Supporting Information

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SI Materials and Methods

Cell Culture and Infection and Preparation of Parasite Lysate. Peritoneal exudate cells, conveniently named macrophages (Mϕs), were harvested from BALB/c, C57BL/6, Toll-like receptor $2^{-/-}$ ($TLR2^{-/-}$), $TLR4^{-/-}$, and IL-$10^{-/-}$ mice by lavage 48 h after i.p. injection of 2% (wt/vol) soluble starch (SIGMA) (1). Mϕs were plated on 90-mm tissue culture Petri dishes or in 24-well plates (Nunc) at a density of 1×10^7 or 1.5×10^6, respectively, and also on sterile 22-mm square coverslips in 35-mm disposable Petri plates at a density of 1.5×10^5 per coverslip in RPMI medium 1640 (SIGMA) supplemented with 10% (vol/vol) heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (GIBCO) (i.e., RPMI complete medium). The cells were left to adhere for 48 h at 37 °C under 5% (vol/vol) CO_2 before infection. Mϕs were infected with stationary-phase *Leishmania donovani* (LD) promastigotes at a ratio of 1:10 for 6 h, washed to remove free parasites, and incubated for another 24 h or 48 h to determine the infection rate. Supernatants obtained from Mϕs infected with stationary-phase LD promastigotes at a ratio of 1:10 for 48 h were subsequently used for ELISA experiments unless otherwise stated. Parasite lysates were prepared as described by Kapler et al. (2) with slight modifications. The protein content of the lysate was estimated, and an equal concentration of the lysate protein was directly used for the experiment without further centrifugation steps.

Treatment. Stationary-phase LD promastigotes were centrifuged at 1,800 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in a minimum volume of PBS and treated with galactosidase (0.2 units; SIGMA) for 1 h at 37 °C to remove surface sugar. The parasites were washed and fixed with 1% paraformaldehyde for 15 min and washed five times with cold PBS (3). These paraformaldehyde-treated parasites were then resuspended in complete medium before infecting the Mϕs. In some experiments, Mϕs were treated with 10 μM ERK1/2 inhibitor U0126 (4), 20 μM PD 98059 (5), 1 μM PI3K inhibitor (wortmannin) (6), 20 μM IκB kinase inhibitor BAY 11-7082 (7), 25 μM JNK inhibitor SP 600125 (8), or 10 μM p38 inhibitor SB 203580 (9) for 45 min before infection in serum-free medium. In some experiments, Mϕs were treated with murine IL-10 (2, 20, or 200 pg/mL) for 12 or 48 h in the presence or absence of 10 μg/mL αIL-10 Ab/isotype control Ab, which was preincubated for 1 h before infecting the Mϕs. In some experiments, Mϕs were stimulated with LPS (1 μg/mL) and used as a positive control.

Generation of Galactosyltransferase Knockdown Antimony-Resistant LD: Construction of Expression Vectors and Transfection in *Leishmania*. The antisense construct of the galactosyltransferase (GalT) gene was generated by PCR amplification of the 1,414- to 1,653-nt region of the phosphoglycan β-1,3 galactosyltransferase 3 gene (*LdGalT3*) from the genomic DNA of LD parasites using the sense primer 5′-CGGGATCCCTGTGGCAGGAGGCGCTGCACCACCGCAA-3′ and the antisense primer 5′-TCCCCCGGGGTCGCTCAAGTACTGTGGCACCTTCAGGTA-3′ and was then cloned in an antisense orientation in the SmaI/BamHI sites of the pXG-B2863 vector (a kind gift from S. M. Beverley, University of Washington, Seattle, WA); this was termed anti-*LdGalT*. The construct, along with the empty vector pXG-B2863, was transfected into LD promastigotes by electroporation as described previously (2). Briefly, late-log-phase promastigotes were harvested and washed twice in OPTI-MEM (GIBCO). Cells were then suspended at a density of 1×10^8 cells/mL, and 0.4 mL of this was taken into a 0.2-mm ice-chilled electroporation cuvette. Thirty micrograms of plasmid DNA was taken in 100 μL of electroporation buffer and added to the cells. After 10 min on ice, the cells were electroporated with a single pulse by a Bio-Rad Gene Pulsar apparatus using 450-V and 550-μF capacitance. The cells were incubated on ice for a further 5 min and then added to 10 mL of drug-free growth medium. After 24 h of survival, 10 μg/mL G418 was added and kept at 22 °C. The transfected cells were monitored visually by microscope, and the drug concentration was increased gradually. Finally, the transfected cells were routinely maintained in medium containing 200 μg/mL G418.

Dye Uptake and Retention Assay. Antimony-sensitive LD (Sb^SLD)– and antimony-resistant LD (Sb^RLD)–infected Mϕs were washed and resuspended (2×10^5 cells/mL) in serum-free RPMI, incubated with an optimum concentration (250 ng/mL) of free Rh123 for 32 h, washed, and further incubated in media free of Rh123. In some experiments, Mϕs were incubated with Rh123 for 16 h, after which they were washed and further incubated in Rh123-free medium with an increasing concentration of either recombinant IL-10 (rIL-10) or rIL-2 (2, 20, or 200 pg/mL) for another 2 h. 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 fluorescence intensity of the cell lysates.

Flow Cytometry and Confocal Microscopy. Stationary-phase LD parasites (2×10^6 cells/mL each), were stained with FITC-labeled *Dolichos biflorus* agglutinin and compared with their mannose level specific to Con A lectin. Samples were subjected to flow cytometry analysis in a FACS Aria II cell sorter (Becton Dickinson). The mean fluorescence intensity was analyzed by FACS DIVA software (Becton Dickinson), and, accordingly, the relative expression of surface sugar was determined. Multidrug-resistant protein 1 (MDR1) was stained using a primary anti-MDR1 Ab (Santa Cruz Biotechnology), followed by a secondary FITC-conjugated anti-rabbit whole-IgG molecule (SIGMA). The appropriate isotype control was used for each experiment. Staining was performed 48 h after washing the unpermeabilized infected cells in cold PBS, specifically to check the expression of MDR1 on the host cell surface (10).

EMSA. DNA probes specific for murine IL-10 promoter containing WT or mutant NF-κB binding sites, represented by the WT mIL-10 probe 5′-GCCCTCTCGGGGTTTCCTTTGGG-3′ and the mutant (Mut) mIL-10 probe 5′-GCCCTCT*CCTCT*TTT*AA*T-TTGGG-3′ (NF-κB binding site is underlined, and mutated bases are italicized), respectively, were used. Supershift EMSA was carried out using the following Abs: αp50, αc-Rel (Santa Cruz Biotechnology), and αp65 (Cell Signaling Technology). For the MDR1 promoter, WT or mutant activator protein-1 (AP-1) binding sites, represented by the WT mMDR1 probe 5′-CAA-TAATACTTGAGTCAAGCTGG-3′ and the Mut mMDR1 probe 5′-CAATAATACT*AC*AGTG*T*AGCTGG-3′ (AP-1 binding site is underlined, and mutated bases are italicized), respectively, were used. Supershift EMSA for the MDR1 promoter was carried out using the following Abs: αc-Fos, αc-Jun, and αFra1 (Cell Signaling Technology). Bands were visualized using a phosphoimager.

Western Blot Analysis. 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 SuperSignalR West Pico or West Dura Chemiluminescent substrate (Pierce).

Reporter Assay. The murine IL-10 promoters −17/−1,576 (1.57 kb; 5′-GCTGGGTCTTGAGCCTCTTCTGG-3′ and 5′-CTGCAAGGCTGCCTTGTGGCTTTG-3′), −17/−292 (275 bp; 5′-GAGGTAGCCCATACTAAAAATAGC-3′ and 5′-CTGCAAGGCTGCCTTGTGGCTTTG-3′), and −864/−1,138 (274 bp; 5′-GGAAGGACAGCCCGGGAGTGTACC-3′ and 5′-CCTGGGAGATCTCTGGTAGTCC-3′) and the murine MDR1 promoters +37/−154 (191 bp; 5′-CCTGTTTCGCAATTTCTC-3′ and 5′-GATGGGACCAGCAGCTCC-3′) and +37/−116 (153 bp; 5′-AGCTGGGCCGGGAGCTGG-3′ and 5′-GATGGGACCAGCAGCTCC-3′) were PCR-amplified and cloned into a pGL3-Basic vector (Promega). Using the IL-10 promoter construct (1.57 kb) and a QuickChangeII PCR-based site-directed mutagenesis kit (Stratagene), the Mut IL-10 promoter construct, containing a deletion at NF-κB binding site −583/−593 of IL-10 promoter, was generated. A Mut MDR1 promoter construct containing a deletion at AP-1 binding site −117/−123 was also generated using the MDR1 promoter construct (191 bp). All the inserts were confirmed by sequencing. RAW264.7 cells were transiently transfected with these IL-10 or MDR1 promoter constructs (2 μg) using Lipofectamine 2000 (Invitrogen), rested for 12 h, and either treated with rIL-10 or infected with Sb^RLD, Sb^SLD, or GalT knockdown Sb^RLD for 48 h. Luciferase activity in cell extracts was measured using the Dual Luciferase Reporter kit (Promega) according to the manufacturer's protocols, and the luciferase activity was normalized to the level of the protein content.

ChIP Analysis. Immunoprecipitation was performed using rabbit IgG or NF-κB Abs, such as αp50, αp65, and c-Rel, overnight at 4 °C, followed by DNA extraction. A PCR assay was performed to amplify the −482/−645 region of IL-10 promoter using primers 5′-GCCCCACAGCACACATATCC-3′ and 5′-CCTGGGTTGAACGTCCG-3′.

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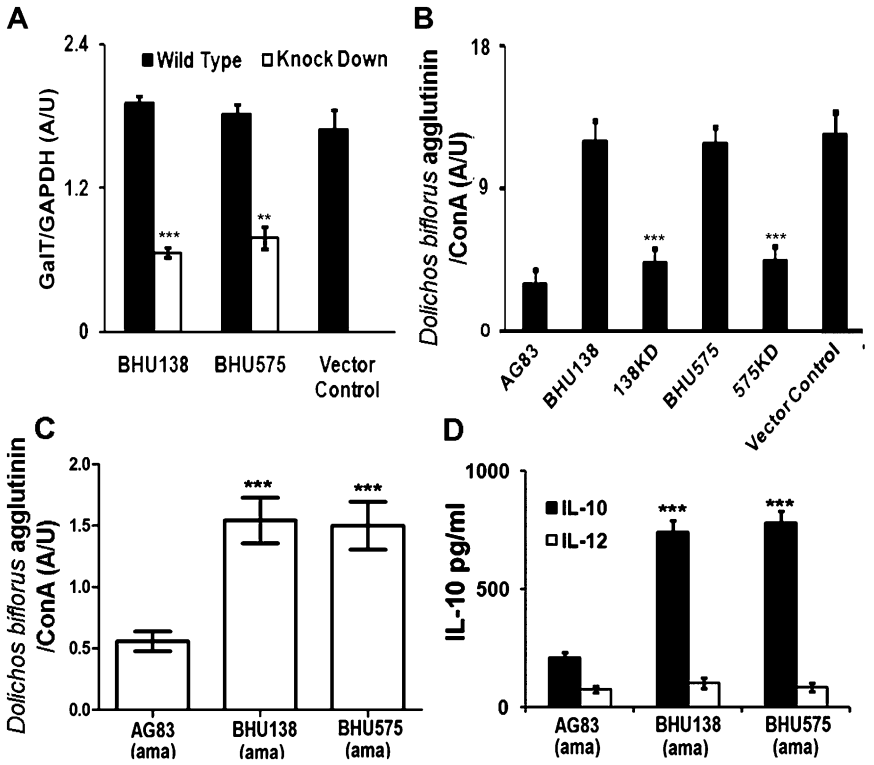

Fig. S1. GalT knockdown Sb^RLD generation and determination of the status of *N*-acetylgalactosamine in Sb^RLD amastigotes. (*A*) GalT mRNA expression was determined by RT-PCR with GAPDH as the internal control. Data are represented as the mean GalT-GAPDH ratio ± SD. (*B*) Flow cytometric determination of *N*-acetylgalactosamine residue by using FITC-linked *Dolichos biflorus* agglutinin, where mannose-specific FITC-linked Con A agglutinin was used as the internal control. Data are represented as the mean of mean fluorescence intensity of the *D. biflorus* agglutinin ($MFI_{Dolichos\ biflorus\ agglutinin}$)/$MFI_{Con\ A}$ ratio ± SD. (*C*) Flow cytometric determination of *N*-acetylgalactosamine residue in amastigotes of Sb^SLD (AG83) and Sb^RLD (BHU138/BHU575). Data are represented as the ratio of the MFI of FITC-linked *D. biflorus* agglutinin to FITC-linked mannose-specific Con A agglutinin, which was used as an internal control. (*D*) Quantification of IL-10 and IL-12 production from Mϕs infected with either amastigotes of Sb^SLD (AG83) or amastigotes of Sb^RLD (BHU138/BHU575) by ELISA. ***$P < 0.001$ (extremely significant). ama, amastigote; A/U, arbitrary unit.

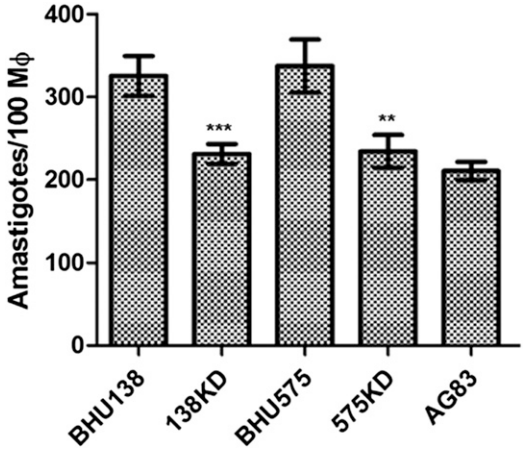

Fig. S2. Surface glycoconjugates affect infectivity of Sb^RLD at an early time point. The number of intracellular amastigotes per 100 Mϕs is shown in response to infection with either Sb^RLD (BHU138/BHU575) or the corresponding GalT knockdown (KD) Sb^RLD (138KD/575KD), or with Sb^SLD (AG83) 24 h postinfection, respectively. Results are presented as the mean ± SD and are representative of three independent experiments. **$P = 0.001–0.01$ (very significant); ***$P < 0.001$ (extremely significant).

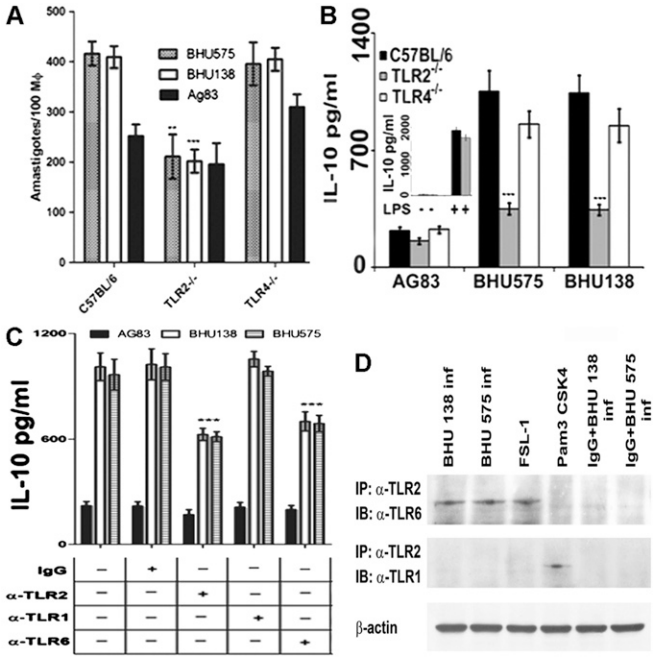

Fig. S3. $Sb^{R}LD$ exploits TLR2/TLR6 heterodimer to up-regulate host IL-10. (*A*) Number of intracellular amastigotes per 100 Mϕs of C57BL/6, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice on infection with either $Sb^{R}LD$ (BHU575/BHU138) or $Sb^{S}LD$ (AG83) 24 h postinfection, respectively. (*B*) Assay of IL-10 in the supernatant in response to infection of Mϕs of C57BL/6, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice with either $Sb^{S}LD$ (AG83) or $Sb^{R}LD$ (BHU575/BHU138) by ELISA. (*Inset*) IL-10 produced from Mϕs on stimulation with LPS treatment. (*C*) Assay of IL-10 in the supernatant in response to treatment with α-TLR2, α-TLR1, or α-TLR6 Ab before infection of Mϕs of BALB/c mice with either $Sb^{S}LD$ (AG83) or $Sb^{R}LD$ (BHU138/BHU575). (*D*) Lysates of Mϕs infected with $Sb^{R}LD$ or treated with FSL-1 or Pam3CSK4 were used for immunoprecipitation with an anti-TLR2 Ab, and the resulting immunoprecipitates (IP) were blotted with anti-TLR6 and anti-TLR1 Abs. Western blotting of total lysates using anti–β-actin Ab serves as a loading control, and whole-rabbit IgG is used as a negative control. IB, immunoblot; inf, infection. Results in *A*–*C* are presented as the mean ± SD. $^{**}P = 0.001$–0.01 (very significant); $^{***}P < 0.001$ (extremely significant).

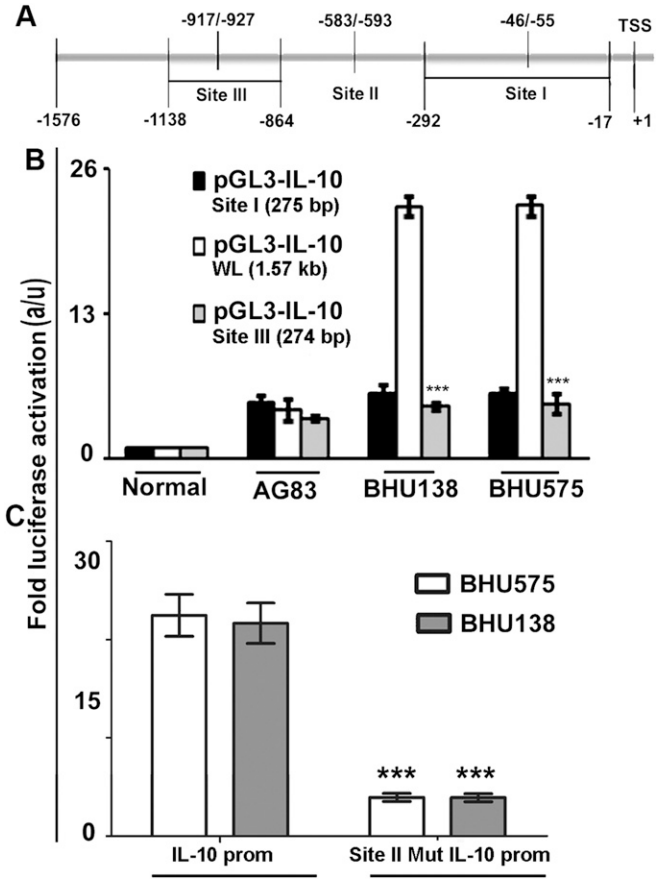

Fig. S4. Mapping of IL-10 promoter for Sb^RLD-driven IL-10 induction. (*A*) Schematic representation of the IL-10 promoter −17/−1,576 (1.57 kb), containing three potential NF-κB binding sites (−46/−55, −583/−593, and −917/−927 defined as sites I, II, and III, respectively). Region −17/−292 (275 bp) containing site I and region −864/−1,138 (274 bp) containing site III were individually cloned in pGL3-basic vector. TSS, transcription start site. (*B*) Comparison of luciferase activity of lysate of RAW264.7 cells transfected with either pGL3–IL-10 promoter construct (275 bp) containing site I or 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 (274 bp) containing site III. a/u, arbitrary unit. (*C*) RAW264.7 cells were transfected either with WT IL-10 promoter construct (prom) containing all three sites (1.57 kb) or with site II Mut IL-10 promoter construct (−583/−593 deleted) and then infected with Sb^RLD (BHU575/BHU138); luciferase activity was measured in cell lysate. Results in *B* and *C* are presented as the mean ± SD. ***$P < 0.001$ (extremely significant).

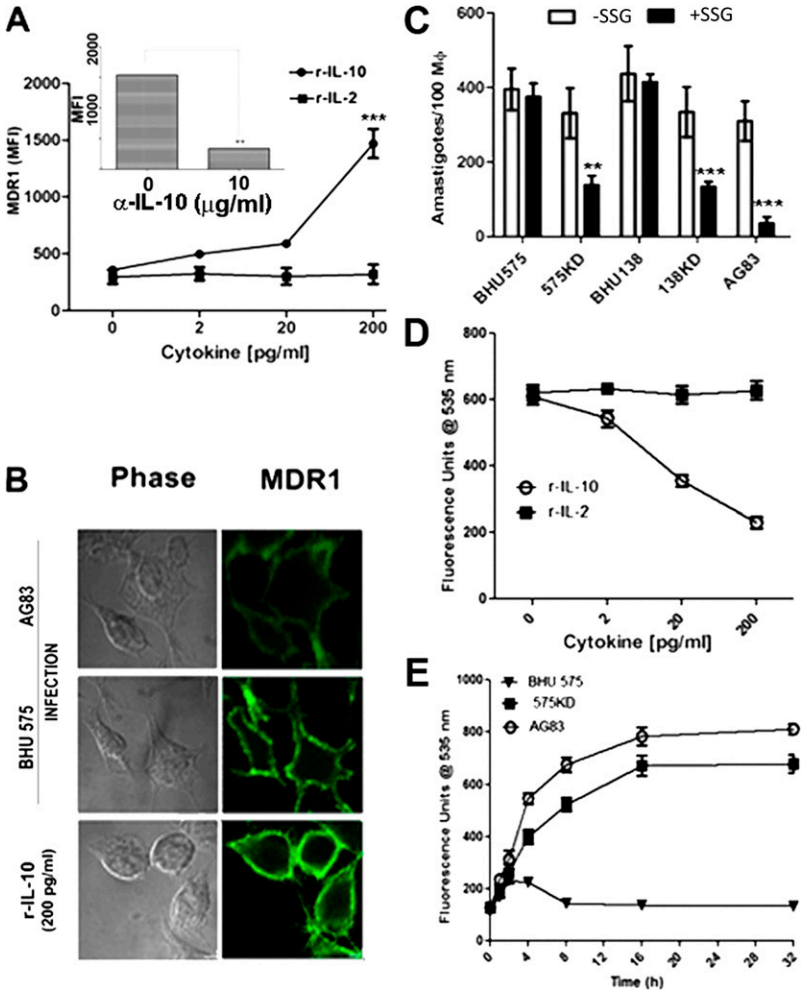

Fig. S5. IL-10–dependent MDR1 overexpression in Mϕs. (*A*) MDR1 up-regulation in terms of mean fluorescence intensity (MFI) in Mϕs of BALB/c origin as a function of rIL-10 (r-IL-10) and rIL-2 (r-IL-2) concentration. (*Inset*) Inhibition of IL-10–driven MDR1 up-regulation in Mϕs in the presence of Ab to IL-10. (*B*) Confocal images of Mϕs infected with $Sb^{S}LD$ (AG83) or $Sb^{R}LD$ (BHU575), or in the presence rIL-10. Panels (left to right) indicate the phase contrast images. (Magnification: 60×.) (*C*) Number of intracellular amastigotes per 100 Mϕs on infection with either or $Sb^{R}LD$ (BHU138/BHU575) or knockdown (KD) $Sb^{R}LD$ (575KD/138KD), or with $Sb^{S}LD$ (AG83) 48 h postinfection in the presence or absence of 60 μg/mL sodium stibogluconate (SSG). $^{**}P = 0.001$–0.01 (very significant); $^{***}P < 0.001$ (extremely significant). (*D*) Intracellular Rh123 retention in Mϕs was studied as a function of either rIL-10 or rIL-2 concentration using fluorescence spectroscopy at an emission wavelength (λ_{em}) of 535 nm. (*E*) Time kinetic study of intracellular Rh123 retention in Mϕs after infection with either $Sb^{R}LD$ (BHU575), KD $Sb^{R}LD$ (575KD), or $Sb^{S}LD$ (AG83). Results in *A* and *C*–*E* are presented as the mean ± SD, and results in *B* are representative of three independent experiments.

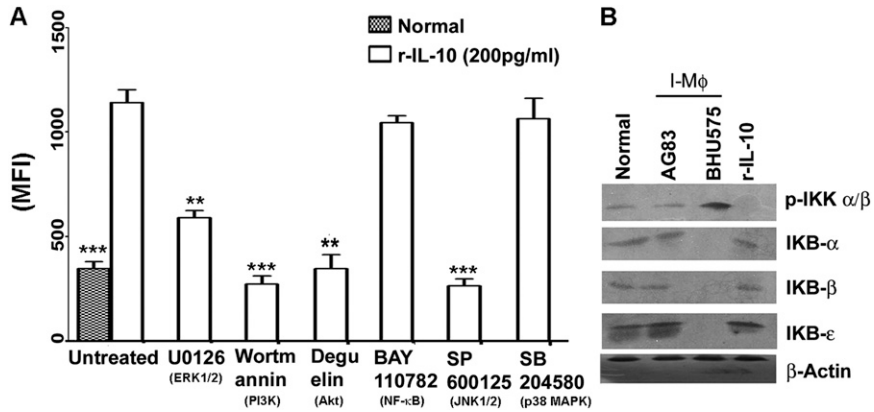

Fig. S6. MDR1 expression is independent of NF-κB. (*A*) MDR1 expression in terms of mean fluorescence intensity (MFI) in Mϕs on treatment with rIL-10 in the presence and absence of array pharmacological inhibitors (U0126, wortmannin, degulin, BAY110782, SP600125, and SB203580) by flow cytometry analysis and expressed as MFI values. (*B*) Western blot analysis of cytoplasmic extracts derived from Mϕs infected (I-Mϕ) with either Sb^5LD (AG83) or Sb^RLD (BHU575), or treated with rIL-10 (r-IL-10), with Abs specific to phospho IκB kinase (p-IKK) α/β and IκB (α, β, ε), where β-actin was used as an internal control. Results in *A* are presented as the mean ± SD, and results in *B* are representative of three independent experiments. $^{**}P = 0.001–0.01$ (very significant); $^{***}P < 0.001$ (extremely significant).