Imipramine Exploits Histone Deacetylase 11 To Increase the IL-12/IL-10 Ratio in Macrophages Infected with Antimoney-Resistant *Leishmania donovani* and Clears Organ Parasites in Experimental Infection

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Imipramine Exploits Histone Deacetylase 11 To Increase the IL-12/IL-10 Ratio in Macrophages Infected with Antimony-Resistant *Leishmania donovani* and Clears Organ Parasites in Experimental Infection

Sandip Mukherjee,*† Budhaditya Mukherjee,*† Rupkatha Mukhopadhyay,* Kshudiram Naskar,* Shyam Sundar,‡ Jean-Claude Dujardin,‡ and Syamal Roy*

The efflux of antimony through multidrug resistance protein (MDR)-1 is the key factor in the failure of metalloid treatment in kala-azar patients infected with antimony-resistant *Leishmania donovani* (SbRLD). Previously we showed that MDR-1 upregulation in SbRLD infection is IL-10–dependent. Imipramine, a drug in use for the treatment of depression and nocturnal enuresis in children, inhibits IL-10 production from SbRLD-infected macrophages (SbRLD-M). It abrogates SbRLD-mediated p50/c-Rel binding to IL-10 promoter and preferentially recruits p65/RelB to IL-12 p35 and p40 promoters, causing a decrease in IL-10 and overproduction of IL-12 in SbRLD-M. Histone deacetylase 11 per se does not influence IL-12 promoter activity. Instead, a imipramine-mediated decreased IL-10 level allows optimal IL-12 production in SbRLD infection. The efflux of antimony through multidrug resistance protein (MDR)-1 is the key factor in the failure of metalloid treatment in kala-azar patients infected with antimony-resistant *Leishmania donovani* (SbRLD). Previously we showed that MDR-1 upregulation in SbRLD infection is IL-10–dependent. Imipramine, a drug in use for the treatment of depression and nocturnal enuresis in children, inhibits IL-10 production from SbRLD-infected macrophages (SbRLD-M). It abrogates SbRLD-mediated p50/c-Rel binding to IL-10 promoter and preferentially recruits p65/RelB to IL-12 p35 and p40 promoters, causing a decrease in IL-10 and overproduction of IL-12 in SbRLD-M. Histone deacetylase 11 per se does not influence IL-12 promoter activity. Instead, a imipramine-mediated decreased IL-10 level allows optimal IL-12 production in SbRLD-M. Furthermore, exogenous rIL-12 inhibits intracellular SbRLD replication, which can be mimicked by the presence of Ab to IL-10. This observation indicated that reciprocity exists between IL-10 and IL-12 and that imipramine tips the balance toward an increased IL-12/IL-10 ratio in SbRLD-M. Oral treatment of infected BALB/c mice with imipramine in combination with sodium stibogluconate cleared organ SbRLD parasites and caused an expansion of the antileishmanial T cell repertoire where sodium stibogluconate alone had no effect. Our study deciphers a detailed molecular mechanism of imipramine-mediated regulation of IL-10/IL-12 reciprocity and its impact on SbRLD clearance from infected hosts. The Journal of Immunology, 2014, 193: 4083–4094.

R esistance to sodium stibogluconate (SSG) or pentavalent antimonials in the treatment of visceral leishmaniasis (VL) or kala-azar is a major problem in the Indian subcontinent (1). In Bihar, the epicenter of Indian kala-azar, traditional pentavalent antimonial treatment was abandoned not because of toxicity but owing to high resistance (2). Pentavalent antimonials remain the treatment of choice in Africa, South America, Bangladesh, Nepal, and India (except north Bihar) (3).

The resistance is due to upregulation of the multidrug resistance protein (MDR)-1 pump, which causes efflux of antimonials (4). New drugs such as miltefosine and amphotericin B are in use for the treatment of antimony-resistant (SbRLD) kala-azar cases, but relapse cases are also increasing (5, 6). Because antimonials and miltefosine use the same efflux pump (7), this raises serious concern about the efficacy of miltefosine, either alone or in combination, in view of the rampant antimony resistance in the field. Recent studies from our group showed that ~78% of the recent clinical isolates are resistant to antimonials although the drug has not been in use for more than a decade in Bihar (8). Moreover, there is an increasing number of reports of post–kala-azar dermal leishmaniasis after successful treatment with miltefosine (9, 10). In 2010, the World Health Organization’s expert committee on leishmaniasis “strongly recommended not to use miltefosine monotherapy” owing to a serious form of leishmaniasis relapse in Nepal after a year when one in five people treated developed the disease (11). The question is how the pre-existing rampant antimony resistance in the field would influence the efficacy of future drugs. Considering the health problem in the Indian subcontinent and Sudan, one would expect that antileishmanial drugs must ideally have the following criteria: they must be orally active, inexpensive, and less toxic and show immunostimulatory properties. Previously we showed that most of the antileishmanial drugs possess immunostimulatory ability (12). The emergence of drug resistance in leishmania is however hampering the control program.

One of the strategies to discover new drugs is to reposition or reemploy existing drugs that will reduce the cost and time for drug research.
IMIPRAMINE ACTIVATES HDAC11 IN LEISHMANIA-INFECTED CELLS

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Development (13). Fleximidazole, a nitroimidazole recently tested in a phase I clinical trial for treating African trypanosomiasis (14), could potentially be used to treat VL (15). Thiiodizole, belonging to the class of antipsychotic drugs, shows promising activities against multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis in a mouse model (16). Most importantly, it shows unique activity against nonreplicating Mycobacterium tuberculosis culture grown under hypoxic conditions (17). Similar to phenothiazines, imipramine, another antipsychotic drug used for treating depression (18) and pediatric nocturnal enuresis (19), alters the proton motive force in Leishmania donovani promastigotes (20) and kills anti-apoptotic-sensitive (Shβ) and Shβ intracellular amastigotes without affecting the host cell viability. Additionally, oral treatment of imipramine clears organ parasites in hamsters (21). Imipramine is a potent inducer in macrophages (Mβs) of TNF-α (22), which is an important cytokine for antileishmanial defense (23) and is a competitive inhibitor of trypanothione reductase, an enzyme upregulated in Shβ L. donovani (ShβLD) (8).

Two important cytokines, IL-10 and IL-12, play a crucial role in Leishmania infection. IL-10 promotes intracellular infection and persistence (24) and increases the risk of relapse (25); IL-12 is the key cytokine for IFN-γ-producing Th1 cells (26, 27). Endogenous IL-12 is also required for the enhancement of the leishmanicidal effect of conventional antileishmanial drugs (28). It acts as a potent histone modulator in the brains of maternally deprived adult rats (29). Previously we have shown that the upregulation of MDR-1 in Mβs by ShβLD is IL-10–dependent and requires recruitment of p50/c-Rel at the IL-10 promoter site (4). Imipramine has been shown to reverse multidrug resistance phenotype in acute myelogenous leukemia cell lines (30). There is a recent report that histone deacetetylase (HDAC)11, one of the newly added members of the HDAC family, regulates IL-10 expression in mouse and human APCs (31). We showed that hamsters infected with ShβLD show a decreased IL-12/IL-10 ratio that is reversed upon oral imipramine treatment (21). The mechanism by which imipramine activates the ShβLD-infected host to increase IL-12 levels with a concomitant decrease in IL-10 is a matter of considerable interest. In this study, we show that imipramine inhibits IL-10 by overexpressing HDAC11, which in turn inhibits MDR-1 upregulation in ShβLD-infected Mβs. A decrease in IL-10 favors IL-12 expression, which in turn favors expansion of antileishmanial T cells, leading to parasite clearance.

Materials and Methods

Animals

BALB/c mice (Mus musculus) and hamsters (Mesocricetus auratus) were maintained and bred under pathogen-free conditions. Use of animals was approved by the Institutional Animal Ethics Committee of Indian Institute of Chemical Biology (IIC) and the Animal facility of the National Center for Cell Science (Pune, India). All animal experiments were performed in accordance with the Indian and international guidelines for the Care and Use of Laboratory Animals, Ministry of Environment and Forest, government of India.

Ethics statement

Use of human subjects was approved by the Ethical Committee of Human Subjects of the Indian Institute of Chemical Biology. Blood samples were drawn from normal healthy individuals after obtaining their written informed consent.

Parasite cultures and maintenance

ShβLD (MHOM/IN/2009/BHU575/0 and MHOM/IN/2005/BHU138) and Shβ L. donovani (ShβLD; MHOM/IN/83/AG83 and MHOM/NP/03/BPK206) (8) maintained in golden hamsters (32) were used for this study.

Preparation of soluble leishmanial Ag

Soluble leishmanial Ag (SLA) was prepared from stationary phase L. donovani promastigotes (BHU 575 and Ag 83) following the published protocol (33). The protein concentration was determined by the Bradford protein assay method (Bio-Rad, Hercules, CA). The prepared Ag was stored at −70°C until further use.

Preparation of macrophages from human PBMCs and infection

Peripheral blood was collected from three healthy donors by venipuncture, and mononuclear cells were separated by centrifugation over Ficoll-Hypaque as described previously (34). Cells were washed using PBS supplemented with 0.5% FCS and harvested in tissue culture plates for 3 h. Nonadherent cells were discarded by washing, and adherent cells were cultured for 3 d and designated as Mβs. Cells were then incubated in the presence or absence of L. donovani (10× the cell number) for 48 h in complete RPMI 1640 media. Imipramine treatment was carried out for 24 h more in selected sets. The supernatant was collected and stored frozen for subsequent experiments.

Cell culture and infection

Peritoneal exudate cells (PECs), conveniently named PEC-Mβs, were harvested from BALB/c mice and purified as described previously (8). PEC-Mβs were infected with stationary-phase L. donovani promastigotes at a ratio of 1:10 for 6 h, washed to remove free parasites, and incubated for another 24 h. Treatments were given where needed. Supernatants obtained from infected and treated PEC-Mβs were used for cytokine analysis by ELISA. In some experiments PEC-Mβs were infected with adenovirus for overexpression of HADCA6 or HDAC11 or GFP (SignaGen Laboratories) following the manufacturer’s protocol. Transfection experiments were performed using the RAW264.7 cell line, defined as RAW-Mβs.

Infection of BALB/c mice

To infect BALB/c mice (6 wk old), amastigotes from ShβLD strain Ag83 and ShβLD strain BHU 575 L. donovani were purified as described (35) and inoculated (107 parasites in 100 μl) via intracardiac routes as reported previously (33).

Preparation of drug stocks for drug assays

Imipramine hydrochloride (Sigma-Aldrich, St. Louis, MO) and sodium stibogluconate (SSG; provided by Albert David, Kolkata, India) solutions were prepared at 1 mg/ml in PBS (Sigma-Aldrich), then subjected to sterile filtration using 0.22-μm filters (Millipore) when required.

Treatments

PEC-Mβs were treated with imipramine at several concentrations (25, 50, and 75 μM). In some experiments PEC-Mβs were treated in serum-free medium with 10 μM ERK1/2 inhibitor U0126 (36), 1 μM P38 inhibitor SB 203580 (37), 20 μM hKb of the kinase inhibitor BAY 11-7082 (8), 25 μM JNK inhibitor SP 600125 (38), or 10 μM p38 inhibitor SB 203580 (39) for 45 min before infection. PEC-Mβs were also treated with rIL-12 (100 and 200 pg/ml), rIL-10 (2, 20, and 200 pg/ml; BD Pharmingen), or neutralizing IL-10 Ab (1 ng/ml; BD Pharmingen) (40). Cells were then incubated in the presence or absence of L. donovani (10× the cell number) for 48 h in complete RPMI 1640 media. Imipramine treatment was carried out for 24 h more in selected sets. The supernatant was collected and stored frozen for subsequent experiments.

Real-time quantitative PCR to estimate expression of HADCA in imipramine-treated ShβLD-infected PECs

Total RNA was isolated from normal and ShβLD-infected PEC-Mβs with or without imipramine treatment using a total RNA isolation kit (Roche Biochemicals) and following the manufacturer’s protocol (Roche Biochemicals) and following the manufacturer’s protocol. cDNA synthesis and real-time quantitative PCR were done as described elsewhere (8). ShβLD-infected PEC-Mβs were defined as ShβLD-PEC-Mβs for convenience. The quantitative PCR contained 2× SYBR Green Supermix (Applied Biosystems, Foster City, CA) diluted twice and forward and reverse primers. The sequences of the forward and reverse primers are presented in Table I. Reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR System. Experiments on negative controls of cDNA synthesis (i.e., without reverse transcriptase) and no-template controls (i.e., without cDNA template) were also done for each gene. All reactions were done in duplicate, and their arithmetic average threshold cycle (Ct) was used for data analysis. The fold of gene expression compared with the control was calculated as 2−ΔΔCt by the 7500 Fast System SDS software, version 1.4 (Applied Biosystems).

Reporter assays

All of the transfection experiments were performed with the RAW264.7 cell line. The murine IL-12 p35 promoters −614 to −1141 (1.4 kb; 5′-GGT GTC

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CTT CTG ATT GCC TTG-3' and 5'-CCG GCA CTG AGA GGA GCT GC-3') and p40 promoters −105/-116S (1.06 kb; 5'-CAG GAC AGG AAT GGA GAA GCC GC-3' and 5'-GGT AGC GAC AGG GAA GAG GAG AG-3') were PCR amplified and cloned into a pGL3-Basic Vector (Promega). Using the IL-12p35 promoter construct (1.4 kb) and a Quick-Change II PCR-based, site-directed mutagenesis kit (Stratagene), three mutated IL-12p35 promoters constructs, containing a deletion at the NF-κB binding site −100/−110, or at the NF-κB binding site −220/−230, or a double deletion at NF-κB binding sites −100/−110 and −220/−240 of IL-12p35 promoter were generated. A mutated IL-12p40 promoter construct containing a deletion at NF-κB binding site −121/−132 was also generated using the IL-12p40 promoter construct (1.06 kb). All of the inserts were confirmed by sequencing. RAW-Ms were transiently transfected with 2 μg of these IL-12 or IL-10 (1.5 kb) or MDR-1 promoter constructs (4) using Lipofectamine 2000 (Invitrogen), rested for 12 h, and treated with rIL-10, rIL-2, or imipramine for 24 h. In some experiments luciferase activity was measured in imipramine-treated RAW-Ms 24 h after ShbLD infection. Luciferase activity in cell extracts was measured using the Dual-Luciferase reporter kit (Promega) according to the manufacturer’s protocols and normalized to the level of the protein content.

Chromatin immunoprecipitation assay

Using a commercially available kit (Upstate Biotechnology), a chromatin immunoprecipitation (ChIP) assay was performed as recommended by the manufacturer with minor modifications. Chromatin was immunoprecipitated at 4˚C overnight with Abs to rabbit IgG or NF-κB as such as anti-p50, anti-p65, RelB, and c-Rel, and DNA was extracted. A PCR assay was performed to amplify the region of the IL-10 promoter (using primers 5'-GCC CCA CAG ACA TAT CC-3' and 5'-CCT GGG TTG AAC GTC CG-3'), the regions of the IL-12 p35 promoter (using primers 5'-CCG GAA AAG AGT GGC TAC TCG C-3' and 5'-GGT AGC GAC AGG GAA GAG GAG AG-3'), or the region of the IL-12p40 promoter (using primers 5'-CCT CTG TAT GAT AGA TGC AC-3' and 5'-GGT TTG TTG TTC-3').

Confocal microscopy

The levels of expression of MDR-1 in rIL-10 or imipramine-treated PEC-Ms were determined by immunostaining followed by confocal microscopy (LSM 510; Carl Zeiss), as described elsewhere (4).

Dye uptake and retention assay

After treatment with several concentrations of imipramine, ShbLD- and ShbLD-infected as well as rIL-10 or MDR-1 promoter constructs (4) were infected with 2 × 10^6 cells/ml in serum-free RPMI 1640 incubated with an optimum concentration (250 ng/ml) of free rhodamine-123 (Rh123) for 32 h, washed, and further incubated in media free of Rh123. At indicated time points, cells were washed three times in PBS and finally lysed in 0.1% Triton X-100. The intracellular dye concentrations were determined by measuring the fluorescent intensity of the cell lysates.

Western blot

Cytoplasmic and nuclear proteins were prepared and Western blotting was performed for p50, p-p65, RelB, HDAC6, HDAC11 (Santa Cruz Biotechnology), p65, p-p38/p-38, p-c-Fos/c-Fos, p-c-Jun/c-Jun, and histone (Cell Signaling Technology) as described elsewhere (4). In brief, blots were probed with specific Abs. Binding of secondary HRP-labeled goat anti-rabbit or goat anti-mouse Abs (Cell Signaling Technology) was analyzed using SuperSignal West Pico or West Dura chemiluminescent substrate (Pierce).

In vitro parasite clearance

To determine in vitro parasite clearance, PEC-Ms were infected with either ShbLD (AG83) or ShbLD (BUH 575) for 24 h and either left untreated or treated. At the endpoints, the coverslips were washed with PBS, dried, fixed with 100% methanol (Merck), stained with 10% Giemsa (Sigma-Aldrich), and examined microscopically. One hundred Ms per coverslip were scored and the amastigotes were enumerated.

In vivo parasite clearance

The 6-wk-infected BALB/c mice were randomly divided into four groups (groups I-IV). Group I received only saline, group II received only SSG at the dose of 20 mg/kg/d once in a week for 4 wk (40), and group III received imipramine at the dose of 0.1 mg/kg/d for 4 wk by oral route using a feeding needle as described by others (41). Group IV received imipramine (0.1 mg/kg/d) by oral route for 4 wk and, 6 h thereafter, i.p. SSG (20 mg/kg) once a week for 4 wk. Two days after the completion of treatment, mice were sacrificed to determine splenic and hepatic parasite burdens by the stamp smear method as described elsewhere (42), as well as by the serial dilution method (33).

Cytokine measurement

Various cytokine levels (IL-10 and IL-12p70) in the splenocytes or peritoneal Ms were measured using a sandwich ELISA kit (BD Pharmingen, San Diego, CA) as per the manufacturer’s protocol. The level of IL-10 from human Ms was determined using commercially available monoclonal anti-human IL-10, anti-human biotin, and rIL-10 (BD Pharmingen) according to standard protocol. IL-12p70 in the same supernatant was measured using sandwich ELISA kit (BD Pharmingen).

T cell proliferation assay

A T cell proliferation assay was performed as described elsewhere (33). Briefly, spleens were isolated from different experimental groups of mice. Single-cell suspensions of splenocytes prepared after Ficoll density gradient centrifugation were suspended in complete RPMI medium. Cells were plated in triplicate in 96-well plates at a concentration of 10^5 cells per well and allowed to proliferate for 3 d at 37˚C in a 5% CO2 incubator either in the presence or absence of SLA (5 μg/ml). At 4 h before harvest, cells were incubated with MITT and T cell proliferation was measured with a nonradioactive MTT cell proliferation assay using an ELISA plate reader (DTX 900 multimode detector, Beckman Coulter, Brea, CA).

Statistical analysis

Each experiment was performed three times and the data represent means of three independent experiments (±SD) unless noted otherwise. Statistical significance between means of various groups was determined using a two-tailed Student t test. Only p values <0.05 were considered to be statistically significant. Values were considered extremely significant (p < 0.001), very significant (p = 0.001–0.01), or significant (p = 0.01–0.05) as indicated. Error bars indicate means ± SD. Data were analyzed using Prism 5.0 (GraphPad Software, San Diego, CA).

Results

Imipramine abrogates ShbLD- or rIL-10-driven MDR-1 overexpression

Imipramine treatment results in a significant dose-dependent increase in the accumulation of Rh123, a surrogate marker for antimony, in ShbLD (BUH 575 or BHU 138)-infected or rIL-10–treated PEC-Ms. However, in ShbLD (AG83 or BPK 206)-infected PEC-Ms, Rh123 accumulation was found to be high without imipramine treatment (Fig. 1A). Because both BHU 575 and BHU 138 showed similar results for the rest of the experiments, only BHU 575 has been used and defined as ShbLD for convenience. Similarly, AG83 was selected as a representative of ShbLD infection.

The MDR-1 reporter assay showed a decrease in IL-10–driven MDR-1 promoter activity as a function of imipramine concentration in RAW-Ms (Fig. 1B). All transfection experiments were performed in the RAW264.7 cell line; for all other experiments, primary Ms were used unless noted otherwise. The upregulation of MDR-1 is specific to IL-10 because rIL-2, an unrelated cytokine, failed to show the above effect. Furthermore, confocal microscopy of rIL-10–treated PEC-Ms stained with FITC-labeled Ab to MDR-1 showed upregulation of MDR-1, which was downregulated in the presence of imipramine (Fig. 1C). Western blot analysis showed that imipramine inhibited nuclear translocation of c-Fos/c-Jun, the subunits of AP-1 transcription factors important in MDR-1 upregulation in ShbLD-PEC-Ms (Fig. 1D).

Imipramine inhibits IL-10 promoter and histone acetylation

Because imipramine treatment abrogated IL-10 production in ShbLD-infected PEC-Ms, we checked the status of IL-10 promoter activity. Imipramine treatment led to abrogation of ShbLD-mediated histone acetylation and binding of p50/c-Rel at the IL-10 promoter...
FIGURE 1. (A) Increase in Rh123 accumulation in Sb^LD (BHU 575, BHU 138)-infected and rIL-10–treated normal M\(_f\)s as a function of imipramine concentration. In the control set, Sb^SLD (Ag 83, BPK 206)-infected M\(_f\)s show accumulation of Rh123 regardless of absence or presence of imipramine. (B) Reporter assay showing inhibition of MDR-1 promoter activity as a function of imipramine concentration. The RAW264.7 cell line was transfected with MDR-1 reporter construct, stimulated with rIL-10 (200 pg/ml) or IL-2 (200 pg/ml), and either treated with an increasing concentration of imipramine or left untreated. A progressive decrease in luciferase activity was observed as a function of imipramine concentration as compared with untreated control. (C) Confocal imaging of MDR-1 expression in rIL-10–treated PEC-M\(_f\)s in the absence and presence of imipramine. Murine PEC-M\(_f\)s were harvested in eight-well chambered slides and adhered for 48 h. After rIL-10 treatment for 24 h, M\(_f\)s were kept with imipramine (25 \(\mu\)M) for another 24 h. The cells were stained with FITC-conjugated anti–MDR-1 Ab and subjected to confocal imaging (original magnification \(\times 100\)). Imipramine treatment abrogated rIL-10–mediated MDR-1 overexpression. (D) Expression of subunits of AP-1 transcription factors in rIL-10–stimulated PEC-M\(_f\)s in the presence and absence of imipramine. M\(_f\)s were stimulated with rIL-10 for 24 h and then treated with imipramine for another 24 h. Expression of p-c-Fos/c-Fos and p-c-Jun/c-Jun in cytoplasmic as well as the nuclear extract was analyzed by Western blot analysis. Histone was used as nuclear loading controls. (E) ChIP analysis showing imipramine treatment prevents p50/c-Rel binding to IL-10 promoters. Nuclear extract from PEC-M\(_f\)s infected with Sb^LD (BHU 575) for 24 h was assessed with Abs to hyperacetylated histone H3 (Ace H3), p65, p50, c-Rel, and RelB and then subjected to PCR amplification. Chromatin was immunoprecipitated by whole-rabbit IgG. No Ab was used as a negative control, and input DNA (5%) was used as an internal control. Results in (A) and (B) are presented as means ± SD, with statistical significance being determined with respect to imipramine untreated control. (C)-(E) are representative of three independent experiments. ***\(p < 0.001\).
confirmed that imipramine treatment induced HDAC11 in normal as well as ShbLD-PEC-Ms but not in untreated ShbLD-PEC-Ms (Fig. 2B). HDAC6 was expressed in ShbLD-PEC-Ms, but this was reduced to some extent after imipramine treatment.

Functional analysis of the role of HDAC11 and HDAC6 in IL-10/IL-12 reciprocity

The relationship, if any, between the altered expressions of HDAC11 and HDAC6 and the production of IL-10 and IL-12 was evaluated. It was observed that overexpression of HDAC11 but not of HDAC6 resulted in an ∼2-fold decrease in IL-10 and an ∼3-fold increase in IL-12 promoter activity in ShbLD-RAW-Ms. Interestingly, imipramine treatment of ShbLD-RAW-Ms overexpressing HDAC11 further decreased IL-10 promoter activity to ∼3-fold with respect to infected control, whereas IL-12 promoter activity was increased by ∼8-fold (Fig. 3A). The cytokine assay revealed an ∼2-fold decrease in IL-10 and an ∼4-fold increase in IL-12 level in ShbLD-PEC-Ms overexpressing HDAC11. Imipramine treatment of ShbLD-PEC-Ms overexpressing HDAC11 showed further reduction of IL-10 by ∼4-fold, whereas IL-12 level was increased by ∼9-fold (Fig. 3B). Interestingly, an IL-12 reporter assay showed similar luciferase activity in both HDAC11 overexpressed and non-overexpressed RAW-Ms in the presence of imipramine (Fig. 3C). Furthermore, we analyzed IL-12 level in imipramine-treated ShbLD-PEC-Ms in the presence and absence of IL-10-neutralizing Ab to determine whether IL-10 has any influence on imipramine-mediated IL-12 production (Fig. 3D). Note that the presence of IL-10-neutralizing Ab further increased imipramine-mediated IL-12 generation in ShbLD-PEC-Ms (Fig. 3D).

Imipramine recruits p65/RelB for IL-12 induction in ShbLD-Ms

Using an array of pharmacological inhibitors, we observed that imipramine-mediated IL-12 generation from ShbLD-PEC-Ms was significantly inhibited ( ∼3.5-fold) only in the presence of p38 and NF-κB inhibitors, whereas inhibitors of ERK, PI3K, and JNK did not show any such inhibitory effect (Fig. 4A).

Table I. Sequences of primers used for real-time PCR

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<th>Gene</th>
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<tr>
<td>HDAC1</td>
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<td>HDAC2</td>
<td>5'-AACTCAACCAGCTACCCAC-3'</td>
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<tr>
<td>HDAC11</td>
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FIGURE 2. (A) Quantitative real-time RT-PCR analysis of HDAC1–HDAC11 mRNA. PEC-Ms were infected with ShbLD (BHU 575) for 24 h and either left untreated or treated with imipramine for an additional 24 h before total RNA isolation. Results are normalized to β-actin expression and presented relative to those of control cells. (B) Western blot analysis of HDAC11 and HDAC6 expression. Nuclear extract was isolated from normal or 24-h-infected ShbLD (BHU 575) PEC-Ms either in the presence or absence of imipramine (24 h). Expression of HDAC6 and HDAC11 in nuclear extract was analyzed by Western blot analysis. Histone was used as nuclear loading controls. Results in (A) are presented as means ± SD, with statistical significance being determined between means of imipramine untreated and treated groups. (B) is representative of three independent experiments. ***p < 0.001.
Promoter scan analysis revealed two NF-κB binding sites at positions −100/−110 and −240/−229 of IL-12 p35 promoter designated as sites A and B, respectively, and one NF-κB half site at position −121/−132 of the IL-12p40 promoter designated as site C (Fig. 4B). To determine the specific promoter site involved in imipramine-mediated IL-12 generation, RAW-Mφs were transfected with IL-12p35 or IL-12p40 or their combination, and luciferase activity was determined after imipramine treatment (Fig. 4C). Deletion of either site A or site B of the IL-12p35 promoter results in an ∼45% reduction of luciferase activity, whereas deletion of both the sites (A and B) caused an ∼80% reduction. Interestingly, although the IL-12p40 promoter failed to show significant luciferase activity alone, presence of it resulted in a significant increase in luciferase activity of the IL-12p35 promoter. Similar experiments in the presence of SbRLD infection also revealed that the presence of all three sites (A–C) is required for optimal imipramine-mediated IL-12 generation (Fig. 4D). To determine the NF-κB subunits critical for imipramine-mediated IL-12 generation in SbRLD-PEC-Mφs, ChIP of the IL-12 promoter was performed using Abs for acetylated histone, p65, p50, c-Rel, or RelB. The results revealed that histone acetylation and binding of p65/RelB at all three sites of the IL-12

FIGURE 3. (A) Overexpression of HDAC11 abrogates IL-10 and induces IL-12 production in SbRLD (BHU 575)-infected Mφs after treatment with imipramine. RAW-Mφs were transfected with either IL-10 or IL-12 reporter construct, infected with SbRLD (BHU 575), and then either transfected with adenovirus encoding HDAC6 or HDAC11 or GFP for 24 h, or left untreated or treated with imipramine for another 24 h. Finally, cells were lysed and luciferase activity was measured. Results are normalized to protein concentrations. (B) IL-10 and IL-12 levels were measured in the culture supernatant of PEC-Mφs under the same experimental condition as above but without transfecting IL-10 or IL-12 reporter constructs and in the presence of an uninfected and untreated control. (C) Luciferase activity in IL-12 reporter construct transfected RAW-Mφs infected with adenoviral encoding either HDAC11 or GFP for 24 h and the cells were either left unstimulated or stimulated with imipramine for another 24 h. Results are normalized to protein concentrations. (D) IL-12 level in the culture supernatant of Mφs infected with SbRLD (BHU 575) and then either left untreated or treated with imipramine for 24 h in the presence and absence of IL-10–neutralizing Ab. Results in (A)–(D) are presented as means ± SD. For (A) and (B) statistical significance was determined between the infected control group and the HDAC11 overexpressed group and also between the HDAC11 overexpressed group and the imipramine-treated HDAC11 overexpressed group. For (D), statistical significance was determined between the infected control group and the imipramine-treated group and also between the imipramine-treated group and the anti–IL-10 Ab treated group. **p = 0.001–0.01, ***p < 0.001.
Reciprocity between IL-10 and IL-12 generation

We studied the influence of IL-10 over imipramine-activated IL-12 promoter activity. Low doses of rIL-10 (2 or 20 pg/ml) failed to alter the status of imipramine-mediated p65/RelB binding to the IL-12 promoter. However, at 200 pg/ml rIL-10 there was a partial abrogation of p65/RelB binding with the IL-12 promoter (Fig. 5A). We also studied the influence of IL-12 or of neutralizing IL-10 Ab on Sb9LD-mediated IL-10 generation from PEC-Mf. Pre-incubation of PEC-Mf with rIL-12 or neutralizing IL-10 Ab

promoter occurred upon imipramine treatment in Sb9LD-PEC-Mfs (Fig. 4E).

Reciprocity between IL-10 and IL-12 generation

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resulted in reduced IL-10 production (Fig. 5B). We observed that the number of amastigotes per 100 PEC-Mφs in the case of SbRLD infection was significantly less when PEC-Mφs were pretreated with rIL-12 or neutralizing IL-10 Ab as compared with untreated control (Fig. 5C). Furthermore, the IL-10 level was significantly higher in SbRLD-infected Il12−/− Mφs as compared with infected wild-type Mφs upon imipramine treatment (Fig. 5D).

**Cotreatment with SSG and imipramine eliminates organ parasites of BALB/c mice infected with SbRLD**

Having seen that imipramine is a potent inhibitor of MDR-1, we asked whether a combination of imipramine with SSG would be effective in clearing SbRLD from infected BALB/c mice. Thus, SbRLD-infected BALB/c mice were treated with SSG (20 mg/kg) in the absence or presence of imipramine (0.1 mg/kg; Fig. 6). Although, SSG alone could clear ~43% of splenic as well as

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**FIGURE 5.** Reciprocal regulation between IL-10 and IL-12 induction. (A) ChIP analysis of IL-12 promoters p35 and p40 with nuclear extract derived from PEC-Mφs infected with SbRLD (BUH 575), treated with rIL-10 in a dose-dependent manner (2, 20, or 200 pg/ml), and assessed with Abs to hyperacetylated histone H3 (Ace H3), p65, c-Rel, and RelB followed by PCR amplification. ChIP by whole-rabbit IgG. No Ab was used as a negative control, and input DNA (5%) was used as an internal control. (B) IL-10 production in the culture supernatant of PEC-Mφs, preincubated with either rIL-12 or rIL-2 and infected with SbRLD, by ELISA. (C) Number of intracellular amastigotes per 100 Mφs in PEC-Mφs preincubated with either rIL-12 or neutralizing IL-10 or rIL-2 and infected with SbRLD. (D) IL-10 production in the culture supernatant of wild-type or Il12−/− PEC-Mφs either left uninfected or infected with BUH 575 in the presence or absence of imipramine. (A) is representative of three independent experiments, and (B)+(D) are presented as means ± SD. For (B) and (C), statistical significance was determined with respect to infected control. For (D), statistical significance was determined between infected IL12−/− Mφs in the presence and absence of imipramine and also between wild-type and the Il12−/− imipramine-treated group. ***p < 0.001.
FIGURE 6. (A and B) Combination of imipramine and SSG clears organ parasites in BALB/c mice infected with Shb5LD. Six-week-old mice were infected with either Shb5LD (BHU 575) or Shb5LD (Ag83) parasites and infection was allowed to be established for the next 6 wk. Treatment was carried out as described in Materials and Methods. Two days after the last treatment, mice were sacrificed and the hepatic (A) as well as the splenic (B) parasite loads were determined by the stamp-smear method. Total parasite load in each organ is expressed in Leishman–Donovan unit (LDU) where 1 LDU = amastigote per nucleated cell × organ weight in milligrams. (C and D) Profile of IL-10 and IL-12 in BALB/c mice as in (A). Splenocytes isolated from different groups of mice were harvested in six-well plates, kept for 48 h, stimulated with SLA (5 μg/ml) or left untreated for 24 h, and the resulting supernatant was assessed for cytokine by ELISA. (E) Imipramine treatment along with SSG favors expansion of antileishmanial T cells. Splenocytes isolated from infected and drug-treated animals were stimulated with SLA (5 μg/ml) or left untreated and the resulting T cell proliferation was measured using a nonradioactive MTT cell proliferation assay. The mice received SSG alone (20 mg/kg), imipramine (0.1 mg/kg) alone, or a combination of both. All of the above experiments were repeated three times with five animals per group, and data are represented for 15 animals per group.
hepatic parasite load, imipramine alone at 0.1 mg/kg dose failed to clear any parasite. However, their combination cleared 98% of the organ parasite burden (Fig. 6A, 6B).

**Cotreatment favors expansion of antileishmanial T cell repertoire with increased IL-12 and decreased IL-10 production from splenocytes**

Splenocytes were derived from SbLD-infected BALB/c mice and stimulated with SLA ex vivo and the expansion of antileishmanial T cells was monitored. We observed that infected mice or infected as well as imipramine (0.1 mg/kg)-treated mice failed to show any anti-SLA–specific T cell expansion whereas the infected and cotreated group (antimony along with imipramine) showed expansion of anti-SLA T cells (Fig. 6E). The resulting cytokine (IL-10 and IL-12) production in the culture supernatant was assayed. Cotreatment of imipramine and antimony was found to be effective to reduce the IL-10 level (10-fold) as well as to increase the IL-12 level (8-fold) as compared with SbLD infection alone (Fig. 6C, 6D).

Similar experiments were performed in BALB/c mice infected with SbLD. In this case SSG alone could clear the hepatic and splenic parasites essentially completely (Fig. 6A, 6B). There was expansion of the anti-SLA–specific repertoire (Fig. 6E) with a surge of IL-12 but not of IL-10 in the group receiving only SSG (Fig. 6C, 6D).

**Discussion**

This study clearly identifies imipramine as a potent inhibitor of MDR-1 pump (Fig. 1A–C) and favored accumulation of Rh123, a surrogate of antimonials in SbLD-Mðð. Thus, imipramine would favor accumulation of antimonials, which would kill intracellular parasites by generating superoxide and NO (43). It is tempting to speculate that imipramine in combination with antimonials may be useful for the treatment of Shkala-azar cases. Pentavalent antimony remains the first line of treatment for VL in sub-Saharan Africa and Brazil (44), but not in the Bihar state in India where continuous exposure to arsenic contamination in drinking water may have contributed to the lower efficacy of antimonials because of cross-resistance (45, 46). Kala-azar patients in the endemic zone of Bihar show overexpression of multidrug resistance–associated protein 1 and permeability glycoprotein in monocytes (40).

Imipramine offers several advantages: it is a drug in use (18), it is orally active, it kills intracellular ShLD and ShLD parasites (21), and it is immunostimulatory (22). Previously, we showed that infection of Mðð with ShLD, but not with ShLD, produces IL-10, which in turn upregulates MDR-1 (4). In the present study we show that imipramine activates HDAC11 (Fig. 2A, 2B) whose overexpression inhibits IL-10 and upregulates IL-12 in ShLD-PEC-Mðð (Fig. 3A, 3B). Our results also suggest that imipramine inhibits IL-10 and upregulates IL-12 in ShLD-infected human Mðð (Supplemental Fig. 1), although further experiments are required to determine whether a similar HDAC-dependent mechanism is in operation in the case of human Mðð. There is a previous report that HDAC11 is a negative regulator of IL-10 (31). We found that imipramine treatment leads to deacetylation of the IL-10 promoter and prevents binding of NF-kB transcription factors to the promoter site (Figs. 1, 2). Lack of acetylation of the IP-10 promoter is already known to cause reduced binding of NF-kB transcription factors (47).

Interestingly, IL-12 promoter activity remains unaltered in imipramine-treated, HDAC11 overexpressed RAW-Mðð (Fig. 3C), suggesting that HDAC11 may not have any direct influence on IL-12 promoter activity. It has been shown that overexpression of HDAC11 does not affect IL-12 mRNA or IL-12 promoter activity (31). We show that p38 and NF-kB are important in imipramine-dependent upregulation of IL-12 in ShLD-PEC-Mðð (Fig. 4). It is also evident from our study that p65/RelB binds to three different sites in the IL-12 promoter for optimal IL-12 generation (Fig. 4D, 4E). Although imipramine treatment resulted in p65/RelB binding with the IL-12p40 promoter, it failed to show any significant luciferase activity. It is therefore possible that although the binding of transcription factor with the IL-12p40 promoter fails to cause its transcriptional activation, it might result in optimal transcriptional activation of the IL-12p35 promoter through functional interaction resulting in optimal transcriptional synergy as previously observed in the case of other transcription factors (48). However, further experiments are required to completely address the role played by the IL-12p40 promoter in imipramine-mediated IL-12 production. Additionally, neutralizing Ab to IL-10 enhances imipramine-mediated IL-12 generation from ShLD-PEC-Mðð, suggesting the inhibitory role of IL-10 on IL-12 generation (Fig. 3A, 3B, 3D). This is in agreement with other reports (49). We also show that imipramine-mediated recruitment of p65/
ReLB is abrogated at 200 pg/ml IL-10 (Fig. 5A). Serum IL-10 levels have been found to attain very high values (480 pg/ml) under some pathological conditions such as metastatic malignant melanoma (50). Thus it seems that high IL-10 may influence IL-12 promoter activity. Interestingly, note that IL-10 has been shown to inhibit IL-12p40 gene transcription (51, 52). Naturally one may ask whether IL-12 may also have any inhibitory influence on IL-10 production. In rIL-12–pretreated SbLD-PEC-Mds, the IL-10 level was found to decrease in culture supernatant, and a significant decrease was noted in the number of SbLD amastigotes per 100 Mds as compared with untreated control (Fig. 5B, 5C), suggesting an inhibitory influence of IL-12 on IL-10. Our observation suggests that infection of wild-type Mds resulted in significantly higher IL-10 production as compared with H12–/– Mds (Fig. 5D). However, the situation was different upon imipramine treatment of infected Mds. This resulted in a ~5-fold decrease in IL-10 production in infected wild-type Mds as opposed to only an ~2-fold decrease in IL-12–/– Mds. Our data thus suggest that there may be a role of IL-12 in inhibiting IL-10 production upon imipramine treatment. This is in agreement with other reports that there is a negative influence of IL-12 over IL-10 induction (53, 54).

IL-12 is the key cytokine for the initiation and maintenance of Th1 responses and IFN-γ production from Th1 cells (26, 55). Endogenous IL-12 is also required for the expression of the leishmanicidal effect of conventional chemotherapy (28). Having established that imipramine is a potent inhibitor of MDR-1, we studied its efficacy in combination with SSG to clear SbLD from the organs of infected mice. Previously we showed that in SbLD-infected hamsters, SSG is effective in combination with the antileishmanial drugs. This resulted in an ∼10-fold decrease in IL-10 production from Th1 cells (26, 55).

In summary, SbLD infection leads to IL-10 surge from host cells, which proceeds further to MDR-1 overexpression and suppression of IL-12 promoter activity. Imipramine treatment activates HDAC11, which inhibits the binding of p50c-Rel with IL-10 promoter, leading to transcriptional inactivation of SbLD-induced IL-10 production. As a consequence, there is an inhibition of IL-10–mediated MDR-1 upregulation. The compound activates IL-12 transcription, which in turn may inhibit IL-10. The mechanism of imipramine-mediated inhibition of IL-10 production and upregulation of IL-12 is presented schematically in Fig. 7.

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**Disclosures**

The authors have no financial conflicts of interest.

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