Antimony resistant but not antimony sensitive *Leishmania donovani* upregulates host IL-10 by recruiting NF-κB at specific site of IL-10 promoter.

**Introduction**

Kala-azar or visceral leishmaniasis (VL), caused by the protozoan parasite *Leishmania donovani* (LD), is (re-)emerging and spreading worldwide, essentially because of human-made and environmental changes, immune suppression and drug resistance (Desjeux et al., 2004, Rabello et al., 2003). To combat the disease, organic pentavalent antimonials were introduced in the Indian subcontinent almost nine decades back (Brahmachari, et al., 1989) with dramatic clinical success. However, the toxicity together with the high treatment failure rate (up to 65%) and the emergence of resistance in the Bihar State, India, made the drug obsolete in the Indian subcontinent (Lira et al., 1999, Croft et al, 2006). Nevertheless, it is still used in first line in Africa and Latin America (Chakravarty et al., 2010). Interestingly, 78% of the recent clinical isolates from the hyperendemic zone of Bihar still showed *in vitro* resistance to antimonials, (Mukhopadhyay et al., 2011) which might be explained by an increased fitness of the corresponding parasites (Downing et al., 2011).

Recent reports have pointed out that antimony sensitive LD (Sb\textsuperscript{5}LD) and antimony resistant LD (Sb\textsuperscript{R}LD) differ markedly in their biochemical and biophysical property, which was also reflected in the variation of their gene copy number (Mukhopadhyay et al., 2011, Downing et al., 2011). Moreover, recent reports also suggest that unlike Sb\textsuperscript{5}LD, Sb\textsuperscript{R}LD upon infection modulates host differentially leading to different disease outcome (MookerjeeBasu et al., 2008, Haldar et al., 2010). The present study also deals with this unique ability of Sb\textsuperscript{R}LD to modulate host to induce significantly higher amount of IL-10 resulting in progressive pathogenesis. We decipher the cascade of molecular events likely involved in the regulation of this phenomenon and identified critical role of NF-κB that allows Sb\textsuperscript{R}LD to modulate the host in favour of its own survival.

**Result**

**Infection with Sb\textsuperscript{R}LD results in significantly higher induction of IL-10 as compare to infection with Sb\textsuperscript{5}LD**

Previously, we have shown that infection of bone marrow DCs with Sb\textsuperscript{R}LD induces higher IL-10 production compared with Sb\textsuperscript{5}LD isolates (Haldar et al., 2010). It was important to determine whether this is a generalised phenomenon for all Sb\textsuperscript{R}LD and also whether this holds true for Mφs, as these are regarded as primary targets for Leishmania infection. Murine Mφs were therefore infected with different isolates and levels of IL-10 protein and mRNA were measured. It was observed that IL-10 production was found to be 3–4-fold higher in protein level and about 1.5-2 fold higher in the mRNA level in case of Mφs infected with Sb\textsuperscript{R}LD compared with Sb\textsuperscript{5} (Fig. 1A, 1B). There was a high
correlation ($r = 0.88$) between SSG resistance of the isolates and IL-10 generation by infected host cells (Fig 1A, Inset).

**Figure 1:** Analysis of IL-10 expression from macrophages (Mφs) infected either with antimony sensitive (Sb$^{S}$) or antimony resistant (Sb$^{R}$) isolates of *Leishmania donovani*. Mφs were infected either with Sb$^{S}$LD or Sb$^{R}$LD isolates for 24 h or left uninfected. (A) The culture supernatants were harvested to determine IL-10 content by ELISA, Inset showing co-relation between EC$_{50}$ of LD isolates and IL-10 induced by them from infected Mφs. (B) RNA was prepared and PCR amplified with specific primer for IL-10 and β-actin and densitometry analysis was represented (C). Results in A and Care presented as mean ± SD.

**Sb$^{R}$LD driven IL-10 production is NF-κB dependent**

In order to identify the most likely signalling molecule in the Sb$^{R}$LD driven IL-10 production, Mφs were treated with a series of inhibitors prior to infection with Sb$^{S}$LD or Sb$^{R}$LD. We observed that Sb$^{R}$LD driven IL-10 production was largely compromised ($p<0.0001$, 3-5 fold) in case of Mφs pre-treated with ERK or NF-κB inhibitors (PD98059, U0126 and BAY110782, respectively; Fig 2A), but not with PI3K (Wortmannin), p38 (SB203580) or JNK (SP600125) inhibitor. A similar picture emerged after western blot analysis: this revealed that infection of Mφs with Sb$^{R}$LD (in contrast with Sb$^{S}$LD) resulted in the activation of ERK1/2 and the degradation of IκB (IκBα, IκBβ, IκBε) proteins. This corresponds to enhanced IκBα phosphorylation due to increased activity of upstream IKK complex, as evidenced from phosphorylation of IKKα/β in Mφ infected with Sb$^{R}$LD (Fig 2B). Unlike
Sb<sup>8</sup>LD mediated infection, there was no phosphorylation of IKKα/β and IκBα when Mφs were infected with Sb<sup>8</sup>LD. Also, Sb<sup>8</sup>LD induced IL-10 production remained unaffected regardless of the presence or absence of inhibitors. Together, these results demonstrate that Sb<sup>8</sup>LD induced IL-10 production by Mφ is NF-κB dependent.

**Figure 2: Sb<sup>8</sup>LD driven IL-10 production from Mφs is NF-κB dependent.** (A) IL-10 production from Mφs infected with either Sb<sup>5</sup>LD (AG83) or Sb<sup>8</sup>LD (BHU575/BHU138) in the presence and absence of an array of pharmacological inhibitors (PD98059, U0126, BAY110782, Wortmannin, SP600125, and SB203580). (B) Western blot analysis of whole cell lysate derived from the corresponding Mφs preparation; expression of phosphorylated ERK1/2 and ERK1/2 was measured in whole cell lysates via Western blot using the same membrane. Cytoplasmic IκBα, IκBβ, IκBe, phospho- IκBα & phospho- Iκκα/β were detected via western blot and the same blot was reprobed for β-actin. Results in A are presented as mean ± SD. B is representative of 3 independent experiments.

**Mapping of IL-10 promoter**

To determine whether Sb<sup>8</sup>LD mediated activation of NF-kB leads to modulation of IL-10 promoter, Mφs were pre-treated with Cyclohexamidine before infecting them with Sb<sup>5</sup>LD or with Sb<sup>8</sup>LD. There was significant reduction in Sb<sup>8</sup>LD induced IL-10 mRNA level in cylohexamide treated Mφs as compared to untreated counterparts suggesting Sb<sup>8</sup>LD infection leads to transient remodelling of IL-10 promoter (Fig 3).
Figure 3: Sb^8LD infection results in transient remodeling of IL-10 promoter. RNA prepared from Mφs infected either with Sb^5LD (Ag83) or Sb^8LD (BHU575/BHU138) in presence or absence of cyclohexamide and PCR amplified to check the IL-10 mRNA level with respect to β-actin. LPS is used as positive control.

There are three potential NF-κB binding sites at the positions -46/-55, -583/-593, and -917/-927 of IL-10 promoter, defined as Site I, Site II, and Site III respectively (Fig 4A). In order to identify the promoter site involved in the Sb^8LD driven IL-10 induction, we transfected Mφs with several constructs (truncated or not) cloned in pGL3-basic vector (Fig 4B) and assessed luciferase activity upon Sb^8LD stimulation.

Figure 4: Mapping of IL-10 promoter. (A) Schematic representation of the IL-10 promoter -17/-1576 (1.57kb), containing three potential NF-κB binding sites, (-46/55, -583/-593, and -917/-927 defined as Site I, II and III respectively). (B) IL-10 promoter +1/-1576 (1.57Kb), region, -17/-292 (275bp) containing Site I, and region, -864/-1138 (274bp) containing Site III, was individually cloned in pGL3-basic vector.

The truncated promoter constructs containing site I (-46/-55) and III (-917/-927) were unable to show enhanced luciferase activity (p<0.0001, 3-4 fold) under Sb^8LD stimulation, in contrast to the untruncated construct (Fig 5A). Although, the IL-10 promoter construct carrying Site I has been reported to show luciferase activity upon LPS stimulation which indicates that specific stimulus might result in differential modulation of host promoter (Cao et al, 2006). Moreover, compared to the WT plasmid carrying the IL-10 promoter construct (1.57 kb), the deleted mutant (Mut) IL-10 promoter construct generated by deleting the Site II (-583/-593) elicited significantly low luciferase activity (p<0.0001, 4-5 fold) in transfected cells.
when infected with Sb\textsuperscript{8LD} (Fig 5B). This observation indicated that the sequence -583/-593 (Site II) is critical for NF-κB mediated transcriptional activation of IL-10 gene under Sb\textsuperscript{8LD} stimulation.

**Figure 5: Specific activation of IL-10 promoter under Sb\textsuperscript{8LD} stimulation.** (A) Comparison of luciferase activity of lysate of RAW264.7 cells transfected either with pGL3-IL-10 promoter construct (275bp), containing Site I or with whole length (WL) pGL3-IL-10 promoter construct (1.57 kb), containing all three sites (I, II and III), or with pGL3-IL-10 promoter construct (274bp), containing Site III; (B) RAW264.7 cells were transfected either with wild type IL-10 promoter construct containing all three sites (1.57 kb) or with Site II-Mut IL-10 promoter construct (-583/-593 deleted), then infected with Sb\textsuperscript{8LD} (BHU575/BHU138); luciferase activity was measured in cell lysate. Results in B and Care presented as mean ± SD.

**Characterization of NF-κB subunits**

In order to determine the NF-κB subunits involved in IL-10 promoter activity, a ChIP assay was performed. Immuno precipitation with acetylated H3, p50, c-Rel and p65 antibodies followed by subsequent PCR amplification revealed that p50 and c-Rel, but not p65, were bound to Site II (-583/-593) when infected with Sb\textsuperscript{8LD} but not with Sb\textsuperscript{5LD} (Fig 6A). This observation was further substantiated by western blot analysis. There was a detectable expression of phosphorylated p50 and c-Rel in the nuclear fraction, when Mφs were infected with Sb\textsuperscript{8LD} but not with Sb\textsuperscript{5LD}, although cytoplasmic expressions of whole p50 and c-Rel were almost equivalent upon infection with all strains (Fig 6B). Finally, specific binding of NF-κB complexes consisting of p50 and c-Rel subunits to the sequence -583\textsuperscript{GGGGTTTCCT} 593 (SiteII) in Mφs infected with Sb\textsuperscript{8LD} was confirmed with EMSA using murine IL-10 probes containing the WT sequence -583\textsuperscript{GGGGTTTCCT} 593 (WT-
mIL-10 probe) or a mutant sequence \(-^{583}CTCTTTAAT^{593}\) (Mut-mIL-10 probe) (Fig 6C). Furthermore, supershift analysis with Ab for p50, c-Rel and p65 revealed that supershift occur only in presence of p50 and c-Rel Ab but not with p65 Ab (Fig 6D).

**Figure 6: Sb^5LD activates p50/c-Rel to bind with IL-10 promoter.** (A) Chip analysis of IL-10 promoter (-482/-645) with nuclear extract derived from Mφs infected with Sb^5LD (AG83) or Sb^6LD (BHU575/BHU138), assessed with antibodies to hyperacetylated histone H3 (Ace H3), p65, p50 and c-Rel, followed by PCR amplification. Chromatin immunoprecipitated by whole rabbit IgG and no antibodies were used as negative controls, and input DNA (5%) as internal control. (B) Nuclear and cytoplasmic extracts derived from Mφs infected either with Sb^5LD (AG83) or with Sb^6LD (BHU575/BHU138), to analyse the expression of phosphorylated-p50 (P-p50) and c-Rel in the nuclear extract (Nu), and the expression of whole p50, c-Rel in the cytoplasmic extract (Cyt) by western blot, where Histone was used as internal control. (C) Analysis of Nuclear NF-κB DNA binding to IL-10 promoter- specific probes containing wild type NF-κB binding site (WT-mIL-10 probe) or mutant NF-κB binding site (Mut-mIL-10 probe) by EMSA. (D) Characterization of DNA binding of different NF-κB complexes to WT-mIL-10 probe by supershift analysis using antibodies specific for the indicated NF-κB subunits. A-D are representative of 3 independent experiments.

Transfection of EGFP-p65 along with whole IL-10 promoter construct (1.57 kb) does not resulted in any further increase in luciferase activity under Sb^5LD stimulation which further suggest p65 sub unit of NF-kB have got no role in Sb^5LD mediated IL-10 generation (Fig 7). Altogether, these results
demonstrate that Sb\textsuperscript{R}LD infection specifically modulates Site II (-583/-593) of IL-10 promoter to induce p50/c-Rel binding thereby initiating IL-10 transcription.

**Figure 7:** Sb\textsuperscript{R}LD infection does not involve binding of p65 with IL-10 promoter. Comparison of luciferase activity of lysate of RAW264.7 cells transfected either with whole length (WL) pGL3-IL-10 promoter construct (1.57 kb), containing all three sites(I, II and III), with or without EGFP-p65 transfection in response to Sb\textsuperscript{R}LD or Sb\textsuperscript{S}LD stimulation.

Sb\textsuperscript{R}LD infection leads to nuclear translocation of p50/c-Rel

In order to confirm the presence of p50/c-Rel in nuclear extracts of Sb\textsuperscript{R}LD infected cells, confocal microscopy was performed. Confocal microscopy showed nuclear translocation of p50/c-Rel, in Sb\textsuperscript{R}LD infected cells (Fig 7). These observations detected p50/c-Rel as an important molecule in Sb\textsuperscript{R}LD mediated IL-10 over production in host.

**Figure 8:** p50/c-Rel is translocated into the nucleus of Sb\textsuperscript{R}LD infected M\textsubscript{Φ}. Cells infected with either Sb\textsuperscript{R}LD for 24h were permeabilised for nuclear staining, stained with Ab specific for p50/c-Rel,
mounted in nuclear staining DAPI containing medium and subjected to confocal microscopy. Result is representative of three independent experiments.

Discussion

Research on *Leishmania* drug resistance focused so far essentially on the parasite itself and the direct molecular adaptations within the parasite (Croft et al., 2006). Recent studies showed that *Leishmania* is a master in manipulation of its host cell; consequently, mechanisms of drug resistance should also be addressed at the level of the infected Mφ. In a previous report, we showed that antimonials resistant *L. donovani* could interfere with the signalling system of the Mφ and counter its oxidative burst (Mookerjee Basu et al., 2006). In present study, we explored another domain of the parasite/host cell interaction that modulates host IL-10 promoter resulting in IL-10 induction.

Our study reviled that Sb^R^LD infected Mφs results in higher IL-10 induction in host as compared to Sb^S^LD infection. Our study suggest that this ability of Sb^S^LD to induce higher IL-10 is a generalised event for all LD isolates which shows resistance to antimony and is highly co-related with their EC_{50}. Sb^R^LD infection of DC has also been reported to induce higher IL-10 as compare to Sb^S^LD infection (Halder et al., 2010). There are previous reports that kala-azar patients harbouring Sb^R^LD show much higher parasite burden and IL-10 production as compared to those harbouring Sb^S^LD (Thakur et al., 2003, Verma et al., 2010).

We characterised the cascade of molecular events likely involved in IL-10 induction in Sb^R^LD infection. Previous reports suggested that the activation of ERK is the key step for IL-10 induction (Loscher et al., 2005). Interestingly, infection with Sb^S^LD results in the inactivation of host ERK, NF-κB and JNK, thereby favouring the establishment of the parasite (Contreras et al., 2010). In our study we also did not observe any activation of ERK or NF-κB in Mφs infected with Sb^S^LD, although Sb^S^LD activate ERK and nuclear translocation of NF-κB involving p50/c-Rel leading to IL-10 induction, through interaction with IL-10 promoter site II (−583/−593). Moreover previous reports have suggested a possible role of PKC in IL-10 induction from Mφs infected with Sb^S^LD (AG83) (Bhattacharyya et al., 2001). It is already known that specific stimulations with LPS lead to unique modifications of the IL-10 promoter (Saraiva et al., 2005). Treatment of Mφs with cyclohexamide prior to Sb^R^LD infection resulted in abrogation of IL-10 induction suggesting modulation of host IL-10 promoter is a key event of Sb^R^LD infection. There is a report that Site I (−46/−55) of the IL-10 promoter binds with NF-κB under LPS stimulation (Cao et al., 2006). Our results (Fig 3) are in agreement with a previous report stating that the p50/c-Rel activated upon infection by *L. major* amastigotes is involved in IL-10 secretion in fresh human monocytes (Guizani-Tabbane et al., 2004).
This study broadens our view on the panel of molecular mechanisms involved in antimonial resistance, highlighting their complementarity. Our study provided the molecular insights as to how Sb\textsuperscript{R}LD differentially scrutinised a specific site of host IL-10 promoter, thereby upregulating IL-10. Our study also suggests that this ability of Sb\textsuperscript{R}LD to induce higher level of IL-10 in host can be utilized as critical factor to discriminate between Sb\textsuperscript{R} and Sb\textsuperscript{S} form of infection.
References


