using *il-12*$^{-/-}$ mice infected with *L. donovani*, which showed increased susceptibility during late time points of infection in both the spleen and liver [9].

IFN-γ produced mostly by Th1 cells during the adaptive phase of the immune response is critical for host antiparasitic activity and subsequent control of *L. donovani* infection in resistant mice [30]. Previous studies have shown that IL-12 signaling via STAT4 is essential for IFN-γ production and Th1 cell development [31], and IL-12 stimulated T cells from *stat4*$^{-/-}$ mice produce low levels of IFN-γ compared to WT T cells [25]. Our studies with *L. donovani* infected *stat4*$^{-/-}$ BALB/c and C57BL6 mice at days 40 and 60 post-infection also

showed low levels of IFN-γ compared to WT counterparts. Similar defects in the production of IFN-γ were also observed in previous studies with *L. donovani* infected *il-12*$^{-/-}$ mice [9], *L. major* infected *stat4*$^{-/-}$ mice [32] and *L. mexicana* infected *stat4/stat6*$^{-/-}$ mice [33]. Furthermore, in this study, we observed low levels of IL-12 induction at days 40 and 60 post-infection in *stat4*$^{-/-}$ BALB/c and C57BL6 mice compared to WT counterparts. In the absence of STAT4, compromised IL-12 mediated IFN-γ production by Th1 cells could lead to a subsequent attenuation of IL-12 production by macrophages and B cells [34].

*Stat4−/−* mice also failed to develop mature hepatic granulomas which are critical to the containment of the *Leishmania* parasite. This is not surprising since the formation of granulomas during *L. donovani* infection requires the action of endogenous IFN-γ [30] and IL-12 [3, 9], which activate macrophages and promote the development of Th1 immune responses important in resistance against VL.

Although IFN-γ signaling is largely mediated by STAT1 [35], and *stat1* $^{-/-}$ mice are highly susceptible to *L. major* infection [36], we previously showed that *stat1*$^{-/-}$ mice are surprisingly highly resistant to *L donovani* through mechanisms which are still not fully understood [12]. Interestingly, others have shown that both *il-12*$^{-/-}$ and *stat4*$^{-/-}$ mice are susceptible to *L. major* infection [32], and *il12*$^{-/-}$ mice are susceptible to *L. donovani* [10]. Our current study demonstrates that *stat4*$^{-/-}$ mice are also susceptible to *L. donovani* infection. It is therefore evident that cytokines and their signaling mediators may not always play similar roles in determining the outcome of experimental VL in mice, which highlights the importance of a thorough evaluation of the role of each signaling molecule in immunity to infection.

Previous studies by Kaplan et al showed that STAT4 deficiency favors the development of Th2 cells [25]. However, in *L. donovani* infection, we do not observe significant differences in IL-10 or IL-4 production by WT and *stat4* $^{-/-}$ splenocytes from BALB/c mice restimulated with LdAg at day 60 post-infection. Further, in C57BL/6 mice, IL-4 and IL-10 production by restimulated splenocytes was higher in WT than in *stat4*$^{-/-}$ mice at the same late stage of infection. It appears from this study that STAT4 deficiency does not enhance the production of Th2 cytokines during *L. donovani* infection. Multiple factors, including the dynamics of host-pathogen interactions govern the development of Th2 cells. In WT C57BL/6 mice, a recovery from the acute stage of infection followed by the abrogation of *L. donovani* induced immune responses might lead to higher Th2 cytokine production than in *stat4*$^{-/-}$ C57BL/6 mice which still contain chronic infection [2]. However, it is the deficiency in the induction and maintenance of Th1 responses, and not the production of Th2 cytokines, that account for the development of progressive and non-healing disease in *L. donovani* infected *stat4*$^{-/-}$ mice.

Our evaluation of the role of STAT4 in SSG chemotherapy showed that STAT4 deficiency did not affect the outcome of SSG treatment in *L. donovani* infected BALB/c mice. Both SSG treated WT and *stat4*$^{-/-}$ mice showed similar levels of parasite clearance from the spleen and liver. The ability to respond to SSG chemotherapy has been shown to require a fully competent T cell response. In T cell deficient mice antimonial treatment is completely inactive [37]. Previous studies with *il-12*$^{-/-}$ and *ifn-γ*$^{-/-}$ mice have also shown that endogenous IL-12 and IFN-γ are essential for optimal activity of SSG based chemotherapy during *L. donovani* infection [27]. In contrast with IL-12, our results demonstrate that STAT4 is not required for optimal activity of SSG based chemotherapy in the management of VL. One possible explanation for this contrasting effect of SSG in *stat4*$^{-/-}$ and *il12*$^{-/-}$ mice could be the influence of the immunosuppressive cytokine IL-35 which has recently been shown to signal through STAT1 and STAT4 [38]. In the absence of IL-12, the immunosuppressive effect of IL-35 could abrogate the antileishmanial activity of SSG,

whereas in *stat4*$^{-/-}$ mice, the effect of IL-35 is lost, rendering them more responsive to SSG chemotherapy. Not surprisingly, treatment with SSG lowered IL-4 and IL-10 production in *stat4*$^{-/-}$ mice at the same rate as in WT mice. Further, similar rates of increase in the ratio of IFN-γ to IL-10 were observed in SSG treated WT and *stat4*$^{-/-}$ mice compared to PBS treated controls. These factors could contribute to the optimal activity of SSG in *L. donovani* infected WT mice and such effects do not seem to be compromised in *stat4*$^{-/-}$ mice.

In conclusion, we demonstrate for the first time the role of STAT4 in disease progression during experimental VL. Our findings reveal that STAT4 is essential for immunity against *L. donovani* infection. During the late phases of infection, the absence of STAT4 results in progressive and non-curing infection in the spleen and liver of both BALB/c and C57BL6 mice due to defects in the induction of Th1 cytokines. On the other hand, unlike IL-12, STAT4 is dispensable for antimonial based chemotherapy in *L. donovani* infected mice.

## Materials and Methods

### Animals

Wild type BALB/c and C57BL/6 mice were purchased from Harlan Laboratories. *Stat4*$^{-/-}$ BALB/c and C57BL/6 mice were gift from Dr. Mark Kaplan (Indiana University). Mice were maintained and bred in hepa filter cages in a facility at the Ohio State University. The experiments were performed using 8-10 weeks old sex matched mice according to the institutional guidelines for animal research.

### Parasites and Infection Protocol

*Leishmania donovani* (LV82 strain) was maintained by serial passage of amastigotes in Golden Syrian hamsters. Amastigotes were isolated from the spleen of sick hamsters and experimental mice were injected with $10^7$ *L. donovani* amastigotes in 100μl by intravenous injection into the tail vein. Groups of three or five mice were sacrificed at various time points post infection for further analysis.

### Treatment of mice

*L. donovani* infected wild type (WT) or *stat4*$^{-/-}$ mice on BALB/c background were treated (i.p) with SSG (Albert David, India) at a dose of 500mg/kg body weight of the animal or phosphate buffered saline (PBS) at 2 weeks post infection. Following two weeks of treatment, the animals were sacrificed for further analysis.

### Parasite Burden Calculation

Livers and spleens were harvested, weighed and sectioned to prepare impression smears that were stained with Giemsa to enumerate the number of amastigotes per thousand nucleated cells. The parasite loads were expressed as Leishman-Donovan Units (LDU) = Number of amastigotes per 1000 nucleated cells × organ weight (grams). Effect of SSG therapy was expressed as mean parasite suppression which was determined by comparing percentage parasite reduction in SSG treated mice with the corresponding mean control value [15, 28].

### Cytokine ELISA

Splenocytes isolated from WT and *stat4*$^{-/-}$ mice were plated at a concentration of $0.5 \times 10^6$ cells per well in quadruplicates in a sterile 96-well tissue culture plates. Cells were then stimulated with freeze thawed *L. donovani* antigen (20μg/ml). Supernatants were collected after 72h of incubation at 37°C and analyzed for the production of IFN-γ, IL-12p70, IL-4, and IL-10 by ELISA (BD Pharmingen).

### Quantification of transcript levels by RT-PCR

Total RNA was extracted from 50mg of spleen tissue using TRIZOL Reagent. mRNA was reverse transcribed and cDNA was amplified by real-time PCR as described previously [39]. Primers and reaction conditions were found using the PRIMER BANK website (Massachusetts General Hospital. Primer Bank. http://pga.mgh.harvard.edu/primerbank). Data were normalized to the housekeeping gene β-actin and presented as fold induction over infected WT mice using the delta-delta CT method.

### Histopathology

Tissue sections from the liver of *L. donovani*-infected mice were stained using H&E and subjected to histopathology. Liver granulomas were enumerated and scored as follows: 1) no reaction; 2) developing; 3) mature; and 4) empty [26]. At each time point, livers from at least 4–5 individual mice were analyzed in each group.

### Intracellular cytokine staining

Spleens of infected mice were recovered, homogenized using 70-μM strainers and re-stimulated in the presence of brefeldin A for 5 hours. Cells were then stained with fluorescently labeled anti-CD4 antibody (Biolegend San Diego, CA), permeabilized and intracellular IFN-γ production was measured by flow cytometry using PE conjugated anti-IFN-γ antibody (BioLegend, San Diego, CA) on cells gated on $CD4^+$ populations.

### Statistical analysis

Student's unpaired *t* test was used to determine statistical significance of differences in the values. A value of $p < 0.05$ was considered significant. The statistical significance of antibody titers and IFN-γ: IL-10 ratios were determined by non parametric tests using Mann-Whitney *U*-test.

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## Abbreviations

| | |
|---|---|
| **VL** | visceral leishmaniasis |
| **WT** | wild type |
| **SSG** | sodium stibogluconate |

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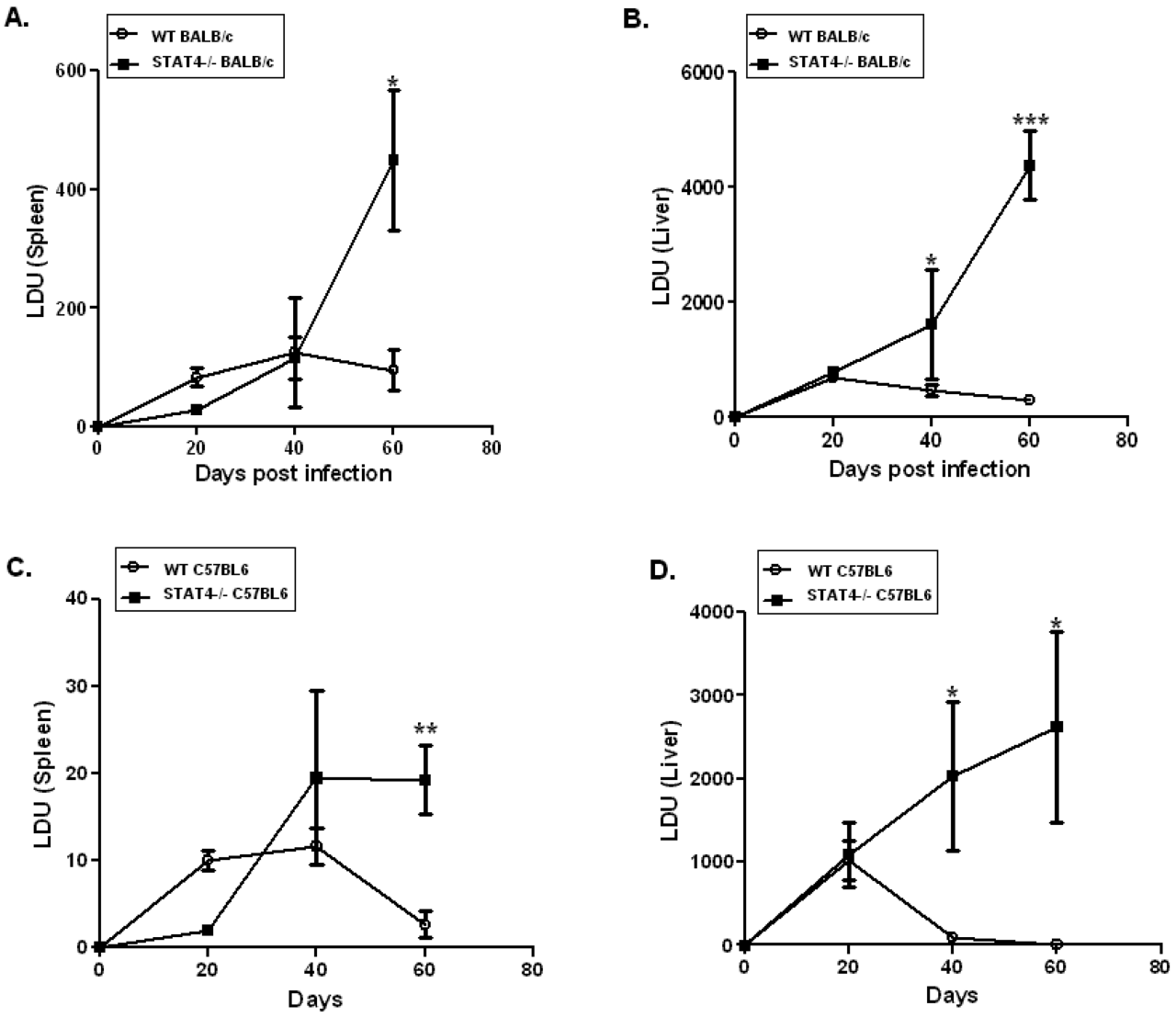


**Figure 1. *stat4*$^{-/-}$ mice are highly susceptible to *L. donovani* infection**

Spleen and liver parasite loads in WT and *stat4*$^{-/-}$ BALB/c (A-B) or C57BL/6 (C-D) mice were determined 20, 40 and 60 days following intravenous inoculation of $10^7$ *Leishmania donovani* amastigotes. Parasite burdens in spleen of and liver were expressed as mean LDU ±SE. The data are the mean values from four or five individual mice per group at each time point in three independent experiments pooled with similar results. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ using unpaired t test.

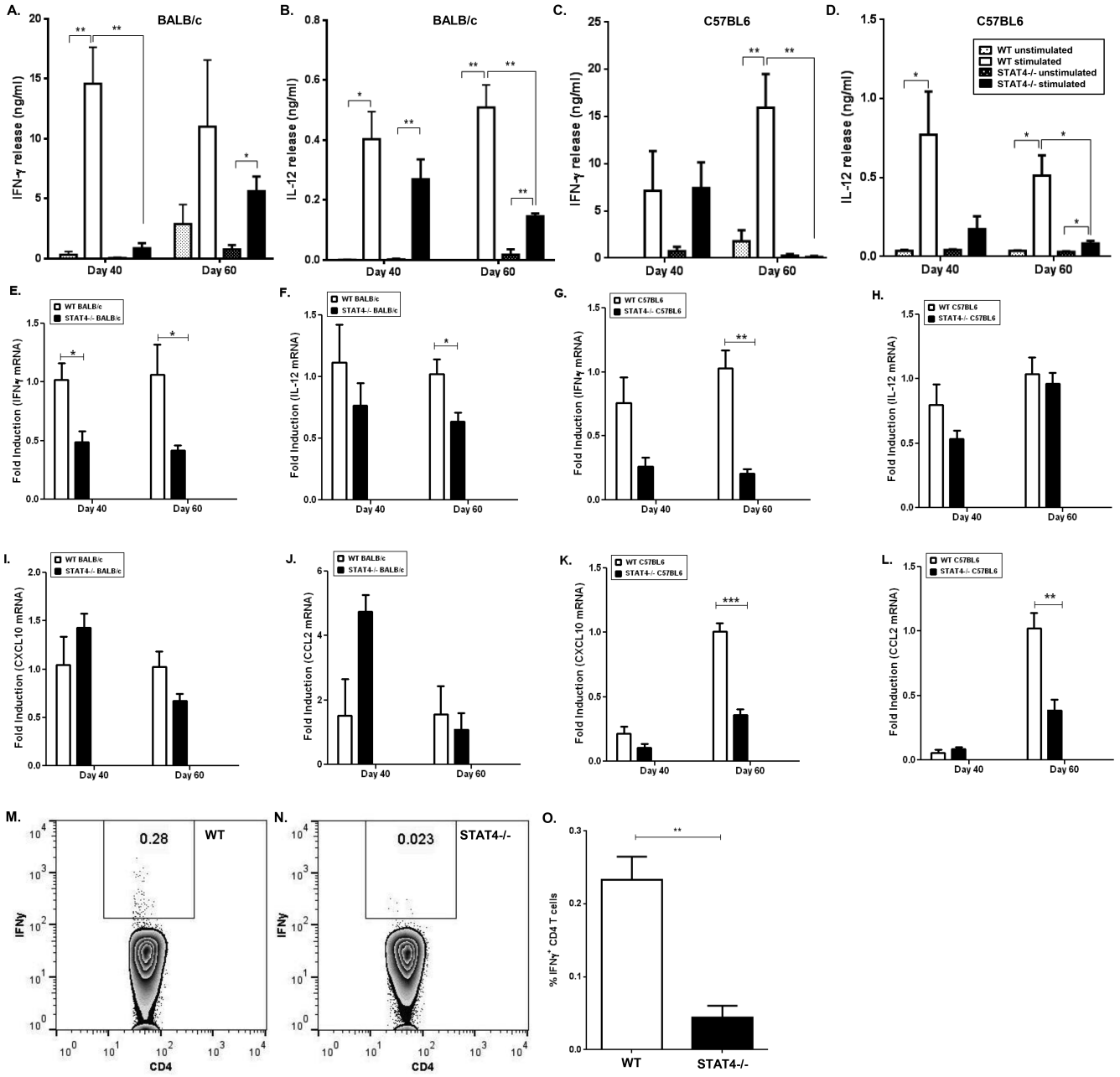


**Figure 2. *stat4*$^{-/-}$ mice are defective in generating a Th1 response during *L. donovani* infection**

Th1 cytokine production by splenocytes from *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice or WT and *stat4*$^{-/-}$ C57BL/6 mice stimulated with 20μg/ml LdAg. IFN-γ (A and C) and IL-12p70 (B and D) were measured by ELISA. Data shown were the mean ± SE of triplicates from four or five individual mice per group and are representative of three individual experiments. *, $P < 0.05$ and **, $P < 0.01$ using unpaired t test.

Th1 cytokine and chemokine mRNA expression by spleen tissue from *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice or WT and *stat4*$^{-/-}$ C57BL/6 mice. IFN-γ mRNA (E and G), IL-12p35 mRNA (F and H), CXCL10 mRNA (I and K) and CCL2 mRNA (J and L) were measured by Real Time PCR. Intracellular IFN-γ production by CD4$^{+}$ T cells isolated from spleens of *L. donovani* infected WT (M) and *stat4*$^{-/-}$ (N) mice. Average percentage of

IFN-γ producing CD4+ T cells of *L. donovani* infected WT and *stat4*$^{-/-}$ (O) mice. (n=5 mice per group). *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ using unpaired t test.

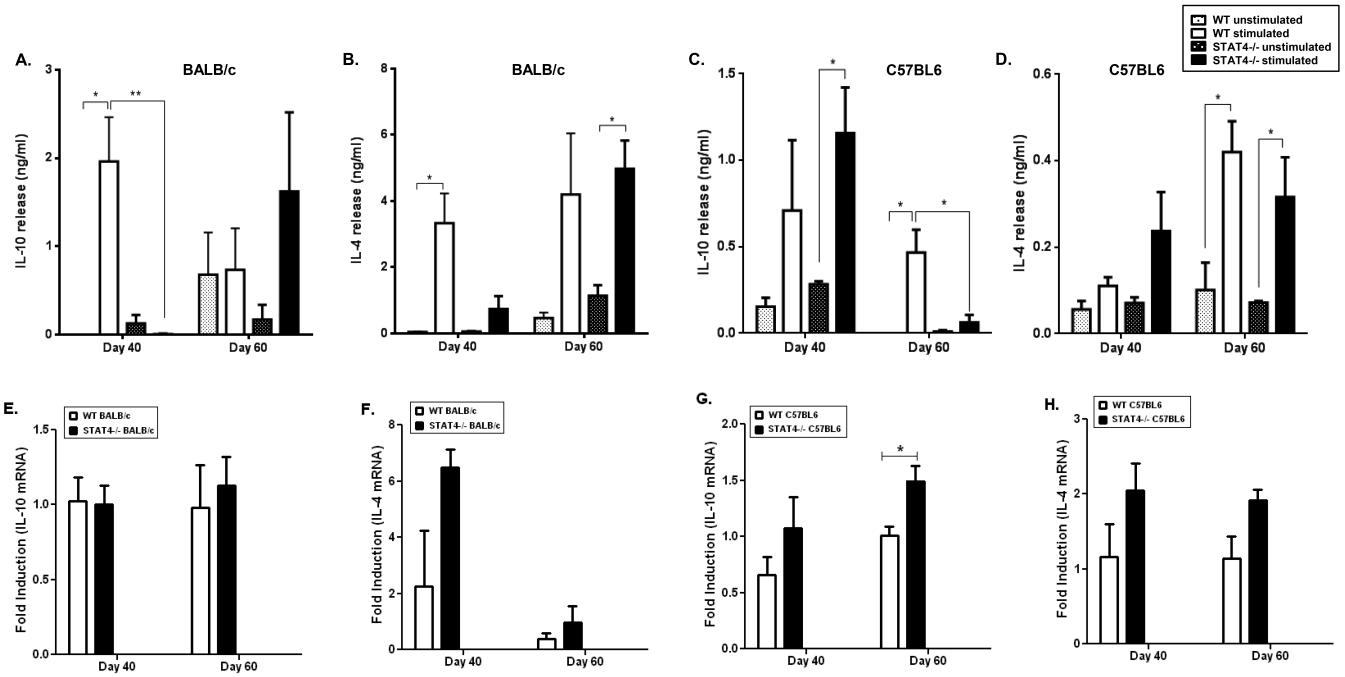


**Figure 3. Th2 responses in *stat4*$^{-/-}$ mice during *L. donovani* infection**

Th2 cytokine production by splenocytes from *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice or WT and *stat4*$^{-/-}$ C57BL/6 mice stimulated with 20μg/ml LdAg. IL-10 (A and C) and IL-4 (B and D) were measured by ELISA. Data shown are the mean ± SE of triplicates from four or five individual mice per group and are representative of three individual experiments. ** $P < 0.01$, * $P < 0.05$ using unpaired t test.

Th2 cytokine mRNA expression by spleen tissue from *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice or WT and *stat4*$^{-/-}$ C57BL6 mice. IL-10 mRNA (E and G) and IL-4 mRNA (F and H) were measured by Real Time PCR. *, $P < 0.05$ and **, $P < 0.01$ using unpaired t test.

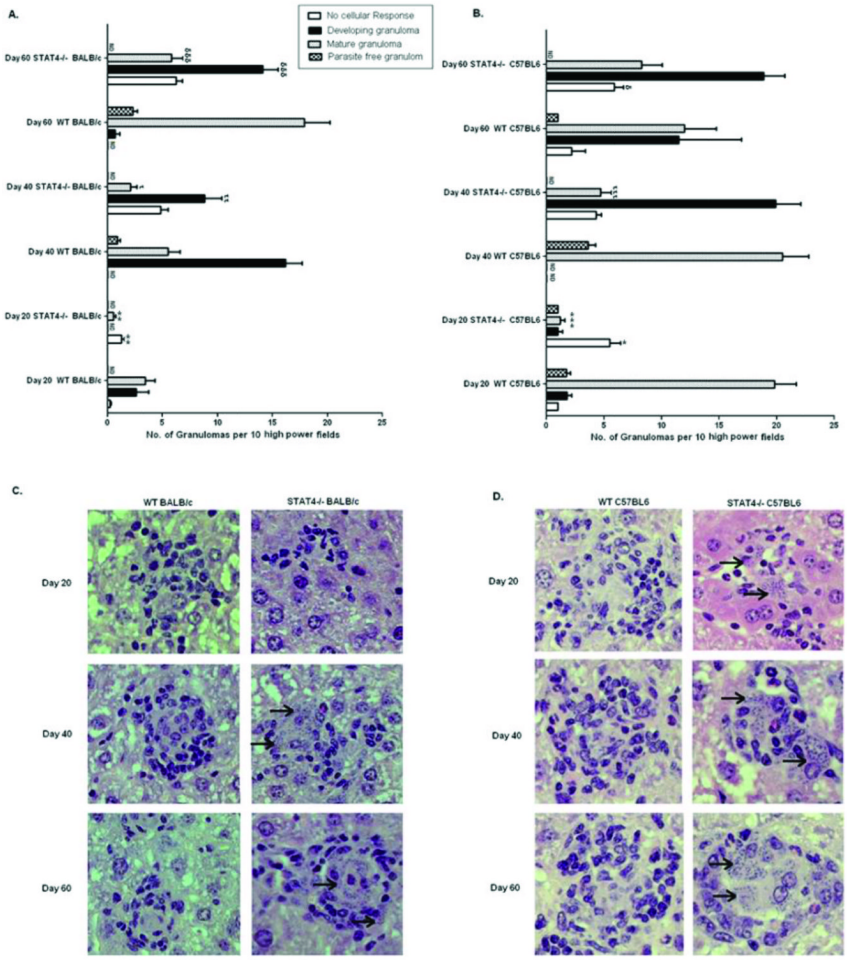


**Figure 4. Immunopathology of *stat4*$^{-/-}$ mice during *L. donovani* infection**

Liver sections from *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice (A) or WT and *stat4*$^{-/-}$ C57BL/6 mice (B) at days 20, 40 and 60 post infection were scored for the extent of granuloma formation. Results were from one representative experiment of three with similar findings. Data were expressed as mean granuloma number per 10 high-power fields (magnification 200×) ±SE. ND, not determined.*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ compared to their respective infected Day 20 WT control; ι, $P < 0.05$, ιι, $P < 0.01$ and ιιι, $P < 0.001$ compared to their respective infected Day 40 WT control; φ, $P < 0.05$, φφ, $P < 0.01$ and φφφ, $P < 0.001$ compared to their respective infected Day 60 WT control. Histopathology of infected livers from WT and *stat4*$^{-/-}$ BALB/c mice (C), or WT and *stat4*$^{-/-}$ C57BL/6 mice (D). WT BALB/c and C57BL/6 mice showed developing granuloma at day 20 post infection and contained matured and parasite free granulomas at days 40 and 60 post infection. In contrast, *stat4*$^{-/-}$ BALB/c and C57BL/6 mice showed marked delay in granuloma formation as there was lack of cellular reaction at day 20 post infection and even at days 40 and 60 post infection, the granulomas were poorly developed and were highly parasitized (arrow). Magnifications 400× for panels C and D.

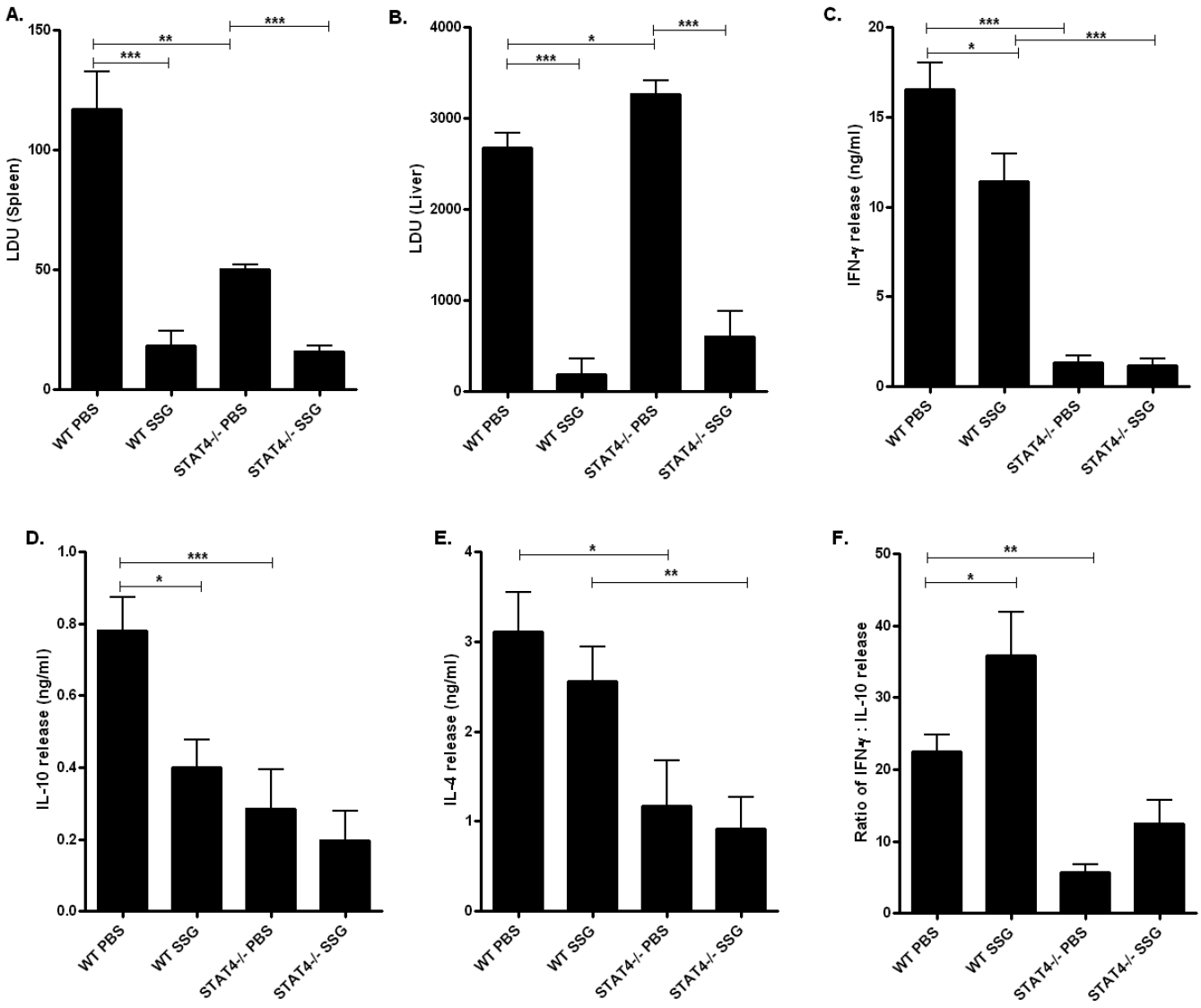


**Figure 5. Activity of SSG based chemotherapy in *stat4*$^{-/-}$ mice**

Spleen (A) and liver (B) parasite loads in SSG or PBS treated *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice. Parasite burdens in spleen of and liver were expressed as mean LDU ±SE. The data were the mean values from four or five individual mice per group at each time point in three independent experiments pooled with similar results. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ using unpaired t test.

Th1 and Th2 cytokine production by splenocytes from SSG or PBS treated *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice stimulated with 20μg/ml LdAg. IFN-γ (C), IL-10 (D) and IL-4 (E) were measured by ELISA. Ratios of IFN-γ to IL-10 production (F) are also shown. Data shown were the mean ± SE of triplicates from four or five individual mice per group and are representative of three individual experiments. *, $P < 0.05$ and **, $P < 0.01$ using unpaired t test.

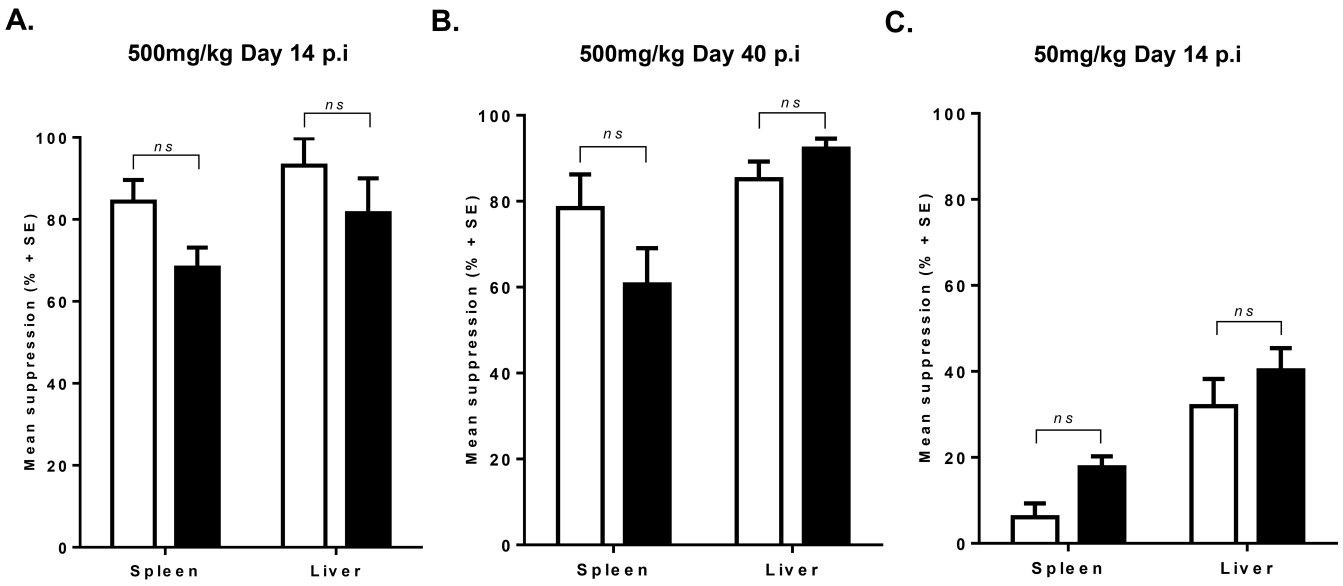


**Figure 6. Mean suppression of parasite burdens in SSG treated WT and *stat4*$^{-/-}$ mice**

The mean suppression (± SE) of parasite burdens in spleen and liver of *L. donovani* infected WT and *stat4*$^{-/-}$ BALB/c mice treated with different doses of SSG or at different times post infection. (A) Mice were treated with 500mg/kg at day 14 post infection and analyzed 14 days later. (B) Mice were treated with 500mg/kg at day 40 post infection and analyzed at day 14 days later. (C) Mice were treated with 50mg/kg at day 14 post infection and analyzed 14 days later. Parasite suppression was measured by comparing each experimental value with the mean control value. Data shown are the mean values from four to six individual mice per group in two independent experiments with similar results. (*n.s: $p < 0.1$*, p.i: post infection).