Use of the ITK/BTK Inhibitor Ibrutinib for the Treatment of Experimental Visceral Leishmaniasis Caused by *Leishmania donovani*

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Abstract

Leishmaniasis comprises a group of diseases caused by protozoa of the genus *Leishmania* that are transmitted *via* the bite of female phlebotomine sand-flies. Infections may be cutaneous or visceral depending on the species, however, visceral leishmaniasis (VL) is significantly more deadly than cutaneous leishmaniasis (CL). Leishmaniasis is present in nearly 100 countries with approximately 200,000 to 500,000 new cases annually for visceral infections, about 50,000 of which are fatal. VL has a mortality rate of roughly 75 to 95% given no treatment and 5 to 10% with standard treatment pentavalent antimonials (SbV) such as sodium stibogluconate (SSG) which are toxic and require prolonged intra-peritoneal or intravenous administration leading to incomplete treatment regimes. Furthermore, the widespread use of antimonials has led to a rampant increase in the prevalence of drug resistant parasites. Ibrutinib is an anticancer drug which has been shown to modify immune cell responses and has previously shown effectiveness against CL. In this study, we sought to evaluate oral treatment with ibrutinib as a host-directed therapy for VL caused by *Leishmania donovani* using mouse models of infection.

Oral ibrutinib treatment resulted in significantly reduced liver and spleen parasite burden as compared to SSG and vehicle solution controls with greater numbers of mature liver granulomas. Ibrutinib-treated mice had a reduced influx of $Ly6C^{hi}$ inflammatory monocytes, known to cause susceptibility to *L. donovani* and treatment was also associated with the release of protective cytokines in the liver and spleen: IFN-γ, tumor necrosis factor α, IL-4, and IL-13. No changes were noted in B cell populations or IgG levels indicative of a B cell independent mechanism. Our results demonstrate oral ibrutinib is substantially more effective than SSG (70 mg/kg) in reducing parasite burden *L. donovani* and acts through alteration of host immunity, therefore, ibrutinib has potential as an immunomodulatory treatment for VL.

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Introduction

Leishmaniasis is a collective term for pathological conditions caused by *Leishmania* parasite infection and represents a large healthcare burden in many developing countries. *Leishmania* are obligate intracellular protozoans that may infect the skin, mucosal membranes or visceral organs. Leishmaniasis is a neglected tropical disease as classified by the World Health Organization, with over 12 million individuals currently infected globally and 2 million new annual cases (WHO 2018). *Leishmania* is present in over 80 tropical and subtropical countries and there are at least 20 distinct *Leishmania* species. Leishmaniasis may be transmitted by over 90 known sand-fly species and more than 70 different animals including humans are potential *Leishmania* reservoirs (WHO 2018, CDC 2013). During a blood feeding, motile *Leishmania* promastigotes are transferred from the sand-fly into a human or animal host where they are phagocytized by neutrophils, macrophages, or dendritic cells (DCs); *Leishmania* parasites differentiate into amastigotes and replicate within phagolysosomes, eventually rupturing the host cell and releasing the parasites. The extracellular amastigotes are again phagocytized by neutrophils, macrophages, DCs or may be taken in by a feeding sand-fly and the infection cycle continues (CDC 2013).

Cutaneous leishmaniasis (CL) is the most prevalent form with an estimated 700,000 – 2 million cases a year, however, VL is the most dangerous type of *Leishmania* infection, surpassed only by malaria in annual fatalities (WHO 2018, CDC 2013, The Center for Food Security and Public Health Factsheets 2017). The primary consequence of cutaneous infections is disfiguring scarring at the lesion sites however VL has a mortality rate of 75 to 95% without proper treatment which is reduced to around 5-10% with correct administration of SSG (WHO 2018,

McGwire and Satoskar 2014). Infection with *Leishmania donovani* or *Leishmania chagasi* is the cause of VL and parasites are dispersed through the blood to the bone marrow, spleen, and liver (de Freitas et al. 2016). Patients with visceral infections develop conditions such as fever, anemia, and hepato-splenomegaly (swelling of spleen and liver) which become chronic and eventually cause death (WHO 2018, McGwire and Satoskar 2014). Up to 20% of VL patients also experience reoccurring infections following successful treatment with SSG which may manifest symptoms in the form of large diffuse parasite-laden skin pustules known as post kala-azar dermal leishmaniasis (Okwor and Uzonna 2016, Zijlstra et al. 2017).

The pentavalent antimonial drug sodium stibogluconate (SSG) was introduced approximately 100 years ago and remains the preferred treatment for all forms of leishmaniasis worldwide despite cardiac, renal, and hepatotoxicity with required daily parenteral injections for at least 20 days (de Freitas et al. 2016). Since *Leishmania* infections commonly occur in remote impoverished areas, treatment may be abruptly discontinued due to cost or supply issues which promotes the selection and expansion of resistant parasite populations. The prolonged and often inappropriate use of antimonial compounds has increased the prevalence of drug-resistant parasites worldwide, particularly *L. donovani* (Sundar et al. 2000, Lira et al. 1999, Clementi et al. 2011, Polonio and Efferth 2008). Other effective medications such as amphotericin B and miltefosine have developed the same problem of promoting the evolution of parasite resistance as both drugs work by targeting parasitic components directly in addition to being less accessible in endemic areas and possessing similar, albeit milder, side effects as SSG (Rai et al. 2013, Rijal et al. 2013, Burza et al. 2014, Sinha et al. 2010, Sunyoto et al. 2018). There is a pressing need to explore new treatment options for VL and therapies that modulate host immune systems are an ideal solution that can be derived naturally or through repurposing previously approved drugs.

Ibrutinib is a potent orally bio-available small molecule irreversible inhibitor of Bruton's tyrosine kinase (BTK) and interleukin-2 inducible kinase (ITK) and is approved by the FDA for the treatment of chronic lymphocytic leukemia and mantle cell lymphoma as well as other B cell malignancies (Wang et al. 2015, Wu et al. 2016). BTK and ITK belong to the TEC-family tyrosine kinases which are essential for intracellular signaling of both B and T lymphocytes respectively in humans (Wang et al. 2015, Wu et al. 2016, Gomez-Rodriguez et al. 2011). BTK is critical to B cell receptor signaling, mediating B cell proliferation which promotes the pathogenesis of leishmaniasis (Arcanio et al. 2017, Silva-Barrios et al. 2016). ITK is present within T cells and participates in proliferation, differentiation as well as the development of specific T helper cell subsets (Gomez-Rodriguez et al. 201, Kosaka et al. 2006). The balance of different helper T cell populations can have drastic effects on *Leishmania* parasite pathogenesis. Previous studies from our lab have shown that ibrutinib mediated ITK inhibition causes suppression of T helper type 2 (Th2) T cell differentiation in favor of T helper type 1 (Th1), preventing disease progression *L. major* induced CL in a mouse model (Dubovsky et al. 2013). Overall, these results suggest that ibrutinib may have potential use as a host targeted therapy to suppress immune response which has been associated with negative outcomes in visceral *Leishmania* infections, namely, B cell proliferation and Th2 differentiation.

Here we determined the effectuality of ibrutinib for experimental *L. donovani* infection in a mouse model. We demonstrate that oral ibrutinib treatment is significantly more effective against *L. donovani* than conventional SSG. Furthermore, our studies show that ibrutinib promotes protective immune response and does not act directly on *Leishmania* parasites.

Methods

6–7-week-old female wild-type BALB/c mice were infected by IV injection of 10^7- *L. donovani* amastigotes taken from infected hamster spleens. Two weeks after infection, the mice were randomized into four groups of five mice: two groups treated with 6mg/kg Ibrutinib dissolved in the vehicle or vehicle alone (0.5% methyl cellulose + 0.1% SDS/SLS) daily *via* oral gavage for 2 weeks or and two groups treated with one dose of 70mg/kg sodium stibogluconate (SSG) or PBS given as an intraperitoneal (IP) injection. On day 14 post-treatment, (two weeks following infection) Leishman-Donovan Units were used to quantify parasite burden by staining tissue sections with Giemsa stain and counting parasites per nucleated cell then multiplying by organ weight (mg).

Analysis of Histopathology

Thin sections of livers taken from infected mice were fixed in formalin, stained with hematoxylin-eosin, and assessed for granuloma formation by a certified pathologist as one of the following: no response, developing granuloma, mature granuloma, granuloma cleared of parasites and tissue cleared of granulomas. Granuloma totals were taken as average of the 5 mice from each group.

Cytokine Assessment by Enzyme-Linked Immunosorbent Assay (ELISA) and Greiss Assay

Spleen cells were isolated from infected spleens into RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% HEPES. The cells were centrifuged, blood cells were lysed using ACK lysis buffer, and remaining viable cells were counted using trypan blue exclusion. 5×10^5 splenocytes/well in RPMI 1640 medium were plated into 96-well tissue culture plates and stimulated with 20 μg/mL *L. donovani* antigen for

three days. The supernatants were collected to assess the levels of the cytokines, interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 12 (IL-12), and interleukin 13 (IL-13). Supernantant nitric oxide (NO) levels were assessed using the Greiss assay.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis

TRIzol extraction (Life Technologies, Carlsbad, CA) was used to isolate RNA from livers and spleens. Complementary DNAs were prepared with an iScript reverse transcription kit, and RT-PCR was done in a CFX 96 RT-PCR cycler using IQ SYBR green super mix (Bio-Rad, Hercules, CA). The primers were selected using the Harvard primer database (http://pga.mgh.harvard.edu/primerbank). Data was normalized by using the housekeeping gene β-actin and presented as fold induction over uninfected mouse gene expression.

Flow Cytometry

To determine the effect of ibrutinib on populations of immune cells in infected organs, a Percoll density gradient with centrifugation was used to isolate the splenocytes and immune cells. 1×10^6 cells were incubated with mouse serum to prevent non-specific binding then stained with fluorescent antibodies for the proteins: CD3, CD4, CD8, Tim3, ST2, DX5, CD19, IFN-γ, IL-4, CD11b, Ly6G, Ly6C, and F4/80 (Biolegend, San Diego). Cells were analyzed using a FACS LSR-II flow cytometer and FlowJo software (BD Biosciences, San Jose) (TreeStar, Ashland, OR).

Statistical Analysis

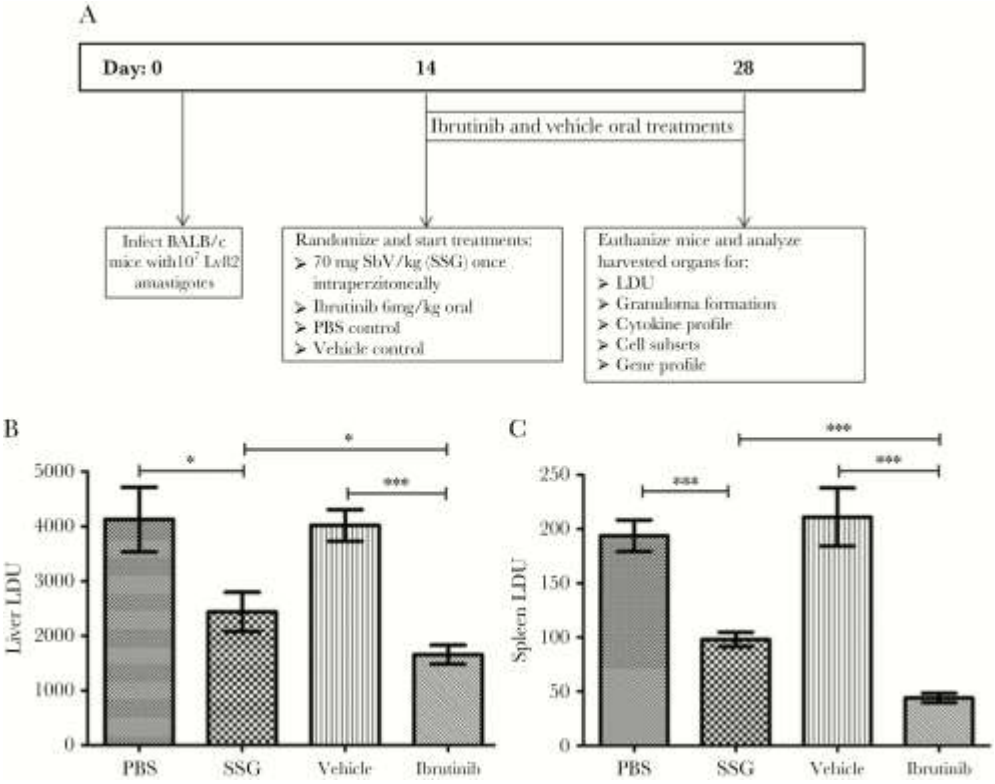
Unpaired student t tests were utilized to determine if differences between experimental and control groups were statistically significant with a P value of greater than .05 considered significant.

Results

Oral Ibrutinib Treatment Is Effective for *L donovani* Infection

To evaluate if ibrutinib treatment is effective for VL, we examined the liver and spleen parasite burdens of mice infected with *L. donovani* that were treated with ibrutinib, vehicle, SSG, and PBS. Mice that were treated with ibrutinib had reduced parasite numbers in the liver and spleen compared to controls and SSG, there was approximately a 50% increased reduction of parasites in the liver and and a 75% increased reduction in the spleen as compared to controls (Figure 1). Together, our results show that oral ibrutinib treatment is an effective therapy for VL and reduces parasite burden more than equivalent human weight doses of SSG.

Figure 1. Treatment with Ibrutinib Reduces Parasite Burden

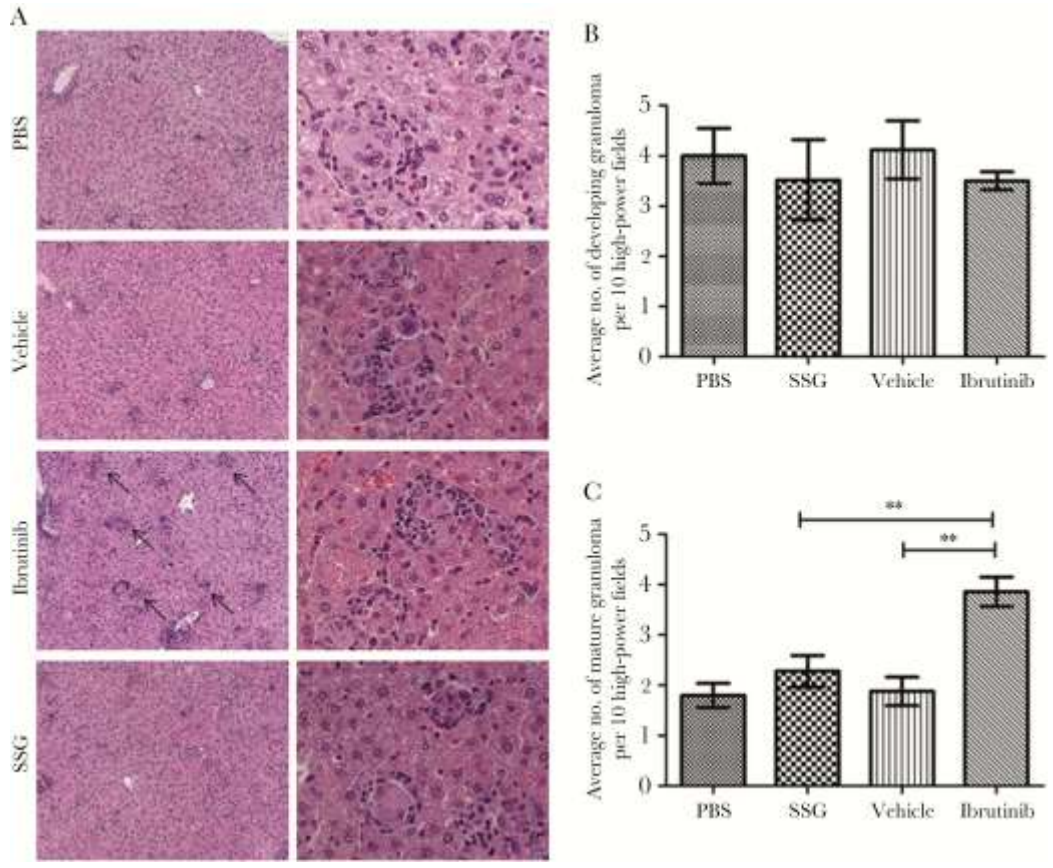


A, Study timeline. 6-7-week-old female BALB/C mice were infected with *L. donovani* amastigotes. Two weeks following infection, the mice were randomized into 4 groups of 5 mice with assigned treatments. Mice in the two different oral treatment groups received ibrutinib (6 mg/kg) or vehicle formulations every day for two weeks while the other groups recieved a single IP injection of 70 mg/kg SSG and PBS. Two weeks after infection, livers and spleens were harvested and parasitic load was assessed. The quantification of parasite load was performed using Leishman-Donovan units (LDU) ± standard error of mean value. **P* < .05 and ****P* < .001, using unpaired t test.

Granuloma Maturation in the Liver

Final parasite clearance in the liver is mediated by mature granulomas and therefore hepatic granuloma formation is a critical step to relapse-free recovery from VL (Murray 2000, Murray 2001). Evaluation of liver sections showed that ibrutinib treated mice had significantly more mature granulomas than vehicle and SSG treated controls with no statistical difference in the number of developing granulomas (Figure 2).

Figure 2. Treatment with Ibrutinib Improves Maturation of Liver Granulomas



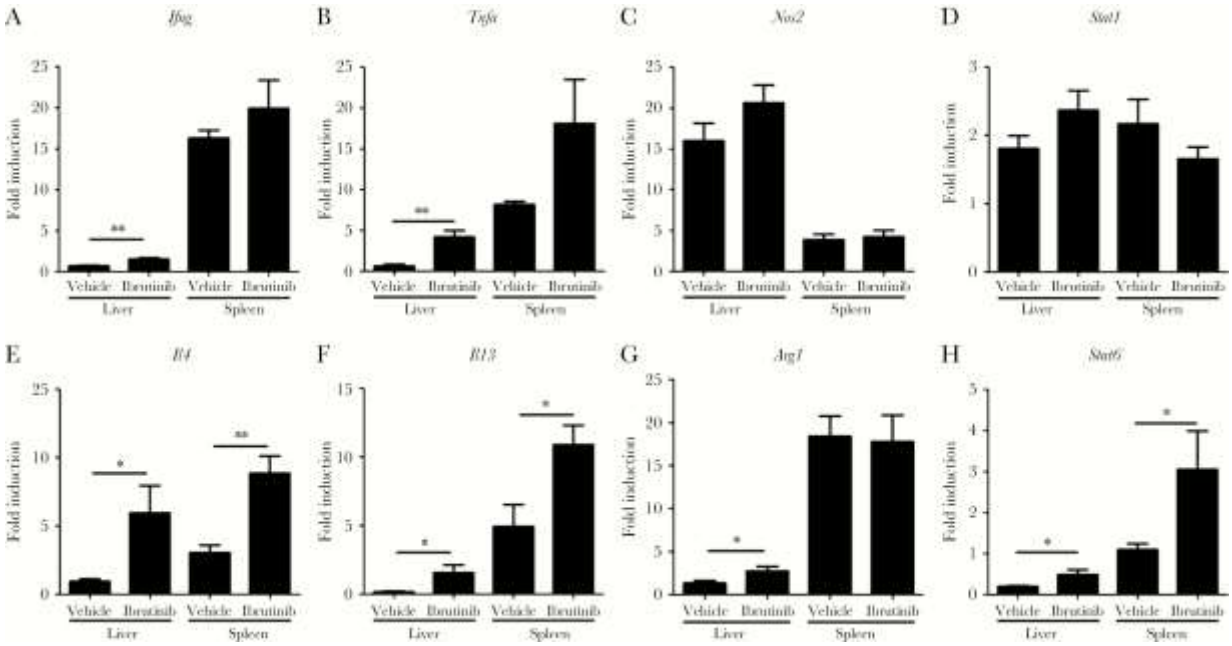
Livers were harvested from each of the treatment groups and assessed for granuloma formation using sections of tissue stained with hematoxylin-eosin. *A,* Representative images of liver sections with granulomas at 100× and 400× magnification. *B,* Granuloma scoring and counting

by certified pathologist. The number of granulomas was assessed per 10 high-power fields at 200× magnification and are indicated as mean ± standard error of the mean $^{**}P < .01$, using unpaired t test.

Treatment with Ibrutinib Increases Protective Cytokine Expression in Livers and Spleens

To discern a potential mechanism of antileishmanial activity caused by ibrutinib treatment, we compared the livers and spleen cytokine profiles of mice infected with *L. donovani*. Ibrutinib-treated mice had increased *Ifng* and *Tnfa* messenger RNA (mRNA) expression in the liver when compared with mice treated with vehicle alone (Figure 3). Elevated expression of *Il4*, *Il13*, and *Stat6* mRNA, was also observed in the spleens and livers of mice treated with ibrutinib (Figure 3). No difference in *Nos2* or *Stat1* mRNA expression were noted (Figure 3).

Figure 3. Protective Cytokine Expression in Livers and Spleens

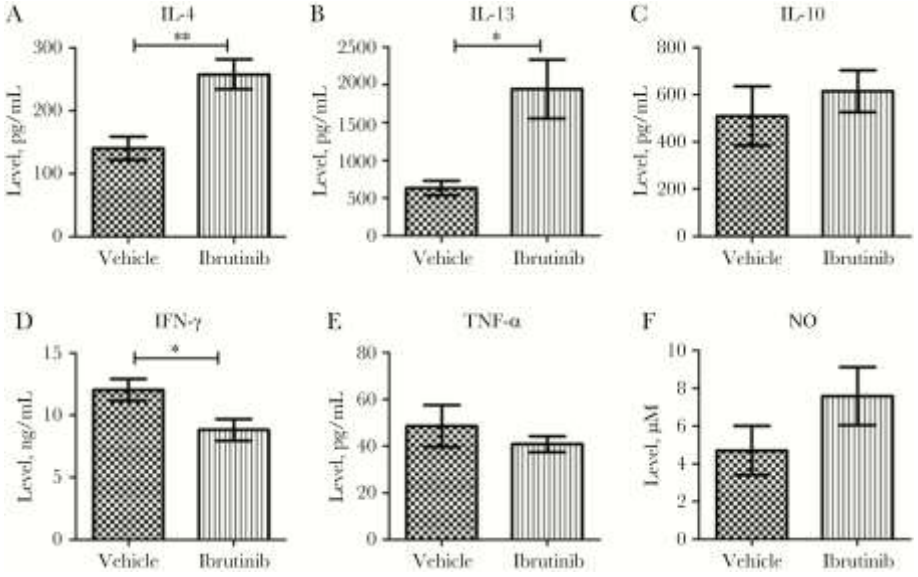


Treatment with ibrutinib treatment invokes simultaneous Th1 and Th2 immune responses during *L. donovani* infection. Liver and spleen gene expression was analyzed using rtPCR. Levels of mRNA in the liver and spleen that encode the following factors were assessed: interferon γ (*Ifng*; *A*), tumor necrosis factor α (*Tnfa*; *B*), Nos2 (*Nos2*; *C*), STAT1 (*Stat1*; *D*), interleukin 4 (*Il4*; *E*), interleukin 13 (*Il13*; *F*), arginase 1 (*Arg1*; *G*), and STAT6 (*Stat6*; *H*). Data is represented as fold expression over values obtained from non-infected wild type mice. $*P < .05$ and $**P < .01$, using unpaired t test.

Increased Expression of IL-4 and IL-13 by Antigen Stimulated Splenocytes

To correlate increased levels of *Il4* and *Il13* mRNA in the spleen with increased levels of the corresponding cytokines, splenocytes isolated from vehicle and ibrutinib treated mice were stimulated with *L. donovani* antigen for three days, and levels of IL-4 and IL-13 in the culture supernatants were assessed using ELISA. Coinciding with *Il4* and *Il13* mRNA expression, splenocytes isolated from mice treated with ibrutinib secreted increased amounts of IL-4 and IL-13 into the supernatant (Figure 4). Decreased IFN-γ was observed in the supernatant of the splenocytes of ibrutinib treated mice as opposed to treatment with vehicle which was unexpected based on previous studies. No significant differences were observed for IL-10, TNF-α, and NO in the supernatant.

Figure 4. Increased Expression of Protective Cytokines by Antigen Stimulated Spleen Cells



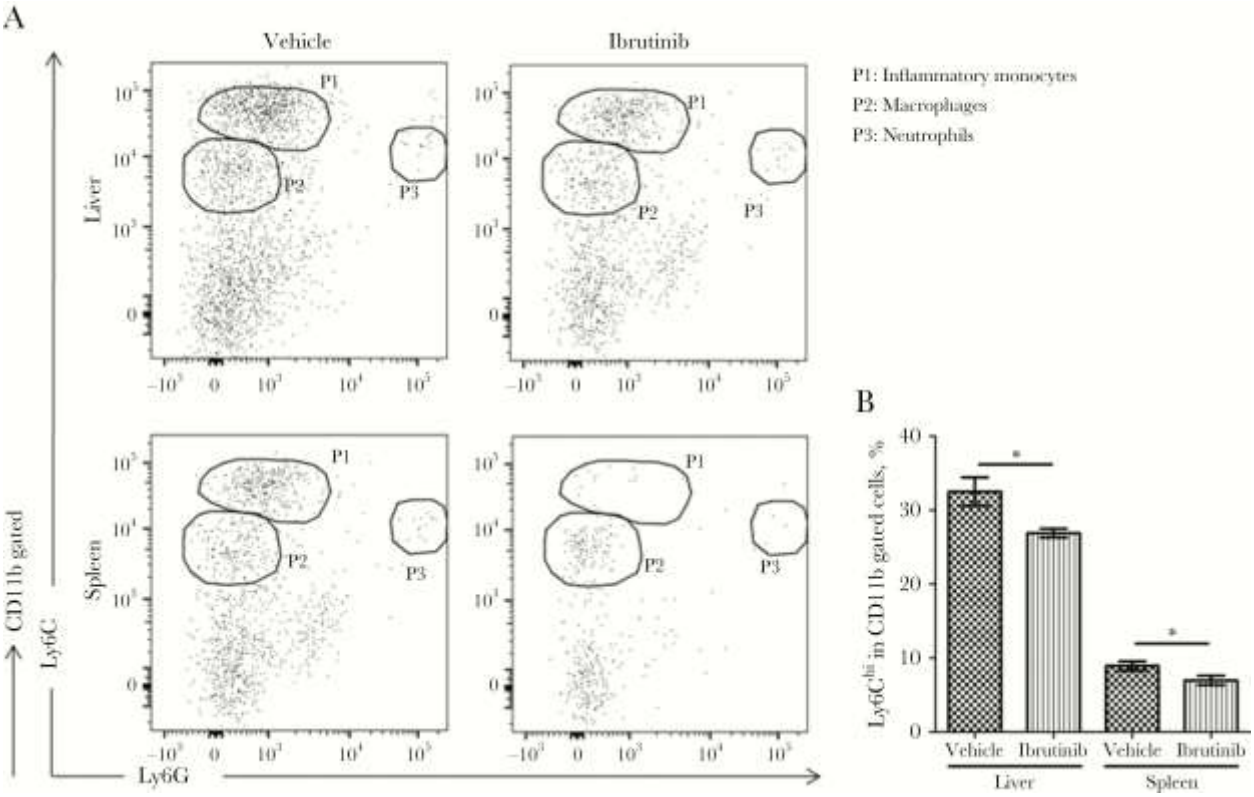
A, B, C, D, E, Splenocytes were harvested and isolated into single-cell suspensions which were re-stimulated with *L. donovani* antigen for three days to assess secretion of cytokines into culture supernatants using ELISA. *F*, A Griess assay was performed to assess NO production by splenocytes. Data is represented as the mean ± standard error of the mean **P* < .05 and ***P* < .01, using unpaired t test.

Ibrutinib Treatment Reduced Accumulation of Ly6C^{hi} Inflammatory Monocytes

Recent work done in our lab demonstrates that Ly6C^{hi} inflammatory mediate pathogenesis in VL, as they are preferentially targeted by *L. donovani* during infection (Terrazas et al. 2017). As it was also previously observed that ibrutinib alters characteristics of other myeloid lineage derived cells such as macrophages and neutrophils, we utilized flow cytometry to assess populations of Ly6C^{hi} inflammatory monocytes in the livers and spleens of mice treated with ibrutinib (Fiorcari et al. 2016). Ibrutinib treated mice had significantly reduced number of

Ly6C^{hi} inflammatory monocytes in the livers and spleens when compared with controls (Figure 5A and 5B).

Figure 5. Reduced Influx of Ly6C^{hi} Inflammatory Monocytes



Treatment with ibrutinib potentially effects many cell populations associated with VL disease progression. Here, myeloid cell populations in the liver and spleen were analyzed utilizing flow cytometry. *A,* Dot plots representing different myeloid cell populations from vehicle and ibrutinib treated mice livers and spleens. P1 shows the inflammatory monocyte population (CD11b+Ly6C^{high}LY6G−), P2 shows macrophages (CD11b+Ly6C^{low} LY6G−), and P3 shows neutrophils (CD11b+Ly6C−LY6G+). *B,* Number of Ly6C^{hi} cells after gating for CD11b in the livers and spleens. *P < .05 using unpaired t test.

Conclusions

The results of this study show that ibrutinib is an effective treatment for VL caused by *L. donovani* and has greater efficacy than traditional SSG therapy in reducing liver / spleen parasitic burden. Treatment with ibrutinib improved mature granuloma formation in the liver, increased protective cytokine production, along with a reduced influx of $Ly6C^{hi}$ inflammatory monocytes known to exacerbate the disease. These observations demonstrate that ibrutinib has potential to be a novel host-directed therapy for VL.

All current therapeutic options for leishmaniasis use drugs which act through direct cytotoxicity to the parasites and administration of these treatments is toxic to the host and often prolonged, resulting in lack of adequate compliance from patients. The evolution of drug-resistant parasite populations is a growing problem promoting the global spread of VL and insights on the mechanisms of host immune response -pathogen interactions can lead to development of treatments for *Leishmania* infection which target the host. Treatments which target host pathways required for survival of parasites or promote host immunity to improve destruction and clearance of parasites may be an effective solution to the increasing threat of parasite resistance. Recent studies done by our group have found that *Leishmania mexicana* entry into phagocytic host cells is mediated by PI3Kγ and inhibition of this enzyme impairs CL progression, additionally, blockage of CCR2 to reduce numbers of $Ly6C^{hi}$ inflammatory monocytes in *L. donovani* infected mice causes significant parasite burden reductions in the viscera (Cummings et al. 2012) (Terrazas et al. 2017). In this current study, ibrutinib was shown to be substantially more efficacious than the conventional antileishmanial drug SSG for treating VL. Mice that were treated with ibrutinib had significantly reduced liver and spleen parasite

burden when compared with SSG, vehicle, and PBS groups. Additionally, ibrutinib treatment was associated with enhanced production in the liver of both Th1 and Th2 associated mRNA transcripts, along with improved capability of splenocytes stimulated with *L. donovani* antigen to produce IL-4 and IL-13 compared to vehicle controls.

The results of this study show that mice infected with *L. donovani* which receive ibrutinib treatment produce increased levels of the Th2 associated cytokines IL4 and IL13 which contrasts with our studies of L. major induced CL where ibrutinib treatment resulted in decreased levels of these cytokines in lymph nodes (Dubovsky et al. 2013). The mechanisms of ibrutinib treatment that induce the profound variety of cytokine responses in CL as opposed to VL remain undefined however these differences could be attributed in part to varying organ specific effects of ibrutinib. It has been demonstrated that IL-4 and IL-13 promote susceptibility to CL caused by *L. major* and *L. mexicana* however it also appears that these particular cytokines are essential to resolve VL, develop mature granulomas in the liver, and promote effective immune responses to particular chemotherapies (Hurdayal and Brombacher 2014, Kopf et al. 1996, Gupta et al. 2013, Satoskar et al. 1995, Stäger et al. 2003, McFarlane et al. 2011, Alexander et al. 2000, Murray et al. 2005). Consistent with these previous studies, livers that were taken from infected ibrutinib treated mice had significantly more mature granulomas than control groups. Our results show that ibrutinib mediated antileishmanial actions in experimental VL are caused by the promotion of protective cytokine release and suppression of immune-cell responses which increase susceptibility to the disease.

Several experimental and clinical studies have demonstrated that B cell proliferation and antibody production contribute to VL progression and patients often experience

hypergammaglobulinemia from activation of polyclonal B cells which is correlated with disease exacerbation, additionally, B cells secrete IL-10 which has been shown to promote susceptibility to VL (Smelt et al. 2000, Gardinassi et al. 2014, Deak et al. 2010). Given ibrutinib's ability to block B cell receptor activation through the inhibition of BTK, we examined the effect of ibrutinib treatment on B cell populations. Contrary to the expected result, analysis of immunoglobulin G levels in the serum as well as populations of B cells in the spleen found there were no significant differences between groups. The results of these analysis suggest the ability of ibrutinib to promote parasite clearance was not due to B cell suppression.

The prototypical host cells of *Leishmania* parasites are phagocytic myeloid cells such as monocytes and macrophages, and recent studies from our lab demonstrated that *L. donovani* preferentially targets Ly6C^{hi} inflammatory monocytes which begin to accumulate in the viscera during infection (Terrazas et al. 2017). The data from this study shows that ibrutinib treated mice livers and spleens have a significant reduction in Ly6C^{hi} inflammatory monocyte influx. Currently it is not clear how simultaneous ITK/BTK inhibition caused reductions in the accumulation of inflammatory monocytes, however, in-progress studies occurring in our lab have shown that ibrutinib induces differentiation of Ly6C^{hi} inflammatory monocytes into DCs. Regardless of the mechanism, a reduction of Ly6C^{hi} inflammatory monocytes is a least in part responsible for parasite clearance from infected organs of mice treated with ibrutinib.

In conclusion, this data shows that ibrutinib treatment is safer and more effective than traditional SSG treatment and demonstrates that anti-parasitic action is mediated by the promotion of a protective immune response. Due to its overall efficaciousness and negligible toxicity, ibrutinib has potential as a novel host immunomodulatory treatment for VL caused by infection with *L. donovani*.

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