**Leishmania donovani** Isolates with Antimony-Resistant but Not -Sensitive Phenotype Inhibit Sodium Antimony Gluconate-Induced Dendritic Cell Activation

Arun Kumar Haldar¹, Vinod Yadav², Eshu Singhal², Kamlesh Kumar Bisht³, Alpana Singh², Suniti Bhaumik¹, Rajatava Basu¹, Pradip Sen², Syamal Roy¹

1 Division of Infectious Diseases and Immunology, Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India, 2 Division of Cell Biology and Immunology, Institute of Microbial Technology, Council of Scientific and Industrial Research, Chandigarh, India, 3 Institute of Microbial Technology, Council of Scientific and Industrial Research, Chandigarh, India

**Abstract**

The inability of sodium antimony gluconate (SAG)-unresponsive kala-azar patients to clear **Leishmania donovani** (LD) infection despite SAG therapy is partly due to an ill-defined immune-dysfunction. Since dendritic cells (DCs) typically initiate anti-leishmanial immunity, a role for DCs in aberrant LD clearance was investigated. Accordingly, regulation of SAG-induced activation of murine DCs following infection with LD isolates exhibiting two distinct phenotypes such as antimony-resistant (Sb<sup>RD</sup>LD) and antimony-sensitive (Sb<sup>SLD</sup>LD) was compared *in vitro*. Unlike Sb<sup>SLD</sup>LD, infection of DCs with Sb<sup>RD</sup>LD induced more IL-10 production and inhibited SAG-induced secretion of proinflammatory cytokines, up-regulation of co-stimulatory molecules and leishmanicidal effects. Sb<sup>RD</sup>LD inhibited these effects of SAG by blocking activation of PI3K/AKT and NF-κB pathways. In contrast, Sb<sup>SLD</sup>LD failed to block activation of SAG (20 μg/ml)-induced PI3K/AKT pathway; which continued to stimulate NF-κB signaling, induce leishmanicidal effects and promote DC activation. Notably, prolonged incubation of DCs with Sb<sup>SLD</sup>LD also inhibited SAG (20 μg/ml)-induced activation of PI3K/AKT and NF-κB pathways and leishmanicidal effects, which was restored by increasing the dose of SAG to 40 μg/ml. In contrast, Sb<sup>RD</sup>LD inhibited these SAG-induced events regardless of duration of DC exposure to Sb<sup>RD</sup>LD or dose of SAG. Interestingly, the inhibitory effects of isogenic Sb<sup>RD</sup>LD expressing ATP-binding cassette (ABC) transporter MRPA on SAG-induced leishmanicidal effects mimicked that of Sb<sup>SLD</sup>LD to some extent, although antimony resistance in clinical LD isolates is known to be multifactorial. Furthermore, NF-κB was found to transcriptionally regulate expression of murine γ-glutamylcysteine synthetase heavy-chain (mγGCS<sub>hc</sub>) gene, presumably an important regulator of antimony resistance. Importantly, Sb<sup>RD</sup>LD but not Sb<sup>SLD</sup>LD blocked SAG-induced mγGCS expression in DCs by preventing NF-κB binding to the mγGCS<sub>hc</sub> promoter. Our findings demonstrate that Sb<sup>RD</sup>LD but not Sb<sup>SLD</sup>LD prevents SAG-induced DC activation by suppressing a PI3K-dependent NF-κB pathway and provide the evidence for differential host-pathogen interaction mediated by Sb<sup>RD</sup>LD and Sb<sup>SLD</sup>LD.

**Introduction**

Kala-azar, caused by **Leishmania donovani** (LD), is regarded as the most severe form of leishmanial infection, which can be fatal in patients when left untreated. In the absence of an effective vaccine, treatment with pentavalent antimonial compounds such as sodium antimony gluconate (SAG) remains as the first-choice therapy for kala-azar. However, therapeutic utility of SAG is now jeopardized by the emergence of antimony-resistant strains of LD [1], which is becoming a major concern of the World Health Organization (www.who.int/infections-disease-report/2000).

Resistance to antimonial drugs, as observed in leishmanial infection, is marked by two independent “checkpoints”. The first is associated with the impaired biological reduction of the pentavalent antimony (Sb<sup>IV</sup>) prodrug to a toxic trivalent (Sb<sup>III</sup>) form, although the site (macrophage (Mφ) and/or parasite) and mechanism of reduction (enzymatic or nonezymatic) are undefined. The second checkpoint involves a regulatory mechanism promoting reduced influx and/or enhanced efflux/sequestration of active drug that lowers its intracellular accumulation [2,3]. Importantly, these two events are largely dependent on the intracellular level of thiol compounds such as glutathione (γ-glutamylcysteinylglycine, GSH) and parasite-specific trypanothione, which in turn are regulated by both host- and LD-γ-glutamylcysteine synthetase, a rate-limiting enzyme in glutathione biosynthesis [2–5]. Although the increased expression of
Author Summary

Kala-azar, a life-threatening parasitic disease caused by Leishmania donovani (LD), is widening its base in different parts of the world. Currently, there is no effective vaccine against kala-azar. The antimonial drugs like sodium antimony gluconate (SAG) have been the mainstay of therapy for this disease. Recently, due to the emergence of antimony-resistance in parasites, SAG often fails to cure kala-azar patients, which is compounding the disaster further. It is still unknown how infection with LD exhibiting antimony-resistant phenotype, in contrast to antimony-sensitive phenotype, is handled by the kala-azar patients upon SAG treatment. This demands an understanding of the nature of host immune responses against these two distinct categories of parasites. Accordingly, we compared the impact of infection with LD exhibiting antimony-resistant versus antimony-sensitive phenotype on dendritic cells (DCs). DCs upon activation/maturation initiate anti-leishmanial immunity. We showed that parasites with antimony-resistant but not antimony-sensitive phenotype prevented SAG-induced DC activation/maturation by blocking activation of NF-κB. The latter is a key signaling pathway regulating DC activation/maturation. Our studies for the first time provide both a cellular and molecular basis for differential response of host cells to parasite isolates with antimony-resistant and antimony-sensitive phenotype, which may influence the outcome of the disease.

γ-glutamylcysteine synthetase (γGCS) gene in antimony-resistant strains of LD is controversial [3–6], inhibition of γGCS by buthionine sulfoxamine (BSO) reverses ShH resistance in LD [7]. Therefore, γGCS expression contributes to antimony resistance in LD by regulating intracellular thiol level.

In addition to the mechanisms noted above, the unresponsiveness of kala-azar patients to treatment with SAG is also believed to be a consequence of skewed type-2 immune response that suppresses interferon (IFN) γ-mediated protective immunity [8]. Nonetheless, IFNγ production by T cells is reduced in non-responders compared to SAG-responders [8,9]. The endogenous production of IFNγ and importantly, its principal inducer IL-12, determines the anti-leishmanial efficacy of SAG in a fully immunocompetent host infected with LD [10,11]. Following LD infection, the early production of IL-12 is exclusively mediated by dendritic cells (DCs) [12]. This tempted us to speculate a possible involvement of DCs in regulating “SAG responsiveness” versus “unresponsiveness” in kala-azar patients.

DCs normally play a key role in initiating and regulating Leishmania-specific T cell reactivity [13,14]. However, the T cell stimulatory capacity of DCs depends on their state of activation and maturation. In contrast to mature DCs, immature DCs exhibit a reduced capacity to stimulate T cells due to low expression of MHC and co-stimulatory molecules, and the lack of production of proinflammatory cytokines. Gene expression associated with the development, activation, maturation and antigen-presenting cell (APC) function of DCs is largely regulated by the transcription factor NF-κB [15–18]. For instance, inhibition of NF-κB activation suppresses DC maturation and APC function [19,20]. NF-κB is a hetero- or homo-dimeric complex of structurally related proteins p50, p32, p65 (RelA), cRel and RelB. In resting cells, NF-κB is sequestered in the cytoplasm by the inhibitory proteins IκBα, IκBβ and IκBe [21]. However, cellular activation with wide range of stimuli such as LPS, TNFα and IL-1 phosphorylates and thereby activates a multisubunit complex IκB kinase (IKK) consisting of IKKα/IKK1, IKKβ/IKK2 and IKKγ/NEMO [22]. Subsequently, activated IKK promotes downstream events, for example, phosphorylation followed by polyubiquitination and 20S proteasome-mediated degradation of IκB proteins [21]. NF-κB dimers then translocate to the nucleus, and bind to consensus sequences to induce gene transcription. Notably, the phosphatidylserinol 3-kinase (PI3K)/AKT pathway has been demonstrated in a variety of models to regulate NF-κB activation [20,23,24].

Studies demonstrated that stimulation with SAG induces the PI3K/AKT pathway and enhances production of proinflammatory cytokines and leishmanicidal effector molecules in Mφ [25]. Furthermore, SAG stimulates NF-κB activation in different cell types, such as CD4+ T cells and peripheral blood mononuclear cells [26]. Importantly, blockade of NF-κB activation is shown to impair γGCS expression in murine Mφ-like cell line [27]. However, direct role of NF-κB in transcriptional regulation of murine γGCS promoter is undefined. With this in mind, the current study was initiated to define the role of SAG in murine DC activation and its regulation by LD isolates with SAG-resistant (ShLD) and SAG-sensitive (ShLD) phenotype. We demonstrate that ShLD but not ShLD infection suppresses SAG-induced activation/maturation and γGCS expression of DCs by inhibiting NF-κB activation in a PI3K/AKT-dependent manner.

Results

ShLD- and ShLD-infected DCs respond differentially to SAG treatment

Although Mφs are regarded as a “primary target” for leishmanial infection, recent studies indicate DC infection with various Leishmania spp, including LD [28,29]. Indeed, LD infection was observed in both immature bone marrow-derived DC (BMDC) (CD11c+CD8α+) and splenic DC (sDC) (Figure 1A). To determine the leishmanicidal effect of SAG on intracellular ShLD and ShLD in DCs, BMDCs and sDCs were infected with GFP expressing promastigotes of ShLD strain 2001 (GFP-2001) or ShLD strain R5 (GFP-R5) for 3 hours, stimulated with SAG (20 μg/ml) for 24 hours, and the frequency of infected DCs measured via flow cytometry. A comparable level of DC infection was observed with both GFP-2001 and GFP-R5 (Figure 1B). However, the percentage of BMDCs or sDCs infected with GFP-2001 (ShLD) was reduced by 5 to 9-fold following SAG treatment (Figure 1B). In marked contrast, SAG treatment failed to exhibit any significant effect on GFP-R5 (ShLD) infection in DCs (Figure 1B). Furthermore, analyses via Giemsa staining demonstrated that intracellular amastigotes of other ShLD strains exhibit similar resistance to the SAG-induced leishmanicidal effect in DCs. For instance, a significant reduction in both percentage of infected BMDCs and intracellular parasite number were observed in AG83 (ShLD)- and to a lesser extent in 39 (ShLD)-infected BMDCs after SAG treatment (10 and 20 μg/ml) for 24 and 48 hours (Figure S1). Titration of parasites demonstrated that parasite to DC ratio (multiplicity of infection; MOI) of 10:1 was the optimum ratio for maximum LD infection in DCs (data not shown). Therefore, this MOI was used for all subsequent experiments unless otherwise stated.

Next, the regulation of SAG-induced activation and maturation of DCs by ShLD and ShLD was investigated. For this purpose, BMDCs and sDCs were infected with ShLD and ShLD promastigotes for 3 hours, washed and cultured with or without SAG (20 μg/ml) for an additional 48 hours. The activation and maturation of DCs were determined by analyzing MHC and co-stimulatory molecule expression and secretion of cytokines.
Figure 1. SAG treatment exhibits differential effect on Sb<sup>SLD</sup>- and Sb<sup>RLD</sup>-infected DCs. (A) BMDCs and ex vivo derived sDCs were infected with 2001 promastigotes (Sb<sup>SLD</sup>) in vitro at a MOI of 10:1 for overnight, washed thoroughly to remove free parasites and localization of intracellular LD parasites was ascertained via Giemsa staining. (B) BMDCs and sDCs were infected in vitro with GFP-2001 (Sb<sup>SLD</sup>) or GFP-R5 (Sb<sup>RLD</sup>) promastigotes, as described above, for 3 hours or left uninfected. Free parasites were removed from DCs by thorough washing. The LD-infected DCs were then cultured with or without SAG treatment (20 \( \mu \text{g/ml} \)) for another 24 hours whereas uninfected DCs were left untreated. DCs were immunostained with \( \alpha \text{CD11c-PE} \) and LD infection in DC was analyzed by FACS. Numbers in upper right quadrant indicate the percentage of LD-infected DCs as represented by \( \text{CD11c}^+ \text{GFP}^+ \) cells. In some experiments (C-F), BMDCs were infected with Sb<sup>SLD</sup> (AG83, 2001) or Sb<sup>RLD</sup> (39, GE1F8R) strains for 3 hours or left uninfected as described above. DCs were then washed to remove free parasites and stimulated with SAG (20 \( \mu \text{g/ml} \)) for 48 hours and (C) IL-10, (D) IL-12p70 and (E) TNF-\( \alpha \) secretion in culture supernatants were measured via ELISA or (F) surface expression of co-stimulatory molecules and MHCs measured via FACS. Open histograms represent untreated DCs and shaded histograms represent DCs plus SAG. For this and all other figures the label "Uninf" represents uninfected DCs. Data are the representative of three independent experiments. *\( p < 0.001 \) versus DC+AG83; **\( p = 0.002 \) and \( p = 0.003 \) versus DC+2001; †\( p < 0.001 \), ††\( p = 0.004 \) and ‡\( p = 0.002 \) versus DC+SAG (Student’s \( t \) test). Error bars indicate mean ± SD.

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Infection of BMDCs and sDCs with SbRLD strains 39 or GE1F8R induced more IL-10 production as compared with SbSLD strains AG83 or 2001 (Figures 1C and S2A). Interestingly, IL-10 secretion from SbRLD-infected DCs was not affected by SAG treatment (Figures 1C and S2A). Furthermore, SAG-stimulated secretion of proinflammatory cytokines such as IL-12p70 and TNFα from DCs were inhibited by SbRLD and not SbSLD infection (Figures 1D-E and S2B-C). Finally, SAG treatment up-regulated CD40, CD80, CD86, MHC-I (H2Kd) and MHC-II (IAd) expression in BMDCs infected with SbRLD but not SbSLD (Figure 1F). Consistent with work by other groups [30,31], co-stimulatory molecule and MHC expression of untreated BMDCs remained unaltered following SbRLD or SbSLD infection (Figure 1F). Together, these results demonstrate that SAG treatment protects DCs from SbSLD but not SbRLD infection. Moreover, stimulation with SAG fails to activate and mature SbRLD-infected DCs, while SbSLD-infected DCs are still capable of activation and maturation upon SAG treatment.

SAG treatment induces NF-κB activation in DCs

Since NF-κB is a key regulator of maturation and APC function of DCs, the effect of SAG treatment on NF-κB activation was investigated. BMDCs were treated with SAG for varying times and DNA binding activity of nuclear NF-κB was determined via electrophoretic mobility shift assay (EMSA). Relative to untreated BMDCs, a 23-fold increase in NF-κB DNA binding activity was initially observed by 0.3 hours, which persisted up to 1 hour after SAG treatment (Figure 2A). Notably, OCT-1 DNA binding was unaltered despite SAG treatment indicating that SAG-induced enhancement of nuclear DNA binding was NF-κB-specific (Figure 2A). Furthermore, supershift analysis using antibodies specific for each Rel family member demonstrated that SAG stimulation of BMDCs induced DNA binding of NF-κB complexes consisting of the p50, p65 and RelB subunits (Figure 2B). In contrast to SAG, BMDC treatment with varying concentrations (25 to 200 µg/ml) of sodium gluconate for 0.3 hours, or 200 µg/ml of sodium gluconate for various times failed to induce DNA binding activity of NF-κB (Figure 2C-D).

Consistent with the EMSA data, SAG treatment for 0.3, 0.5 and 1 hour induced degradation of IκBα proteins in BMDCs (Figure 3A). Importantly, SAG-induced IκBα degradation corresponded to enhanced IκBα phosphorylation (Figure 3B), which could be due to increased activity of upstream IKK complex. To test this hypothesis, BMDCs were stimulated with SAG for 0.3, 0.5 and 1 hour. IKK signalosome was immunoprecipitated from cytoplasmic extracts and kinase activity of the complex determined by measuring phosphorylation of an IκBα-GST substrate in vitro. BMDCs stimulated with SAG for 0.3, 0.5 and 1 hour exhibited approximately 3.6 to 4.7-fold increase in IKK activity compared to untreated BMDCs.

Figure 2. Stimulation with SAG increases nuclear NF-κB DNA binding activity in BMDCs. BMDCs were treated with SAG (20 µg/ml) or specified concentrations of sodium gluconate (SG) for indicated times or left untreated. (A, C-D) DNA binding activity of nuclear NF-κB to H2Kd-specific oligonucleotide probe was measured via EMSA. The OCT-1 DNA binding was used as an internal control. Densitometric analysis represents the ratio of intensity of NF-κB to OCT-1 binding per unit area and is represented as arbitrary units. (B) Binding of different NF-κB complexes to H2K-DNA probe was determined by supershift EMSA using rabbit IgG (Control Ab) or Abs specific for different NF-κB subunits. For this and all other figures, control (Cont) lane represents uninfected DCs without SAG stimulation. Data are representative of three independent experiments. Error bars indicate mean ± SD.

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to untreated BMDCs (Figure 3C). In comparison, the level of IKK1 and IKK2 proteins were similar in all BMDC extracts (Figure 3C). The SAG-induced NF-κB DNA binding, IκBα degradation and IKK activity were also observed in sDCs (Figure S3).

Of note, SAG treatment failed to activate the mitogen-activated protein kinase (MAPK) pathway in BMDCs. In contrast to LPS stimulation, phosphorylation of p38MAPK, ERK1/ERK2 and JNK was not detected in SAG-treated DCs (Figure 3D-F). Collectively, these findings demonstrate that stimulation with SAG induces activation of the IKK complex, phosphorylation and degradation of IκB proteins and downstream nuclear DNA binding of NF-κB in both BMDCs and sDCs, and that the induction of these events in DC is contributed by antimonial moiety of SAG. Furthermore, among different signaling pathways, which are known to regulate DC activation/maturation, NF-κB signaling is selectively induced by SAG treatment.

Figure 3. SAG-treated BMDCs exhibit increased phosphorylation and degradation of IκB proteins and IKK activity. BMDCs were untreated or stimulated either with 20 μg/ml SAG (A-F) or 500 ng/ml LPS (D-F) for specified times. (A) Expression of IκBα, IκBβ, IκBε and β-actin protein in cytoplasmic extracts was detected by Western blot using the same blot. Densitometric analyses represent the ratio of intensity of the corresponding IκB protein to β-actin expression per unit area and are represented as an arbitrary unit. (B) Cytoplasmic phospho-IκBα versus β-actin protein expression was detected via Western blot with the same blot. (C) In vitro IKK activity was determined by measuring phosphorylation of an IκBα-GST substrate. IKK1 and IKK2 protein expression in immunoprecipitated samples was analyzed via Western blot. Densitometric analysis indicates the intensity of phosphorylated (P) IκBα-GST substrate in an arbitrary unit. The levels of (D) phospho-p38MAPK versus p38MAPK, (E) phospho-ERK versus ERK and (F) phospho-JNK versus JNK protein expression in whole cell lysates were determined via Western blot. Data are representative of three independent experiments. Error bars indicate mean ± SD.

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SbLD but not SbLD infection inhibits SAG-induced NF-κB activation in DCs

The effect(s) of SbLD and SbLD infection on SAG-stimulated NF-κB activation in DCs was determined. BMDCs were infected with either promastigotes or amastigotes of 39 (SbLD) or 2001 (SbLD) for varying times, stimulated with SAG for 0.3 hours and nuclear NF-κB DNA binding activity to H2K-specific probe measured via EMSA. SAG-induced NF-κB activity was completely inhibited in BMDCs upon infection with either 39 promastigotes (39Pm) or amastigotes (39Am) (SbLD) at all times of LD infection analyzed (Figure 4A-B). In contrast, BMDC infection with 2001 promastigotes (2001Pm) or amastigotes (2001Am) (SbLD) for up to 6 and 3 hours, respectively, failed to inhibit SAG-stimulated NF-κB DNA binding (Figure 4A-B). SAG-induced NF-κB DNA binding activity was also inhibited in 39Pm (SbLD)-infected sDCs (Figure S4A). The ability of SbLD to block SAG-stimulated NF-κB DNA binding in DCs was not LD strain specific. For example, BMDC infection with promastigotes of SbLD strain GE10/GE19PM, unlike SbLD strain AG313 (AG313Pm), completely prevented SAG-induced NF-κB DNA binding (Figure S4B).

Our finding that SAG-induced NF-κB activity is inhibited selectively in SbLD-infected BMDCs was further confirmed by temporal analysis of IkB protein degradation and activation of IKK. BMDC infection with either 2001Pm or 2001Am (SbLD) for up to 6 or 3 hours, respectively, had no significant effect on SAG-induced IkB degradation (Figure 4C-D). In contrast, IkB degradation stimulated by SAG was persistently inhibited by 39 (SbLD) regardless of the duration of BMDC infection and form of parasite (Figure 4C-D). SAG-induced IkB degradation was similarly inhibited in sDCs infected with 39Pm (SbLD) and BMDCs infected with promastigotes of a different SbLD strain GE10/GE19PM, unlike SbLD strain AG313 (AG313Pm), completely prevented SAG-induced NF-κB DNA binding (Figure S4C-D). Interestingly, inhibition of SAG-induced IkB degradation corresponded with a reduction in IkBa phosphorylation in BMDCs infected with SbLD promastigotes (Figure 4C, E). Furthermore, SAG-induced IKK activation as determined by in vitro IKK activity or phosphorylation of IKK1 and IKK2 was inhibited in extracts prepared from BMDCs and sDCs infected with SbLD but not SbLD promastigotes (Figures 4F and S4E-F). BMDC infection with SbLD and not SbLD amastigotes also blocked SAG-stimulated IKK activity (Figure 4G). Additionally, pretreatment of BMDCs with parasite antigen(s) (SbLD) or culture supernatant (SbLDs) of SbLD-infected NF-κB DNA binding activity, IkB degradation and IKK activity; whereas culture supernatant of SbLD (SbLDs) or SbLD-derived antigen(s) of SbLDs did not (Figure 5).

As demonstrated in Figure 4A-D; SAG-induced NF-κB activation was inhibited in BMDCs infected at a MOI 10:1 (promastigote or amastigote to DC) with SbLD promastigotes or amastigotes for 24 or 6 hours, respectively, similar to SbLD-infected BMDCs. Accordingly, we tested whether the increased intracellular parasite number at these time points of infection rendered 20 μg/ml of SAG insufficient to activate NF-κB. In fact, number of intracellular 2001 (SbLD) or 39 (SbLD) was significantly increased if BMDCs were infected with promastigotes for 24 hours rather than 6 hours (Figure S5A). Likewise, BMDCs infected with amastigotes of the above LD strains for 6 hours exhibited increased intracellular parasite number compared to BMDCs infected for 3 hours (Figure S5B). Notably, SAG (20 μg/ml)-induced NF-κB DNA binding and IkB degradation were detected despite the presence of intracellular parasites in BMDCs infected with 2001Pm and 2001Am (SbLD) for 6 and 3 hours, respectively (Figures 4A-D and S5A-B). Therefore, these two time points of BMDC infection were selected as a “reference” to analyze the basis of defective SAG-induced NF-κB activation in BMDCs infected with 2001Pm or 2001Am (SbLD) for 24 or 6 hours, respectively. Initially, the association of increased intracellular parasite number with impairment of SAG-induced NF-κB activation was verified in both BMDCs infected with 2001Pm or 2001Am (SbLD) for 24 or 6 hours, respectively. For this purpose, BMDCs were infected with 2001Pm (SbLD) or 39Pm (SbLD) for 24 hours at varying MOIs and stimulated with SAG (20 μg/ml) for 0.3 hours. Despite LD infection for fixed duration (24 hours), this approach established varying levels of intracellular parasite number, which was elevated in BMDCs infected at MOI 10:1 compared to other MOIs (Figure S6A). BMDC infection with both 2001Pm (SbLD) and 39Pm (SbLD) at a MOI 10:1 inhibited SAG-induced NF-κB DNA binding activity and IkB degradation (Figure 6A-B). However, SAG-induced NF-κB DNA binding activity and IkB degradation were observed only in 2001Pm (SbLD)-infected BMDCs but not 39Pm (SbLD)-infected BMDCs when BMDC infection was done at MOIs 2.5:1 and 5:1 (Figure 6A-B). Notably, at each of these MOIs both 2001Pm (SbLD)-infected BMDCs and 39Pm (SbLD)-infected BMDCs had comparable level of intracellular parasite number (Figure S6A). Using identical MOIs for BMDC infection, similar results were obtained when the intracellular parasite number and SAG-induced NF-κB activation were analyzed in BMDCs infected for 6 hours with 39Am (SbLD) and 2001Am (SbLD) (Figures 6C-D and S6B). These findings suggest that irrespective of the form of parasite, 2001 (SbLD) and 39 (SbLD) differentially regulate SAG-induced NF-κB signaling in DCs with low intracellular parasite number.

It is possible that with increased intracellular parasite number 2001 (SbLD), similar to 39 (SbLD), developed the capacity to inhibit SAG-induced NF-κB activation and that occurred when BMDCs were infected at a MOI 10:1 with 2001Pm or 2001Am (SbLD) for 24 and 6 hours, respectively. However, this possibility was ruled out when NF-κB activation in response to 20 and 40 μg/ml of SAG treatment was analyzed in BMDCs infected for 6 and 24 hours with 2001Pm (SbLD) or 39Pm (SbLD) at a MOI of 10:1. The effect of SAG (40 μg/ml) stimulation was also verified in BMDCs infected similarly with 2001Am or 39Am for 3 and 6 hours. Stimulation with both 20 and 40 μg/ml of SAG induced NF-κB DNA binding and IkB degradation in BMDCs infected with 2001Pm (SbLD) for 6 hours (Figure 6E-F). In contrast, BMDCs infected for 24 hours with 2001Pm (SbLD) exhibited enhanced NF-κB DNA binding and IkB degradation only when stimulated with 40 μg/ml of SAG (Figure 6E-F). Similarly, the inhibition of SAG-induced NF-κB DNA binding and IkB degradation due to BMDC infection with 2001Am (SbLD) for 6 hours was overcome by increasing the dose of SAG from 20 to 40 μg/ml (Figure 6G-H). On the contrary, 39Pm-infected (SbLD) continued to suppress NF-κB DNA binding and IkB degradation at various durations of infection tested irrespective of dose of SAG used for stimulation (Figure 6E-F). Together, these results demonstrate that SAG-induced NF-κB signaling is impaired by SbLD infection of DCs.

SbLD inhibits SAG-induced NF-κB signaling, DC activation and leishmanial effects by suppressing the PI3K/AKT pathway in an IL-10-independent manner

Stimulation with SAG induces PI3K/AKT activation in Mcφ [25]. Furthermore, PI3K/AKT regulates the NF-κB pathway in DCs via IKK [19,20]. Therefore, the possibility that SAG-induced activation of NF-κB in DCs is PI3K/AKT-dependent was investigated. Initially, the effect of SAG stimulation on AKT activation was assessed by measuring phosphorylation of AKT.
Figure 4. SAG-induced NF-κB signaling is inhibited in Sb\(^{LD}\)- and not Sb\(^{LD}\)-infected DCs. BMDCs were infected in vitro with promastigotes (Pm) (A, C, E-F) or amastigotes (Am) (B, D, G) of specified strains of Sb\(^{LD}\) or Sb\(^{LD}\) at a MOI 10:1 for indicated times or left uninfected. DCs were then stimulated with SAG (20 \(\mu\)g/ml) for 0.3 hours. (A-B) Nuclear DNA binding of NF-κB to H2K-specific probe was measured via EMSA. DNA binding of OCT-1 was used as internal control. Densitometric analyses were determined by measuring the ratio of intensity of NF-κB to OCT-1 binding.
Compared to unstimulated BMDCs, SAG treatment induced a 5 to 7-fold increase in AKT phosphorylation in BMDCs (Figure 7A). Notably, SAG-induced AKT phosphorylation was not observed in BMDCs pretreated with PI3K inhibitors wortmannin (Wort) or LY294002 (LY) (Figure 7A). Next, the effect of PI3K inhibitors on SAG-induced NF-κB signaling was determined. Pretreatment with Wort or LY effectively blocked SAG-induced IKK activity, IkBα degradation and nuclear NF-κB DNA binding in BMDCs (Figure 7B-D). Importantly, infection of BMDCs and SDCs with 39Pm (ShRLD) but not 2001Pm (ShSLD) for 3 hours inhibited SAG (20 μg/ml)-induced AKT phosphorylation (Figures 7E and 7F). SAG (20 μg/ml)-induced AKT phosphorylation was also inhibited due to BMDCs infection for 1 and 3 hours with 39Am (ShRLD) but not 2001Am (ShSLD) (Figure 7E). Similar to 39Pm (ShRLD)-infected BMDCs, SAG (20 μg/ml)-induced AKT phosphorylation, however, was not observed if BMDCs were infected with 2001Pm (ShSLD) for 24 hours (Figure 7G). Interestingly, AKT phosphorylation was observed in these 2001Pm (ShSLD)-infected BMDCs but not 39Pm (ShRLD)-infected BMDCs upon stimulation with 20 μg/ml of SAG (Figure 7G).

Since ShRLD infection stimulated high IL-10 secretion by DCs (Figures 1C and S2A), the possibility that ShRLD inhibited SAG-induced PI3K/AKT (Figures 7E-F and S7) and NF-κB pathways (Figures 4 and S4) in an IL-10-dependent manner was investigated by using a neutralizing zIL-10 Ab. Temporal analyses demonstrated that significant IL-10 production by BMDCs was initially detected after an infection for 12 hours with ShRLD but not ShSLD promastigotes (Figure 7H). Compared to ShRLD-infected BMDCs, IL-10 production was significantly increased in BMDCs infected with ShRLD for 24 and 48 hours (Figure 7H). Strikingly, SAG-induced AKT phosphorylation and NF-κB DNA binding activity were not restored in BMDCs infected with ShRLD for 3 and 24 hours despite zIL-10 Ab treatment (Figure 7I-J). Moreover, zIL-10 Ab treatment effectively prevented inhibition of LPS-induced NF-κB DNA binding in BMDCs pretreated with IL-10 (Figure S8). Therefore, this finding ruled out the involvement of IL-10 in suppression of SAG-induced PI3K/AKT and NF-κB pathways in ShRLD-infected BMDCs.

Consistent with previous report [5], an overexpression of ATP-binding cassette (ABC) transporter MRPA (PGPA) was observed in ShRLD strains 39Pm and GE1F8RPm (Figure S9A). Whether MRPA plays any role in mediating the inhibitory effects of ShRLD on SAG-induced PI3K/AKT and NF-κB activation in DC was then investigated. Accordingly, SAG-induced AKT phosphorylation and NF-κB activation in BMDCs infected for 3 hours with 39Pm (ShRLD), 2001Pm (ShSLD) and isogenic 2001Pm expressing MRPA (2001Pm-MRPA) (Figure S9B) were compared. The latter was developed by transfecting 2001Pm (ShSLD) with a DNA construct expressing MRPA. A complete blockade of SAG-induced AKT phosphorylation and NF-κB DNA binding activity was observed in 39Pm (ShRLD)-infected BMDCs (Figure 8A-B). In contrast, SAG-induced AKT phosphorylation and NF-κB DNA binding activity were detected in 2001Pm (ShSLD)-infected BMDCs (Figure 8A-B). However, infection of BMDCs with 2001Pm-MRPA inhibited SAG-induced AKT phosphorylation and DNA binding activity of NF-κB in BMDCs, albeit partially (Figure 8A-B).

Next, a direct role for PI3K in ShRLD and ShSLD regulation of SAG-induced proinflammatory cytokine secretion and leishmanicidal effects in DCs was investigated using Wort or Ly. In contrast to 39Pm (ShRLD)-infected BMDCs, SAG-stimulated IL-12 and TNFα production were observed in both uninfected and 2001Pm (ShRLD)-infected BMDCs (Figure 9A-B). However, pretreatment of uninfected and 2001Pm (ShRLD)-infected BMDCs with Wort or Ly significantly inhibited SAG-induced secretion of IL-12 and TNFα (Figure 9A-B). Furthermore, PI3K inhibitors prevented SAG (20 μg/ml)-induced reduction of percentage of infected BMDCs and intracellular parasite number in BMDCs infected for 3 hours with 2001Pm (ShRLD) (Figure 9C-D). Importantly, BMDCs infected with 2001Pm (ShRLD) for 24 hours exhibited a significant reduction in both percentage of infected BMDCs and intracellular parasite number only when treated with 40 μg/ml of SAG (Figure S10). Treatment of these 2001Pm (ShRLD)-infected BMDCs with Wort or Ly blocked the leishmanicidal effects of SAG (40 μg/ml) (Figure S10). In contrast to 2001Pm (ShRLD)-infected BMDCs, SAG-induced leishmanicidal effects were not observed in 39Pm (ShRLD)-infected BMDCs regardless of duration of infection and dose of SAG (Figures 9C-D and S10). In addition, BMDC infection for 3 hours with 2001Pm-MRPA, unlike 2001Pm (ShSLD), partly but significantly suppressed SAG-induced leishmanicidal effects (Figure 9E-F). Collectively, these data demonstrate that blockade of PI3K/AKT pathway by ShRLD impairs SAG-induced NF-κB signaling, DC activation and leishmanicidal function and that it is IL-10-independent. Furthermore, these inhibitory effects of ShRLD are partly contributed by MRPA.

Inhibition of NF-κB activation by ShRLD suppresses SAG-stimulated murine γGCS heavy-chain gene expression in DC

Previous studies have demonstrated an association of antimony resistance of leishmanial parasite with γGCS heavy-chain (γGCSγ) gene expression of host [4]. The latter encodes the catalytic subunit of γGCS [32]. In fact, a comparative analysis demonstrated that SAG-induced murine γGCSγ (mγGCSγ) expression was unaffected in 2001Pm (ShSLD)-infected BMDCs but selectively inhibited in 39Pm (ShRLD)-infected BMDCs (Figure 10A). The molecular basis for ShRLD-mediated suppression of mγGCSγ expression in DC was then explored. Despite SAG stimulation, the inhibition of mγGCSγ expression could be due to suppression of NF-κB activation in ShRLD-infected BMDC. To investigate this possibility, the regulatory role of NF-κB in mγGCSγ promoter activity was initially ascertained. An approximately 1.0 kb DNA sequence upstream of the transcriptional start site of mγGCSγ gene (Mas musculus chromosome 9 genomic contig, NT_039474.7; GI:149260095) was selected as the promoter region using Ensembl and UCSC browsers. The mγGCSγ promoter was found to contain a putative NF-κB binding site (AACTT-895 (Figure 10B-C). Furthermore, DNA binding of NF-κB complexes consisting of p50, RelB and p65 subunits specifically to the sequence (GGGGAAACCTT)2055 (WT-mγGCSγ probe) or
mutant sequence -904CTCTAAGAAT-895 (Mut-mGCShc probe) (Figure 10D) and supershift analysis (Figure 10E).

Next, the role of NF-κB binding site -904GGGGAAACTT-895 in regulation of promoter activity of mGCShc gene was tested via luciferase reporter assay using p987-luc and Mut p987-luc, the reporter constructs of mGCShc promoter fragment containing wild-type and mutant NF-κB binding site, respectively. Compared to control vector (pEGFP-C1) transfected cells, expression of NF-κB subunit p65 strongly induced luciferase activity of p987-luc (Figure 10F). This enhanced luciferase activity of p987-luc was completely blocked upon co-transfection with pEGFP-dominant negative IκBα (pEGFP-IκBαΔN) encoding the NF-κB-specific inhibitor, IκBαΔN (Figure 10F). The lack of luciferase activity of Mut p987-luc despite p65 expression (Figure 10F) further

Figure 5. DC pretreatment with antigens or culture supernatant of SbLD inhibits SAG-induced NF-κB pathway. BMDCs were either pretreated with antigens prepared from indicated strains of SbLD (SbLDsAg) or SbRLD (SbRLDsAg) (A-C) or cultured in RPMI 1640 complete medium containing culture supernatants of indicated strains of SbLD (SbLDs) or SbRLD (SbRLDs) at a complete medium to supernatant ratios of 1:1 (D) or 1:3 (D-F) for 3 hours. BMDCs were then washed and stimulated with SAG (20 μg/ml) for 0.3 hours. (A, D-E) Nuclear NF-κB binding to H2K-DNA probe or OCT-1 DNA binding was measured via EMSA. (B, F) Cytoplasmic IκBα, IκBβ, IκBε and β-actin expression were detected by Western blot using the same blot. (C) In vitro IKK activity was determined as in Figure 3 and the same blot was reprobed for IKK1 and IKK2 protein. Data are representative of three independent experiments.

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indicated that the sequence $^{503}$GGGGAACTT$^{955}$ is required for NF-κB-mediated transcriptional activation of $m$GC$\text{Shc}$ gene. Interestingly, SAG-induced NF-κB DNA binding to WT-$m$GC$\text{Shc}$ probe was inhibited in 39Pm (Sb$^{R}$LD)-infected BMDCs (Figure 10G). In contrast, SAG-induced NF-κB DNA binding to WT-$m$GC$\text{Shc}$ probe was readily detected in 2001Pm (Sb$^{S}$LD)-infected BMDCs (Figure 10G). These findings suggest that SbRLD suppresses SAG-induced $m$GC$\text{Shc}$ expression in DC by inhibiting NF-κB DNA binding to the $m$GC$\text{Shc}$ promoter.

**Discussion**

Antimonial drugs activate innate effector cells to promote an anti-leishmanial effect [25]. However, regulation of antimonial
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**Figure 7.** PI3K/AKT suppression by SbLD inhibits SAG-induced NF-κB signaling in an IL-10 independent manner.

BMDCs were infected with promastigotes of SbLD strains 2001(2001Pm) (E, G-J) and AG83 (AG83Pm) (H), and SbRLD strains 39 (39Pm) (E, G-J) and GE1F8R (GE1F8Pm) (H); or amastigotes of 2001(2001Am) and 39 (39Am) (F) for indicated times or left uninfected as described in Figure 4. Subsequently, BMDCs were stimulated or not with 20 (A-J) or 40 (G) μg/ml of SAG for specified times. For experiments (A-D), uninfected BMDCs were treated with 200 nM Wort or 50 μM Ly for 1 hour prior to SAG stimulation. In some experiments (I-J), BMDCs infected with 2001Pm (SbLD) or 39Pm (SbLD) for 3 or 24 hours were treated with...
Drug-mediated immune activation by SbRLD and SbSLD is ill-defined. This is of particular interest in view of the lack of efficacy of antimonial compounds reported for SAG-unresponsive kala-azar patients. Recent studies indicated that DCs play a key role in regulating anti-leishmanial immune response [12–14,33]. Accordingly, the role of SAG in activation of DCs, its regulation by SbRLD and SbSLD and the molecular mechanism involved therein were investigated. Here we provide evidence that SbRLD and SbSLD differentially regulate activation of DCs. Furthermore, SAG-induced signaling pathway associated with DC activation is selectively targeted by SbSLD infection.

In an agreement with an earlier report [28], both BMDCs and ex vivo sDCs were infected in vitro with LD promastigotes (Figure 1A). The “SAG-resistant” phenotype did not significantly affect the efficiency of LD infection, but did impact the susceptibility of LD to the leishmanicidal effects of SAG. In contrast, SAG treatment significantly impaired DC infectivity of SbSLD including reduction in both intracellular parasite number and percentage of infected DCs (Figures 1B and S1). The differential response of SbRLD and SbSLD towards SAG treatment was also noted in their ability to regulate activation and maturation of DCs. In contrast to SbSLD, SbRLD infection

**Figure 8.** 2001Pm-MRPA but not 2001Pm inhibits SAG-induced AKT phosphorylation and DNA binding activity of NF-κB. BMDCs were infected for 3 hours with 2001Pm (SbSLD), 39Pm (SbRLD), 2001Pm expressing MRPA (2001Pm-MRPA) or 2001Pm transfected with empty vector (2001Pm-EV) and stimulated with 20 μg/ml of SAG as described in Figure 4. (A) Expression of phosphorylated (P) AKT and AKT were measured in whole cell lysates via Western blot using the same membrane. Densitometric readings represent the ratio of intensity of phosphorylated (P) AKT protein to AKT expression per unit area and are represented as arbitrary units. (B) Nuclear NF-κB and OCT-1 DNA binding activities were measured via EMSA. Densitometric analysis represents the ratio of intensity of NF-κB to OCT-1 binding per unit area and is represented as arbitrary units. Data are representative of two independent experiments. Error bars indicate mean ± SD.

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Antimony-Resistant *L. donovani* Inhibits DC

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**D**

![Graph D](image)

**E**

![Graph E](image)

**F**

![Graph F](image)
inhibited SAG-induced proinflammatory cytokine secretion and up-regulation of co-stimulatory molecule and MHC expression in DCs (Figures 1D-F and S2B-C). Noteworthy is that SbRLD induced increased IL-10 secretion by DCs compared to SbSLD (Figures 1C and S2A). This finding reinforces the inherent ability of SbRLD and SbSLD to differentially immunoregulate DC activation. Previous studies demonstrated that IL-10, a potent suppressor of anti-leishmanial immunity, minimizes responsiveness to SAG [34,35]. Therefore, increased IL-10 production may play a critical role in disease pathogenesis in the host infected with SbRLD.

The second important finding arising from this study is that both promastigotes and amastigotes of SbRLD and SbSLD differentially regulate SAG-induced NF-κB activation in DCs. Indeed, SbRLD but not SbSLD infection blocks SAG-induced NF-κB signaling by suppressing IKK activation, and IKB protein phosphorylation and degradation (Figures 4 and S4). In this regard, it should be noted that SAG stimulation of uninfected DCs induced concomitant degradation of all three IKB proteins (Figure 3A), although degradation of IKBα generally occurs with delayed kinetics upon cellular activation [36,37]. This finding, however, is consistent with a number of studies reporting rapid degradation of IKBα and IKBε depending on the type of cell and the nature of the stimulation [19,20,36–39]. Nevertheless, the suppression of SAG-induced IKB protein degradation by SbRLD ultimately impaired nuclear NF-κB DNA binding activity (Figures 4 and S4). Surprisingly, BMDC infection at a MOI of 10:1 with SbRLD promastigotes or amastigotes for 24 or 6 hours, respectively, also inhibited SAG (20 μg/ml)-induced NF-κB activation (Figure 4). SAG (20 μg/ml)-induced NF-κB activation was restored in these BMDCs but not SbSLD-infected BMDCs upon lowering the MOIs (<10:1) for BMDC infection, which established reduced levels of intracellular parasite number compared to MOI 10:1 (Figures 6A-D and S6). This finding suggests that intracellular parasite number plays a critical role for differential regulation of SAG-induced NF-κB activation by SbRLD and SbSLD. The early inhibition of NF-κB activation in SbRLD amastigote versus promastigote-infected BMDCs (Figure 4) is in agreement with the fact that DCs internalize amastigotes more efficiently than promastigotes [40–42]. However, SbRLD and SbSLD still retain their ability to differentially regulate SAG-induced NF-κB activation in BMDCs with high intracellular parasite number. This conclusion is supported by results demonstrating that despite stimulation with 40 μg/ml of SAG, NF-κB activation was blocked in BMDCs infected for above durations with SbRLD amastigotes or promastigotes at a MOI of 10:1 but readily observed in BMDCs infected similarly with SbSLD (Figure 6). Here, the SAG dose was increased from 20 to 40 μg/ml keeping in mind that SAG therapy requires multiple dosing schedules to ensure enough antimony accumulation in tissues of kala-azar patients [43]. Furthermore, equivalent and/or increased concentrations of SAG have previously been used by other groups [44,45]. Under identical conditions, the recurrence of NF-κB activation in SbSLD-infected BMDCs by increasing the dose of SAG from 20 to 40 μg/ml (Figure 6) further suggested that 20 μg/ml of SAG was insufficient to activate NF-κB in these BMDCs due to high intracellular parasite number.

Noteworthy is that the inhibitory effect on NF-κB activation was not dependent on live SbRLD. For instance, parasite antigens derived from SbRLD (SbRLDAg) and SbSLD culture supernatant (SbSLDsAg) but not the SbRLD-derived antigens (SbRLDsAg) or culture supernatant of SbSLD (SbSLDs) efficiently inhibited SAG-induced NF-κB activation (Figure 5). These findings suggest that the inhibition of NF-κB activation is specific for SbRLD/SbRLD-derived antigen(s)/factor(s) secreted by SbRLD. Strikingly, this inhibition of NF-κB activation correlated with suppression of SAG-induced proinflammatory cytokine secretion by SbRLD infection (Figures 1, 4, and S2). Studies involving gene transfer of a modified IκBα recombinant into immature DC demonstrated that blockade of NF-κB activation alone prevents up-regulation of co-stimulatory molecule expression and production of proinflammatory cytokines [16,18]. Based on these reports coupled with our own observations, we conclude that the SbRLD blocks SAG-induced NF-κB signaling to prevent DC activation and maturation.

Our results further suggest that SbRLD inhibits IKK and NF-κB activation by blocking SAG-induced PI3K/AKT signaling. SAG stimulation of DCs induced PI3K activation as measured by phosphorylation of AKT, a downstream signaling mediator of PI3K (Figures 7A and S7). Blockade of PI3K/AKT activation by Wort or Ly completely suppressed SAG-stimulated IKK activity and NF-κB signaling (Figure 7A-D), indicating a direct involvement of the PI3K/AKT pathway in NF-κB activation by SAG in DCs. Importantly, PI3K/AKT activation is negatively regulated by Src homology phosphotyrosine phosphatase (SHP-1), which dephosphorylates PI3K [46]. Furthermore, SHP-activity is inhibited by SAG [47]. Therefore, blockade of SHP-activity by SAG may indirectly promote PI3K phosphorylation and activation of downstream AKT, IKK and NF-κB in DCs. Similar to uninfected DCs, SAG treatment induced PI3K/AKT activation in DCs infected with SbRLD promastigotes for 3 hours and SbSLD amastigotes for 1 and 3 hours (Figures 7E-F and S7). Importantly, PI3K inhibitors impaired SAG (20 μg/ml)-induced NF-κB pathway, DC activation and leishmanicidal effects in BMDCs infected with SbSLD promastigotes for 3 hours (Figures 9 and S11). These results suggest the inability of SbRLD to regulate PI3K/AKT and NF-κB pathways. Consequently, SAG continues to exhibit leishmanicidal effects in SbSLD-infected DCs. The inability of SbRLD to regulate SAG-induced leishmanicidal effects was maintained despite BMDC infection for 24 hours with SbSLD promastigotes. This was apparent when increased dose of SAG (40 μg/ml) was used for treatment (Figure S10). In fact, treatment with 40 μg/ml of SAG restored AKT phosphorylation and therefore exhibited leishmanicidal effects in a PI3K-dependent manner in these SbSLD-infected BMDCs (Figures 7G and S10). Interestingly, SbRLD infection mimicked the effects of the PI3K inhibitors in that all SAG-induced events as mentioned above were also blocked by SbRLD regardless of duration of infection, form of parasite and dose of SAG (Figures 7, S9 and S11). One intriguing possibility is that IL-10 produced by SbSLD-infected DCs mediated SbSLD-induced suppression of PI3K/AKT signaling.
Figure 10. Suppression of SAG-induced mGCS_{hc} expression by Sb^{RLD} is NF-κB-dependent. Both uninfected BMDCs (A, C-E, G) and BMDCs infected with 2001Pm (Sb^{SLD}) or 39Pm (Sb^{RLD}) for 3 hours (A, G) were stimulated with SAG (20 μg/ml) for specified times as in Figure 4. (A) The mRNA expression of mGCS_{hc} versus mGAPDH was determined via RT-PCR. Densitometric data represent ratio of intensity of mGCS_{hc} to mGAPDH mRNA expression per unit area and are presented as an arbitrary unit. (B) Schematic presentation of mGCS_{hc} promoter indicating the position of NF-κB binding site and ChIP primers (P1, P2). (C) NF-κB binding to −991/−673 region of mGCS_{hc} promoter was examined by ChIP using Antimony-Resistant L. donovani Inhibits DC PLoS Pathogens | www.plospathogens.org 15 May 2010 | Volume 6 | Issue 5 | e1000907
the primers shown in B and indicated Abs. Amplification of mGAPDH promoter and chromatin immunoprecipitated by rabbit IgG were used as negative controls, and input DNA (2%) as an internal control. (D, G) Nuclear NF-κB DNA binding to mGCSBc promoter-specific probes containing wild-type NF-κB binding site (WT-mGCSBc probe), (D, G) or mutant NF-κB binding site (Mut-mGCSBc probe) (E) was determined via EMSA. OCT-1 DNA binding activity was used as internal control. (F) DNA binding of different NF-κB complexes to WT-mGCSBc probe was determined via supershift analysis using rabbit IgG (Control Ab) or Abs specific for indicated NF-κB subunits. (F) NF-κB-mediated regulation of mGCSBc promoter activity was determined by reporter assay. NIH3T3 cells were co-transfected with Renilla luciferase vector (pRL-CMV) and firefly luciferase reporter plasmid containing mGCSBc promoter with wild-type (p987-luc) or mutant (Mut p987-luc) NF-κB binding site. In addition, all co-transfections contained pEGFP-C1 empty vector or pEGFP-p65 and/or pEGFP-κBα/κBκ. Twenty-four hours later, firefly and Renilla luciferase activities in cell lysates were measured. The data represent the fold induction of firefly/Renilla luciferase activity ratio relative to pEGFP-C1-transfected cells. Data are representative of three independent experiments. p<0.001 versus NIH3T3+p987-luc+pEGFP-C1 and p<0.001 versus NIH3T3+p987-luc+pEGFP-p65 (Student’s t test). Error bars indicate mean ± SD.

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Antimony-Resistant L. donovani Inhibits DC

AKT and NF-κB pathways (Figures 1C, 4, 7, S2A and S7). This correlation can be made because IL-10 inhibits AKT activation and NF-κB pathway in DCs [19]. Furthermore, the inhibitory effects of IL-10 on DCs can be mediated in an autocrine manner [40]. However, this is unlikely since SAG-induced NF-κB activation was inhibited even in BMDCs infected with SbRLD for 1 and 3 hours, when IL-10 production was not detected (Figures 4 and 7H). Moreover, neutralization of IL-10 produced by BMDCs upon SbRLD infection for 24 hours failed to block SbRLD-induced inhibition of PI3K/AKT and NF-κB pathways (Figure 7H). Therefore, SbRLD infection of BMDCs for up to 24 hours inhibits SAG-induced PI3K/AKT and NF-κB pathways in an IL-10-independent manner and eventually impairs DC activation.

Another key finding is that the suppression of NF-κB activation by SbRLD but not SbSLD inhibits not only DC activation but also SAG-induced mGCSBc expression in DC (Figure 10). Importantly, regulation of host γGCSBc expression and therefore host GSH level by SbRLD plays a key role for antimony resistance in LD infection [4]. Although regulation of γGCSBc expression by NF-κB was shown in a murine Mo-like cell line by blocking NF-κB activation [27], our observation establishes a direct role for NF-κB in mediating the promoter activity of the mGCSBc gene (Figure 10C-F). Interestingly, SbRLD blocked SAG-induced myGCSBc expression in DC by preventing NF-κB binding to the mGCSBc gene promoter (Figure 10G). This suggests a key role for NF-κB in SbRLD-mediated suppression of mGCSBc expression in DC. Our findings (Figures 10A and S12) are consistent with the recent work by Carter and colleagues demonstrating that SbRLD transcriptionally down-regulate host γGCSBc expression and up-regulate their own γGCSBc (LD-γGCSBc) expression [4]. Whereas SbRLD-induced inhibition of host γGCSBc expression reduces host GSH level and impairs reduction of SbV to toxic SbIII form; elevated expression of LD γGCSBc by SbRLD restores GSH level that promotes efflux of SAG and confers protection against oxidative stress [4].

The mechanism of antimony resistance in clinical LD isolates is unknown and may differ from laboratory-derived resistant parasites [3]. The true markers of clinical antimony resistance in LD isolates are still lacking [2]. A gene, PG1, is reported to confer antimony resistance in clinical isolates of LD [45,49]. In addition, enhanced expression of several other genes including MRPA and proteophosphoglycans (PPG) was demonstrated in antimony-resistant compared to antimony-sensitive field isolates [Salotra P, Singh R, Nakhshi H. 2005. Clinical Microbiology and Infection. Vol 11, Suppl 2: 47] [5,50]. As an initial effort to determine whether any SbRLD-specific factor(s) mediated the suppression of SAG-induced NF-κB signaling and leishmanicial effects in DCs, the role for MRPA was investigated. Results obtained using 2001Pm and its isogenic strain expressing MRPA (2001Pm-MRPA) showed that the inhibitory effect of 2001Pm-MRPA on SAG-induced PI3K/AKT and NF-κB pathways and leishmanicial activities mimics that of SbRLD to some extent (Figures 8 and 9). However, the real bearing of this observation in naturally occurring antimony-resistant LD isolates is still questionable and needs detailed investigation further. Moreover, the effects of SAG are likely to be inhibited in DCs by other SbRLD-specific factor(s) also. The relative contribution of these parasite-specific factors in SbRLD-mediated inhibition of DC activation is currently under investigation. Furthermore, an association of antimony resistance with genetic variation among LD strains has been proposed [51]. Recent studies demonstrated that due to high genetic polymorphism, strain 39 is remarkably distinct not only from antimony-sensitive strain 2001 but also from other antimony-resistant clinical LD isolates exhibiting homology with antimony-sensitive parasites [51]. On the other hand, the antimony-sensitive strains 2001 and Dd8 exhibit significant genetic similarity [51]. The extreme genetic polymorphism might be a potential cause of antimony resistance in strain 39 [51]. These reports together with our findings emphasize the notion that antimony resistance of clinical LD isolates is “multifactorial” [3].

SAG unresponsiveness in kala-azar patients also entails a number of host-regulated events including dominance of a type-2 T cell response and altered host gene expression [3,4,8,9]. Our findings demonstrate that SAG treatment induces PI3K-dependent NF-κB activation in DCs, which is blocked by SbRLD but not SbSLD infection. Dysregulation of SAG-induced NF-κB activation favors persistent survival of SbRLD in DCs despite SAG treatment by: 1) inhibiting NF-κB-dependent mGCSBc expression, a key mediator of SbV reduction to SbIII, and 2) preventing DC activation and maturation required for the initiation of the anti-leishmanial immune response. Notably, a heterogenous response to SAG treatment may be observed in humans. This possibility is raised by a report demonstrating that SAG treatment of monocyte-derived DCs restores their capacity to respond to LPS in ~60% of type 1 diabetes patients [52]. Importantly, some variability in SAG responsiveness is also reported in kala-azar patients [53]. It is speculated that the genetic differences among individuals may influence the response following SAG therapy in the patients infected with same Leishmania species and living in the same endemic area [54]. Further studies are needed to define how genetic variation, if any, influences the outcome of SAG treatment in kala-azar patients.

Materials and Methods

Animals

BALB/c mice and golden hamsters (Mesocricetus auratus) were maintained and bred under pathogen-free conditions.

Ethics statement

Use of both mice and hamsters was approved by the Institutional Animal Ethics Committees of Institute of Microbial Technology and Indian Institute of Chemical Biology, India. All
animal experimentation was performed according to the National Regulatory Guidelines issued by CPSEA (Committee for the Purpose of Supervision of Experiments on Animals), Ministry of Environment and Forest, Govt. of India.

Parasite cultures and preparation of soluble antigen from LD

ShSLD [GEF8R (MHOM/IN/89/GE1), 39, R5] and ShSLD [AG83 (MHOM/IN/83/AG83), 2001] strains are gifts from Dr. Neeloo Singh (Central Drug Research Institute, India) and Dr. Shyam Sundar (Banaras Hindu University, India) and were maintained in saline as described [45,55–57]. Amastigotes were obtained from spleens of infected hamsters as described [58]. Subsequently, amastigotes were transformed into promastigotes and maintained as described [59]. GFP-2001 and GFP-50 are gifts from Dr. Neeloo Singh and were maintained in M199 complete medium (10% FBS, penicillin/streptomycin) with 720 µg/ml gentamicin disulfate (G418) [Sigma, St. Louis, MO] [60]. Soluble antigens were prepared from ShLD and ShSlD promastigotes (10^7/ml) as described [59].

Transfection of 2001Pm

2001Pm (ShLD) at mid log or stationary phase were washed twice with electroporation buffer (21 mM HEPES, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM NaH2PO4; 6 mM glucose). The parasites were resuspended in ice-cold electroporation buffer to a final concentration of 10^7/ml. An aliquot (400 µl) of parasite suspension was mixed with 35–40 µg of chilled pGEM 7ZF 3-neo-α L. tarentolae MRPA or pGEM 7ZF 3-neo-α DNA (kind gifts from Dr. Marc Ouellette, Laval University, Canada), transferred to a 2-mm gap cuvette and electroporated using BIO-RAD Gene-Pulsor X cell instrument at 450 V and 500 µF (3.5 to 4 milli-seconds pulse time). After electroporation, parasites were immediately placed on ice for 10 minutes and cultured at 22°C for 24 hours in Schneider’s insect medium with 1500 µg/ml G418. Subsequently, parasites were washed twice with electroporation buffer (21 mM HEPES, pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM NaH2PO4; 6 mM glucose). The culture was maintained for one month under drug pressure (once per week) to obtain the stable transfectants. The mRNA expression of MRPA in these stable transfectants was verified via RT-PCR.

DC preparation

BMDCs and sDCs were prepared from male or female BALB/c mice between 8–12 weeks of age as described [19]. Flow cytometric analyses indicated >85% and ~90% purity of BMDCs and sDCs, respectively, based on CD11c expression.

DC infection with promastigotes or amastigotes of LD

DCs (5 × 10^6/well) were infected in vitro at specified MOIs either with amastigotes of ShLD or ShLD; or promastigotes of stationary phase ShLD, ShLD or respective GFP-LD for indicated times in a 6-well plate in RPMI 1640 complete medium (10% FBS, penicillin/streptomycin, L-glutamine, soybean protein, non-essential amino acids, 2-mercaptoethanol). Subsequent-ly, DCs were washed, resuspended in RPMI 1640 complete medium and stimulated with SAG (10, 20 and 40 µg/ml) of clinical grade or sodium gluconate (25, 50, 100 and 200 µg/ml) for specified times. The doses of SAG mentioned here and for all experiments represent the concentration of ShLD. DC infection with GFP-ShLD or GFP-ShLD was determined via flow cytometry.
IKK assay
IKK signalosome was immunoprecipitated from 700 μg of a whole DC lysate using Protein A/G agarose beads (Santa Cruz Biotechnology) and rabbit polyclonal 2iIKK1 Abs. In vitro kinase reaction was performed and kinase activity of immunoprecipitated IKK complex determined as described [20].

Flow cytometry
The following monoclonal antibodies used for flow cytometry were purchased from eBioscience (San Diego, CA): PE-αCD11c, FITC-αCD11b, FITC-αCD40, FITC-αCD80, FITC-αCD86, FITC-αH2Kb and FITC-αmouse IL-10. The fluorescence of stained cells was analyzed on a FACSCalibur (BD Biosciences) using Cell Quest Pro software.

Measurement of IL-12, TNFα and IL-10 secretions from DCs
DCs (1 x 10^6/ml) were infected with promastigotes of SbRLD or SbLD for 3 hours or left uninfected, washed and stimulated with SAG for additional 48 hours in a 24-well plate. Alternatively, DCs (1 x 10^6/ml) were infected with promastigotes of SbRLD or SbLD for varying times or left uninfected. The culture supernatants were analyzed for IL-12, TNFα and IL-10 productions in triplicate using ELISA kits (BD Biosciences) following the manufacturer’s instructions.

Analysis of murine γGCS heavy-chain (mγGCShc) and LD γGCS heavy-chain (LD-γGCShc) expression in SbRLD- and SbLD-infected BMDCs
BMDCs (5 x 10^5/well) were infected with SbRLD or SbLD for 3 hours or left uninfected, stimulated with SAG for 24 and 48 hours as described in Figure 1A. Untreated (24h) and untreated (48h) represent LD-infected DC controls cultured without SAG treatment for 24 and 48 hours, respectively. The percentage of infected BMDCs (A) and number of intracellular amastigotes per BMDC infection with AG83Pm but not 39Pm is shown in Figure S1. Untreated (24h) and untreated (48h) represent LD-infected DC controls cultured without SAG treatment for 24 and 48 hours, respectively. The percentage of infected BMDCs (A) and number of intracellular amastigotes per 1000 BMDCs (B) were determined by Giemsa staining. Open and solid bars represent AG83Pm and 39Pm-infected BMDCs, respectively. Data are representative of three independent experiments. *p<0.001, **p = 0.003, ***p = 0.002 and ****p = 0.0012 versus DC+AG83Pm of respective times (Student’s t test). Error bars indicate mean ± SD.

Detection of MRPA expression in LD
RNA was isolated from LD promastigotes and mRNA expression of MRPA was detected by amplifying the 179 bp cDNA fragment of MRPA via reverse transcription-PCR using primers: 5′-GGCCAGGCATTGGTGTGGTC-3′, 5′-TTGGCTGATCGCCAGATGGCTG-3′ [5]. mRNA expression of LD-tubulin was used as loading control.

Chromatin immunoprecipitation (ChIP)
BMDCs (5 x 10^5/well) were stimulated with SAG for 0.3 hours or left untreated. ChIP was performed using ChIP-IT kit (Active Motif) following the manufacturer’s instructions. After immunoprecipitation using rabbit IgG or NF-κB Abs such as p53, pRelB and p65, followed by DNA extraction; PCR was performed to amplify −991/−673 region of mγGCShc promoter using primers: P1, 5′-GGCTTCCAGGACCTTCCG-3′ and P2, 5′-TT- GTAGACCTCCAACATGGCATG-3′. For a negative control, mGAPDH promoter was amplified by using primers 5′-CAGCTTGGCATTCTTCCCA-3′ and 5′-GACGCAGACCTGAATGCTGAGTGC-3′ [63].

Reporter assay
An approximately 1.0 kb (−987/+25) long 5′-flanking sequence of mγGCShc gene was amplified by PCR using murine genomic DNA (Promega, Madison, WI) and cloned into pGL3-Basic vector. Using this resulting construct, p987-luc, and a Quick-ChangeII PCR-based site-directed mutagenesis kit (Stratagene, Cedar Creek, TX); the construct (Mut p987-luc) containing a mutant NF-κB binding site, similar to that described in EMSA studies, in −987/+25 region of mγGCShc promoter was generated. Both constructs were confirmed by sequencing. NIH3T3 cells were transiently transfected with a DNA mixture containing p987-luc or Mut p987-luc (0.266 μg), pRL-CMV (0.200 μg), pEGFP-IkbAN (1kbZ with amino acids 1–36 deleted) (0.266 μg) using lipofectamine LTX (Invitrogen). The latter two expression vectors are gifts from Dr. Johannes Schmid (Medical University of Vienna, Austria) and Dr. Susan Kandarian (Boston University, USA) respectively [64,65]. The DNA amount in each transfection was kept constant. Cells were grown for 24 hours after transfection. The luciferase activity of the cell lysates was determined using Dual Luciferase Reporter Assay System (Promega) and GLOMAX luminometer (Promega) following the manufacturer’s instructions. The level of luciferase activity was normalized to the level of Renilla luciferase activity.

Supporting Information
Figure S1 BMDC infection with AG83Pm but not 39Pm is inhibited by SAG treatment. BMDCs were infected in vito at MOI 10:1 with AG83Pm (SbRLD) or 39Pm (SbLD) for 3 hours and stimulated with SAG (10 and 20 μg/ml) for 24 and 48 hours as described in Figure 1. Untreated (24h) and untreated (48h) represent LD-infected DC controls cultured without SAG treatment for 24 and 48 hours, respectively. The percentage of infected BMDCs (A) and number of intracellular amastigotes per 1000 BMDCs (B) were determined by Giemsa staining. Open and solid bars represent AG83Pm and 39Pm-infected BMDCs, respectively. Data are representative of three independent experiments. *p<0.001, **p = 0.003, ***p = 0.002 and ****p = 0.0012 versus DC+AG83Pm of respective times (Student’s t test). Error bars indicate mean ± SD.

Figure S2 SbRLD and SbLD differentially regulate cytokine secretion by sDCs. Ex vivo derived sDCs were infected with 2001Pm (SbRLD) or 39Pm (SbLD) for 3 hours or left uninfected. sDCs were then washed to remove free parasites and stimulated with SAG (20 μg/ml) for 48 hours as in Figure 1. Production of (A) IL-10, (B) IL-12p70 and (C) TNFα in the culture supernatants were measured via ELISA. Data are representative of three independent experiments. *p = 0.004 and **p = 0.008 versus DC+2001Pm; 1p<0.001 versus DC+SAG (Student’s t test). Error bars indicate mean ± SD.

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Figure S3  SAG treatment induces NF-κB pathway in sDCs. sDCs were stimulated with SAG (20 μg/ml) for the specified times or left untreated. (A) EMSA was used to measure DNA binding activity of nuclear NF-κB to H2K-DNA probe. OCT-1 DNA binding was used as internal control. (B) Western blot was used to detect cytoplasmic IκBα and β-actin protein using the same blot. (C) In vitro IKK activity was determined as described in Figure 3. Expression of IKK1 and IKK2 were determined via Western blot using same membrane. Data are representative of three independent experiments.

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Figure S4 BMDCs and sDCs infection with ShRLD strains GE1F8R and 39, respectively, induces SAG-induced NF-κB signaling. sDCs were infected with 2001Pm (ShRLD) or 39Pm (ShRLD) (A, C, E) and BMDCs with AG83Pm (ShLD) or GE1F8Rpm (ShRLD) (B, D, F) for 3 hours as described in Figure 4. DCs were then washed and stimulated with SAG (20 μg/ml) for 0.3 hours. (A-B) Nuclear NF-κB or OCT-1 DNA binding activity was determined via EMSA. (C-D) Cytoplasmic IκBα and β-actin protein were detected by Western blot using the same blot. (E) IKK phosphorylation was detected via Western blot and the same blot was repurposed for IKK1 and IKK2 protein. (F) In vitro IKK activity, and IKK1 and IKK2 protein expression were determined as described in Figure 3. Data are representative of three independent experiments.

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Figure S5 Temporal analysis of DC infection with ShRLD and ShLD. BMDCs were infected at a MOI 10:1 with promastigotes (Pm) (A, C) or amastigotes (Am) (B, D) of indicated ShRLD and ShLD strains for specified times. The number of intracellular amastigotes per 1000 BMDCs (A-B) and percentage of infected BMDCs (C-D) were determined by Giemsa staining. Data are representative of three independent experiments.

P<0.01 versus BMDCs infected with 2001Pm for 6 hours, #P<0.01 versus BMDCs infected with 39Pm for 6 hours, **P=0.014 versus BMDCs infected with 2001Am for 3 hours and *P=0.011 versus BMDCs infected with 39Am for 3 hours (Student’s t test). Error bars indicate mean ± SD.

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Figure S6 DC infection varies with MOIs at specified time points of infection. BMDCs were infected at varying MOIs either with 2001Pm (ShRLD) or 39Pm (ShRLD) for 24 hours (A, C) or 2001Am (ShRLD) or 39Am (ShRLD) for 6 hours (B, D). BMDCs were then washed. The number of intracellular amastigotes per 1000 BMDCs (A-B) and percentage of infected BMDCs (C-D) were determined by Giemsa staining. Data are representative of three independent experiments.

P<0.005 versus BMDCs infected with 2001Pm at MOIs 2.5:1 or 5:1 (promastigote:BMDC); #P<0.01 and **P<0.005 versus BMDCs infected with 39Pm at MOIs 2.5:1 or 5:1 (promastigote:BMDC); *P<0.005 versus BMDCs infected with 2001Am at MOIs 2.5:1 or 5:1 (amastigote:BMDC); **P<0.005 and #P<0.01 versus BMDCs infected with 39Am at MOIs 2.5:1 or 5:1 (amastigote:BMDC) (Student’s t test). Error bars indicate mean ± SD.

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Figure S7 ShRLD but not ShLD infection inhibits SAG-stimulated phosphorylation of AKT in sDCs. Ex vivo sDCs were infected with 2001Pm (ShRLD) or 39Pm (ShRLD) for 3 hours at a MOI 10:1 or left uninfected. The sDCs were washed thoroughly and stimulated with SAG (20 μg/ml) for 0.3 hours. Expression of phospho-AKT versus AKT in the cytoplasmic extract was determined via Western blot. Data are representative of two independent experiments.

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Figure S8 Treatment with zIL-10 Ab prevents inhibition of LPS-induced NF-κB DNA binding activity in IL-10-pretreated BMDCs. BMDCs were pretreated with IL-10 (50 ng/ml) for 24 hours in the presence or absence of 10 μg/ml of zIL-10 Ab or isotype control Ab. BMDCs were then washed and stimulated with LPS (500 ng/ml) for 0.5 hours. Nuclear NF-κB binding to H2K-DNA probe or OCT-1 DNA binding was measured via EMSA. Data are representative of two independent experiments.

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Figure S9 An increased expression of MRPA is detected in ShRLD and 2001Pm-MRPA but not ShLD. The mRNA expression of MRPA versus LD-tubulin in ShRLD strains 39Pm and GE1F8Rpm (A); ShLD strains AG83Pm (A) and 2001Pm (A-B); 2001Pm expressing MRPA (2001Pm-MRPA) (B); and 2001Pm transfected with empty vector (2001Pm-EV) (B) was determined via RT-PCR. Densitometric data represent ratio of intensity of MRPA to LD-tubulin mRNA expression per unit area and are presented as an arbitrary unit. Data are representative of three independent experiments. Error bars indicate mean ± SD.

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Figure S10 SAG (40 μg/ml) treatment restores leishmanicidal effects in ShRLD- unlike ShRLD-infected DCs despite prolonged infection. BMDCs were infected with 2001Pm (ShRLD) or 39Pm (ShRLD) at a MOI of 10:1 for 24 hours. BMDCs were then washed and treated with 200 nM Wort or 50 μM Ly for 1 hour. Subsequently, BMDCs were stimulated with 20 or 40 μg/ml of SAG for 24 hours or left untreated. The percentage of infected BMDCs (A) and number of intracellular amastigotes per 1000 BMDCs (B) were determined by Giemsa staining, SAG20 and SAG40 represent stimulation of BMDCs with 20 and 40 μg/ml of SAG, respectively. Data are representative of three independent experiments.

*p<0.005, **p=0.094 and #p=0.13 versus DC-2001Pm; **p<0.005 versus DC-2001Pm+SAG40 (Student’s t test). Findings were considered significant with "p" values ≤ 0.05. Error bars indicate mean ± SD.

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Figure S11 Pretreatment with PI3K inhibitors block SAG-induced NF-κB signaling in ShRLD-infected DCs. BMDCs were infected for 3 hours with 2001Pm (ShRLD) and 39Pm (ShRLD) at MOI 10:1, treated with Wort or Ly and stimulated with SAG (20 μg/ml) for indicated times as in Figure 8. (A) In vitro IKK activity was measured as in Figure 3. The same blot was repurposed for IKK1 and IKK2 protein. (B) Expression of cytoplasmic IκBα and β-actin protein was detected by Western blot using the same blot.

C) Nuclear NF-κB or OCT-1 DNA binding activity was determined via EMSA. Data are representative of three independent experiments.

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Figure S12 LD γGCS heavy-chain (LD-γGCS) expression is more in ShRLD- compared to ShLD-infected BMDCs. BMDCs were infected with 2001Pm (ShRLD) or 39Pm (ShLD) for 3 hours at MOI 10:1. Infected DCs were then stimulated with SAG (20 μg/ml) for 3 hours or left untreated. The mRNA expression of LD-γGCS was determined via RT-PCR. Densitometric data represent ratio of intensity of LD-γGCS to LD-tubulin mRNA expression per unit area and are presented as an arbitrary unit. Data are representative of three independent experiments. Error bars indicate mean ± SD.

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Author Contributions

Conceived and designed the experiments: PS. Performed the experiments: AKH VY ES KKB AS SB RB. Analyzed the data: PS. Contributed reagents/materials/analysis tools: PS SR. Wrote the paper: PS. Contributed to conceptualization of some aspects of antimony resistance in LD infection: SR.

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