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RESEARCH PAPER

TLR9 and MyD88 are crucial for the maturation and activation of dendritic cells by paromomycin–miltefosine combination therapy in visceral leishmaniasis

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BACKGROUND AND PURPOSE

The combination of paromomycin–miltefosine is a successful anti-leishmanial therapy in visceral leishmaniasis (VL). This encouraged us to study its effect on Toll-like receptor (TLR)-mediated immunomodulation of dendritic cells (DC), as DC maturation and activation is crucial for anti-leishmanial activity.

EXPERIMENTAL APPROACH

In silico protein–ligand interaction and biophysical characterization of TLR9–drug interaction was performed. Interaction assays of HEK293 cells with different concentrations of miltefosine and/or paromomycin were performed, and NF-κB promoter activity measured. The role of TLR9 and MyD88 in paromomycin/miltefosine-induced maturation and activation of DCs was evaluated through RNA interference techniques. The effect of drugs on DCs was measured in terms of counter-regulatory production of IL-12 over IL-10, and characterized by chromatin immunoprecipitation assay at the molecular level.

KEY RESULTS

Computational and biophysical studies revealed that paromomycin/miltefosine interact with TLR9. Both drugs, as a monotherapy/combination, induced TLR9-dependent NF-κB promoter activity through MyD88. Moreover, the drug combination induced TLR9/MyD88-dependent functional maturation of DCs, evident as an up-regulation of co-stimulatory markers, enhanced antigen presentation by increasing MHC II expression, and increased stimulation of naive T-cells to produce IFN-γ. Both drugs, by modifying histone H3 at the promoter level, increased the release of IL-12, but down-regulated IL-10 in a TLR9-dependent manner.

CONCLUSIONS AND IMPLICATIONS

These results provide the first evidence that the combination of paromomycin–miltefosine critically modifies the maturation, activation and development of host DCs through a mechanism dependent on TLR9 and MyD88. This has implications for evaluating the success of other combination anti-leishmanial therapies that act by targeting host DCs.

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Abbreviations

CID, compound identity; DS, discovery studio; GM-CSF, granulocyte monocyte-colony stimulating factor; HEK, human embryonic kidney; LAL, limulus amebocyte lysate; MyD88, myeloid differentiation primary response gene 88; MyD88DN, MyD88 dominant negative; PDB ID, protein data base identity; pSV40, plasmid of simian virus 40

Introduction

Visceral leishmaniasis (VL/kala-azar), a common, neglected tropical disease, is prevalent in more than 80 countries including Asia, Africa, southern Europe and South America. *Leishmania donovani* is the main causative parasite for VL in the Indian subcontinent with nearly 0.5 million new VL cases per annum (Desjeux, 2004; Chappuis *et al.*, 2007).

For the last six decades, pentavalent antimonials (Sb^V) have been successfully used to treat kala-azar. However, resistance is now well established, especially in areas of endemicity in Bihar, India (Sundar *et al.*, 2000; Das *et al.*, 2005). The newer oral drug miltefosine is a potent anti-leishmanial drug with a longer half-life, a property likely to promote resistance (Prez-Victoria *et al.*, 2003). Paromomycin has undergone extensive clinical trials in Indian kala-azar patients. However, being an aminoglycoside, acquired resistance is likely to occur when used as a monotherapy. At present, despite several options, the treatment of VL is unsatisfactory and is, therefore, undergoing a transition in terms of the first line of choice. To encounter the problem of treatment failure in VL and to reduce the length of therapy, the combination of at least two effective anti-leishmanial agents is a desirable option. Both paromomycin and miltefosine are proven safe, efficient and affordable monotherapeutic options in India with a cure rate of over 90% (Sundar *et al.*, 2002; 2007). Furthermore, we have recently shown that a short-course combination of these drugs is the best treatment option for VL with a 98.7% success rate (Sundar *et al.*, 2011).

The development of innate and adaptive immunity critically depends on pattern recognition receptors, for example Toll-like receptors (TLRs; Medzhitov and Janeway, 1997). Furthermore, it has been suggested that drugs can also act as patterns to induce cell activation through TLRs, and in turn, activate signalling pathways that result in host resistance to the pathogen (Sau *et al.*, 2003; Bellocchio *et al.*, 2005; Razonable *et al.*, 2005). We have recently demonstrated that a paromomycin/miltefosine combination promotes TLR4-dependent induction of anti-leishmanial immune response *in vitro* (Das *et al.*, 2012) and previous findings have revealed that miltefosine has immunomodulatory properties (Wadhone *et al.*, 2009; Mukherjee *et al.*, 2012). However, the combined interaction of miltefosine/paromomycin with host molecules for effective DC maturation and activation is not well understood.

Immature dendritic cells (DCs) take up antigens efficiently, but express low levels of MHC and co-stimulatory molecules and thus their ability to stimulate T-cells is low (Banchereau and Steinman, 1998; Banchereau *et al*, 2000). Numerous studies have indicated that the recognition of specific patterns by TLRs induces DCs to produce IL-12 (Kaisho *et al.*, 2001). Recently it has been shown that the early NK cell response to infection with *L. donovani* is dependent on TLR9-mediated IL-12 production by myeloid DCs (Schleicher *et al.*, 2007). In the present study, we evaluated the interaction of TLR9 with the combination of paromomycin–miltefosine and its effect on the maturation and activation of DCs that shift the prevailing disease-promoting Th2 response to Th1-mediated protection in VL.

Methods

In silico *protein–ligand interaction*

For *in silico* TLR9–drug interaction studies, we first constructed the 3-D model of TLR9 based on the translated amino acid sequence consisting of 1032 amino acids (Template PDB ID: 3J0A_A) using DS software v2.5 (Discovery Studio 2.5 Accelyrs, San Diego, CA, USA) by a protein-threading/fold-recognition method as described in Bowie *et al.*, (1991). Furthermore, for model validation, we used the Ramachandran plot to evaluate the Φ/Ψ distribution of the backbone conformational angles for each residue of the 3-D structure. The structures of miltefosine (CID 3599) and paromomycin (CID 16580) were downloaded from PubChem as *.sdf file format. To investigate whether paromomycin and miltefosine interact with human TLR9, we performed computational protein–ligand interaction studies using DS software v2.5, which involved docking of the modelled structure of TLR9 protein with miltefosine and paromomycin, as a single or combined ligand.

Parasites and cell lines

The parasite used in the study was *L. donovani* (MHOM/IN/83/AG83). Axenic cultures of the promastigote stage of the parasite were maintained at 22°C as described previously (Das *et al.*, 2011). HEK293 cells stably transfected with TLR9 (293XL-hTLR9A), TLR4 (293-hTLR4A-MD2-CD14) and wild-type HEK cells (293-null) were purchased from InvivoGen (San Diego, CA, USA) and cultured according to the instructions provided.

Patients

Randomly selected, diagnostically confirmed VL patients (n = 10, age: 30.01 ± 5.66; M/F% 70/30) and non-endemic healthy controls (n = 10, age: 28.2 ± 3.11; M/F% 80/20) were enrolled in the study with informed consent as per standard guidelines. Bone marrow (BM) aspirates were drawn from VL patients only before treatment with informed consent and later microscopically analysed for parasite positivity. All clinical investigations were performed as per the Declaration of Helsinki. This study was approved by the institutional Ethics Committee of the Rajendra Memorial Research Institute of Medical Sciences, Patna, India. The patients were treated with a combination of paromomycin and miltefosine as described previously (Sundar *et al.*, 2011) and blood was drawn before and after the treatment.

BM DC culture

Parasitologically positive BM aspirates of VL patients were diluted with 1X PBS (PH 7.4) and layered on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) and BM cells were isolated using density-gradient centrifugation according to the manufacturers' instructions. BM cells were then plated at 1×10^6 cells·mL^{-1} in 24-well plates with 10% FBS-RPMI 1640 supplemented with IL-4 (500 U·mL^{-1}; BD Biosciences, San Jose, CA, USA) and GM-CSF (800 U·mL^{-1}; BD Biosciences) for 6 days. At day 6, loosely adherent cells were harvested by gentle pipetting, plated at 4×10^5 cells·mL^{-1} in 24-well plates, and cultured with or without a combination of paromomycin/miltefosine (30 and 3 μM, respectively; these concentrations had no significant effect on cell viability and therefore were chosen as described earlier Das *et al.*, 2012), 100 ng·mL^{-1} LPS or 0.1 mM CpG 2006 in a fresh medium for 2 more days as described later. The expanded DC culture at day 6 contained 78–82% CD11c+CD11b+ cells.

Culture of monocyte-derived DCs

Peripheral blood mononuclear cells were isolated from heparin-treated venous blood of healthy volunteers (n = 10) or VL patients (n = 10) using density-gradient centrifugation over Ficoll-Paque (Amersham Biosciences). Adherent monocytes were cultured in RPMI 1640 supplemented with 10% FBS and antibiotic-antimycotic (Invitrogen Life Technologies) in the presence of IL-4 (500 U·mL^{-1}; BD Biosciences) and GM-CSF (800 U·mL^{-1}; BD Biosciences) for 6 days. Fresh culture medium with the same supplements was added at day 3 and DCs were harvested at day 6. The DCs were resuspended in fresh cytokine-containing culture medium and transferred to culture plates at a concentration of 0.5×10^6 cells·mL^{-1} and stimulated as described in the subsequent section. The expanded DC culture at day 6 contained 80–84% CD11c+CD11b+ cells.

Infection and treatment of monocyte-derived DCs and cell lines with drugs

L. donovani amastigotes harvested from the spleen of infected male golden hamsters (6 weeks of age) were used to infect human monocyte-derived DCs at an amastigote : DC ratio of 10:1 on chambered slides (Nunc, Roskilde, Denmark) for the indicated time periods as described previously (Das *et al.*, 2012). After incubating the cultures overnight at 37°C and 5% CO_2 in RPMI 1640 medium plus 10% heat-inactivated autologous serum, non-ingested parasites were washed off and drug dilutions added. The infected DCs were stimulated with paromomycin/miltefosine combination for 48 h. CpG2006 stimulation (0.1 mM) was used as a positive control for inducing DC maturation. To correct for potential endotoxin contamination, 10 μg·mL^{-1} polymyxin B (PMB) was added or the endotoxin level was measured by LAL colorometric test. After 48 h of culture at 37°C in the presence of 5% CO_2, culture supernatants were collected. Supernatants were stored at -20°C until the levels of the cytokines were measured.

Drug–cell interaction assay

Stock solutions (10 mM each) of anti-leishmanial drugs miltefosine and paromomycin sulphate (Sigma–Aldrich, St. Louis, MO, USA) were freshly prepared in de-ionized water and later filter sterilized. All subsequent dilutions were prepared in RPMI 1640 with 10% heat-inactivated autologous serum on the day of the assay. To correct for potential endotoxin contamination, 10 μg·mL^{-1} PMB was added in some (as indicated) experiments or the endotoxin level was measured by the LAL colorometric test. Infected DCs were incubated with drug dilutions for a total of 5 days at 37°C and 5% CO_2; the medium was changed once after 48 h. Untreated DCs received medium plus 10% heat-inactivated (56°C for 30 min) FBS (Sigma-Aldrich) and infection was determined on day 5 post-infection. The 5 day post-infection points also served as untreated controls. In some experiments, cells were treated with the human TLR9 inhibitor oligodeoxynucleotide (ODN) (Invitrogen Life Technologies, Carlsbad, CA, USA) before the drug treatment or pre-transfected with small interfering RNA for TLR9 (TLR9-siRNA) as described below. The effects of the drugs on cell viability were tested by use of the trypan blue dye-exclusion method.

ELISA

An interaction ELISA assay was performed to investigate the biophysical interaction between TLR9 and paromomycin and/or miltefosine, as described previously (Wang *et al.*, 2012) with some modifications. Drugs with similar structures, viz. neomycin/framycetin for paromomycin (CID 8378; Sigma-Aldrich) and ilmofosine for miltefosine (CID 55008; Sigma-Aldrich), were used as controls. The drugs were dissolved in 70% ethanol as 10 mM stock solutions. The 96-well ELISA microplate (BD Biosciences) was coated separately with indicated dilutions (50μl per well) of the drugs prepared from stock solutions in sterile de-ionized water as described previously. The plates were then dried overnight. The wells were washed 3 times with PBST buffer [PBS (PH 7.4) with 0.05% Tween-20] and then blocked with 5% BSA solution at room temperature for 1 h. After three times washing with PBST, 10 μg·mL^{-1} of human TLR9 recombinant partial protein (Abnova, Taipei, Taiwan) was added and incubated for 1 h at RT. After washing with PBST five times, the indicated concentration of biotin-TLR9 MAb (Imgenex, San Diego, CA, USA) was added and incubated at room temperature for 1 h. After 5 washings, streptavidin-coupled HRP conjugate (Thermo Pierce, Rockford, IL, USA) was diluted at the ratio of 1:2000 and added into the wells and incubated at RT for 1 h. After a further 7 washings, 100 μL of TMB substrate reagents (Sigma-Aldrich) were added per well and incubated at RT for 10–30 min; 50 μL of 1 M H_3PO_4 was subsequently added to each well to stop the colour reaction and the absorbance at 450 nm was measured on a Beckman-Coulter DTX microplate reader (Beckman Coulter, Fullerton, CA, USA). In addition, human IFN-γ, IL-12 and IL-10 were measured in culture supernatants using BD OptEIA kits (BD Biosciences) according to the manufacturer's instructions.

Transient transfection of cells and measurement of luciferase activity

Transient transfection of HEK293 wild-type (293-null), HEK293-TLR4 (293-hTLR4A-MD2-CD14) and HEK293-TLR9 (293XL-hTLR9A) with plasmids containing NF-κB reporter luciferase and MyD88DN (gifts from Dr Fabio Re, University of Tennessee, USA and Professor Jürg Tschopp, University

of Lausanne, Switzerland) was performed using FuGENE6 (Roche, Basel, Switzerland) transfection reagent following the manufacturer's instructions. Briefly, cells were incubated with plasmids containing 1 μg of NF-κB reporter luciferase plasmid and, to correct for differences in transfection efficiency, each group of cells was transfected with 80 ng of pSV40/LACZ and co-transfected with 3 mg of MyD88DN (in indicated experiments) or 3 μg of empty vector and incubated overnight. The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity. The cell extracts were prepared for determination of luciferase activity on a luminometer by enhanced luciferase assay reagents (Analytical Luminescence Laboratory, San Diego, CA, USA) according to the manufacturer's instructions.

Phenotypical analysis of DCs

The phenotypical analysis of DCs was performed using standard flowcytometry protocols. Briefly, 10^5 DCs were incubated with mAbs (BD Biosciences) against human TLR9, CD14, CD80, CD86 and MHC II for 30 min at 4°C. The cells for intracellular TLR9 staining were pretreated with Permfix solution (BD Biosciences) for 10 min at 4°C for permeabilization and fixing purposes followed by a wash with Perm-wash solution (BD Biosciences) before addition of TLR9 antibodies. Cells were then washed and incubated with FITC-conjugated goat anti-mouse IgG (Jacksons Immunochemicals, West Grove, PA, USA) for 30 min at 4°C in complete darkness. Subsequently, cells were washed and analysed in FACSAriaII (BD Biosciences) for the proportion of positive cells relative to cells stained with relevant IgG isotypes. Cells were gated according to their forward- and side-scattering patterns. For each marker, 10^4 cells were counted in the gate.

Mixed leukocyte reaction (MLR) assay

Unstimulated or stimulated Bone-marrow-derived dendritic cells of VL patients were harvested at day 8, washed, irradiated at a dose of 30 Gy, and plated at twofold serial dilutions in 96-well round-bottom plates (Nunc). These stimulated DCs were incubated for 3 days with 5×10^4 per well of CD4+ T-cells, which were isolated using magnet-associated cell sorter (MACS) with CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from peripheral blood of the same individual (prepared and stored frozen before treatment of the patient). The resulting population of CD4+ T-cells after MACS was >95% CD4+, as determined by flow cytometry. Finally, [^{3}H]-thymidine was added to the cultures during the last 18 h and the incorporation was assessed in a scintillation counter. The supernatant of the coculture was collected and stored at -20°C for IFN-γ estimation by ELISA.

Real-time PCR

Total RNA was isolated from 2×10^6 cells of differentially stimulated DCs using RNAqueous kit (AmBion, Austin, TX, USA) according to the manufacturer's protocol. The extracted RNA was treated with 2 U of Rnase-free DNase (AmBion) followed by the deactivation of DNase. Using the platinum quantitative reverse transcription-PCR Thermoscript One-Step System kit (Invitrogen Life Technologies), 1 μg of RNA was converted into complementary DNA by reverse transcription-PCR. To determine the transcription levels of IL-12 and IL-10 in DCs, real-time PCR was performed using a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen Life Technologies), as per the manufacturer's instructions, in an Applied Biosystems Prism 7000 sequence detection system. The resultant products were normalized to the expression of GAPDH. All analyses were performed in triplicate from at least three independent cell stimulation experiments. The primers (sequences of human origin obtained from the GenBank/EMBL database), designed by Primer 3 software, are GAPDH, 5′-GTCGCTGTTGAAGTCAGAGG-3′ and 5′-GAAACTGTGGCGTGATGG-3′; IL-12 5′-CCAAGAACTTGCAGCTGAAG-3′ and 5′-TGGGTCTATTCCGTTGTGTCT-3′ and IL-10 5′-TGAGAACCAAGACCCAGACA-3′ and 5′-TCATGGCTTTGTAGATGCCT-3′.

RNA-mediated interference

Preconfirmed TLR4- and TLR9-specific small interfering RNA and non-targeting control small interfering RNA was from Dharmacon (Chicago, IL, USA). These were transfected into human cultured DCs with SMARTpool siGENOME siRNA buffer and DharmaFECT transfection reagents according to the manufacturer's protocol. The media was then replaced with fresh antibiotic-free complete RPMI-1640 containing 10% human AB serum (heat inactivated), treated with indicated stimuli for a desired period of time as described previously (Das *et al.*, 2012).

Chromatin immunoprecipitation (CHIP) assay

CHIP assays were conducted using the CHIP Assay kit following the manufacturer's protocol (Millipore, Bedford, MA, USA). Briefly, 2×10^6 BM-derived DCs in six-well plates were treated as indicated in figure legends. Cells were then fixed for 15 min at 37°C in 1% paraformaldehyde and washed on ice with ice-cold 1X PBS containing 1 mM PMSF. The cells were then harvested and lysed in SDS lysis buffer. The DNA was sheared by ultrasonication for 3600 s pulses at 20% amplitude. Thereafter, the lysates were cleared by centrifugation and diluted in CHIP dilution buffer, followed by preclearance using salmon sperm DNA/protein A-agarose. The ' "input DNA" ' was collected and protein–DNA complexes were immunoprecipitated with 5 mg of either antibodies [anti-phospho-H3 and anti-acetyl-H3 Abs (Abcam)] overnight at 4°C. Finally, the Ab–protein–DNA complexes were captured using salmon sperm DNA/protein A-agarose for 1 h at 4°C. After washing beads alternatively with LiCl, and Tris-EDTA buffers, the protein/DNA complexes were eluted using 1% SDS, 0.1 M $NaHCO_3$ buffer and heated at 65°C for 4 h. DNA was then obtained using phenol/chloroform extraction and ethanol precipitation. PCR was conducted using promoter specific primers as described previously (Bhattacharya *et al.*, 2011): IL-10 promoter (STAT3 binding region): 5′-TCATGCTGGGATCTGAGCTTCT-3′ and 5′-CGGAAGTCACCTTAGCACTCAGT-3′ (94°C, 15 s; 56°C, 30 s; 72°C, 1 min, 35 cycles); IL-12p40 promoter (NF-κB binding site): 5′-AGTATCTCTGCCTCCTTCCTT-3′ and 5′-GCAACACTGAAAACTAGTGTC-3′ (initial denaturation at 95°C for 3 min; amplification cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min, 40 cycles). PCR-amplified product was subsequently electro-

phoresed in 2% agarose gel and visualized under UV light following staining with ethidium bromide. Real-time PCR was also performed for relative quantification of promoter levels and samples were normalized to input DNA controls.

Statistical analysis

All experiments were conducted at least in triplicate, and the results are expressed as mean ± SEM. The Mann–Whitney *U*-test was used to determine statistical significance (GraphPad Prism software v5.0; GraphPad Software Inc., La Jolla, CA, USA).

Results

Paromomycin and miltefosine interact with TLR9

Recently, we have shown that the combination of paromomycin and miltefosine trigger innate immune responses by stimulating the production of TLR4-dependent TNF-α and NO (Das *et al*., 2012). However, the role of TLR9 in anti-leishmanial therapy is undisputed. Therefore to probe TLR9-drug interaction studies with paromomycin and miltefosine, we first constructed the 3-D model of TLR9 based on the translated amino acid sequence consisting of 1032 amino acids (Template PDB ID: 3J0A_A) by protein-threading method. For model validation, Ramachandran plot (the Φ/Ψ distribution of the backbone conformational angles for each residue of the 3-D structure) revealed that 74.6% residues are in the core region, 18.9% residues are in allowed region, 3.5% residues lie in generously allowed region and 3.0% residue in disallowed region, followed by loop refinement and side chain refinement protocol of DS software v2.5 program.

To investigate whether paromomycin and miltefosine interact with human TLR9, the computational protein–ligand interaction studies using DS software v2.5 involved docking of modelled structure of TLR9 protein with miltefosine and paromomycin; as single or combined ligand. The parameters used for selection of 3-D models of TLR9 and their interaction with anti-leishmanial ligands are summarized in Table 1. The global energy function/dock score of TLR9 with paromomycin is the highest interaction energy shown to be is –61.04 relative units, this value is considered to be related to free binding energy and higher negative value means higher free binding energy and thus higher interaction probability (Figure 1A, Table 1). Interestingly, hydrophobic and polar intermolecular interactions are present between the C-terminal TIR domain and paromomycin, the residues involved in the formation of H-bonds with paromomycin are Asp^{864}, Asp^{871}, Arg^{931}, Asn^{1017}, His^{1019} (Figure 1C). The different atoms of paromomycin-TLR9 involved in the formation of the H-bond are O12-OD2 (Asp^{864}) O5-OD1 (Asp^{871}), O12-NH1 (Arg^{931}), NH18-O (Asn^{1017}) and O11-NE2 (His^{1019}) with distances 2.4, 3.4, 3.4, 3.6 and 2.7 in Å respectively. Further, the interaction of functional C-terminal TIR domain of TLR9 with miltefosine revealed a dock score of –48.74 and only one amino acid is involved in the formation of one H-bond with atom O1 of Ala^{872} and O4 atom of miltefosine and their H-bond distance 3.2 Å (Figure 1B, Table 1).

The tertiary structures of TLR9 exhibited successful binding with paromomycin/miltefosine into the central cavity of the TIR domain of TLR9, exhibiting –59.83 relative units (Figure 1C). It is important to note that, miltefosine, in different conformations, demonstrated the formation of 10 H-bonds with TLR9 and Arg^{931} was found to be the amino acid frequently involved. The atoms O4 and O5 of miltefosine are involved in the formation of the H-bond with atom NH1 of Arg^{931} and their distances are 2.7 Å and 3.4 Å respectively (Figure 1C). Simultaneously, paromomycin exhibited five H-bonds with TLR9 and the residues involved are Ser^{861}, Leu^{868}, Tyr^{870}, Asp^{871}, Ala^{903}, Gly^{1010} and Thr^{1014}. The bond

Table 1

Interaction of TLR9 with paromomycin and miltefosine

Protein	Ligands	Global energy	Interacting amino acid residues	No of H-bond	aVdW	rVdW	ACE	Inside	Remarks
TLR9	Miltefosine (MF)	–48.74	Ala^{872}	1	–20.36	4.99	–14.15	0.62	One H-bond, which differs from combination docking. Binding energy is –48.74, shows the better interaction.
TLR9	Paramomycin (PM)	–61.04	Asp^{864}, **Asp^{871}**, Arg^{931}, Asn^{1017}, His^{1019}	5	–27.01	10.30	–18.57	1.37	One residue Asp^{871} involved shown in bold is also present in combination docking and shows better interaction.
TLR9	Miltefosine Paromomycin (MF + PM)	–59.83	**MF-(2):** Arg^{931} **PM- (8),** Ser^{861}(2), Leu^{868}, Tyr^{870}, **Asp^{871}**, Ala^{903}, Gly^{1010}, Thr^{1014}	10	–32.41	4.84	–10.84	3.22	Four extra H-bonds are present, residue (in bold) is also present in single docking, which shows interacting residues belong to close proximity with paramomycin.

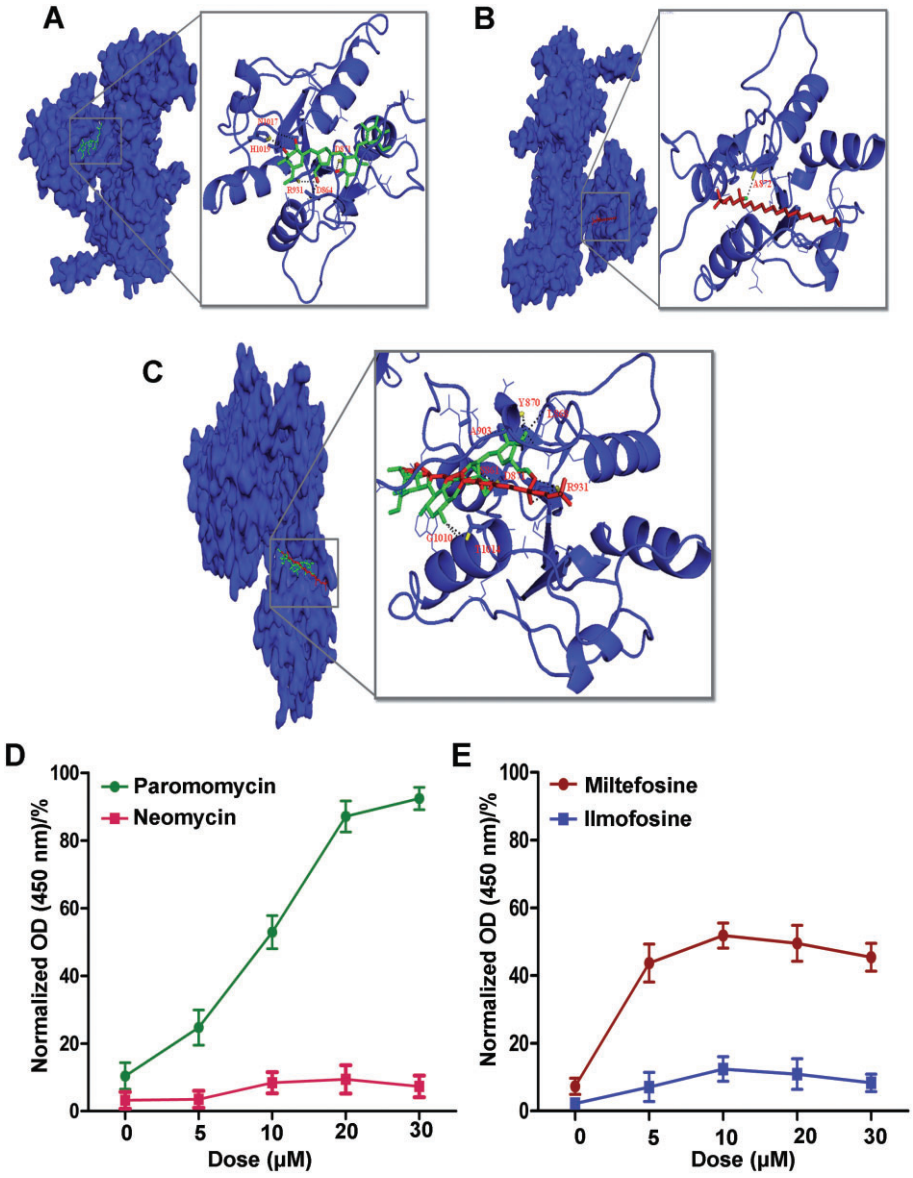

Figure 1

Interaction of TLR9 with paromomycin and miltefosine. (A) Cartoon diagram showing interactions of TLR9 with drugs; TLR9 interacts with anti-leishmanial drugs, paromomycin (A), miltefosine (B) or both drugs in combination (C). The surface representation of TLR9 receptor is shown in blue, paromomycin in green and miltefosine in red respectively. The interacting residues in the inhibitory residual interface region are further enlarged in the box. Side chains of the amino acids contributing to hydrogen bond formation are represented as a stick model with the residue names and numbers shown next to them. TLR9 side chains stick model atom, which interacts are shown in yellow and atoms are in green. Black dotted lines represent the hydrogen bonds. These pictures were generated by PyMol v0.99. (D,E) Biophysical characterization of paromomycin and miltefosine binding with human TLR9. Different concentrations of paromomycin/neomycin or miltefosine/ilmofosine were coated on ELISA plates. Human recombinant TLR9 partial protein (10μg mL^{-1}) was added, and the drug-bound TLR9 was detected by adding biotin-TLR9 monoclonal antibody followed by streptavidin-HRP conjugate substrate. Absorbance with 30 μM paromomycin (in D) and 3 μM miltefosine (in E) was set as 100%.

distances are O13-OG (Ser^{861}) 2.8 Å, O11-OG (Ser^{861}) 3.2 Å, O11-O (Leu^{868}) 3.4 Å, O8-OD2 (Asp^{871}) 2.7 Å, N18-O (Ala^{903}) 2.8 Å, O11-O (Arg^{905}) 2.7 Å, N16-O (Gly^{1010}) 3.3 Å and N16-OG1 (Thr^{1014}) 3.2 Å respectively (Figure 1C). One unique residue Asp^{871} of TLR9 (Table 1) was found to be actively involved in most of the interactions, that is in single ligand mode (paromomycin alone) and combined ligand mode (both miltefosine and paromomycin). Interestingly, it was noted that the combination of the drugs interacted better with TLR9 than when paromomycin or miltefosine was used as a monotherapy in respect of H bonding (Table 1).

The computational data demonstrated the binding of TLR9 to paromomycin/miltefosine. In addition, to test whether paromomycin/miltefosine may biophysically bind

to human TLR9, we performed an interaction-based ELISA (Wang *et al.*, 2012). Our findings suggested that both paromomycin and miltefosine bound to TLR9 in a concentration-dependent manner (Figure 1D,E). Interestingly, it was revealed that TLR9 has more affinity for binding paromomycin compared with miltefosine (Figure 1D,E). By contrast, binding of the drugs to BSA (negative control) instead of TLR9 was negligible, indicating the specificity of TLR9-paromomycin/miltefosine binding (not shown). Additionally, neomycin and ilmofosine demonstrated very low affinity for TLR9 (Figure 1D,E). Interestingly, both neomycin and ilmofosine showed a smaller dock score compared with paromomycin and miltefosine with TLR9 (S. Das *et al.*, unpubl. obs.).

Paromomycin/miltefosine combination stimulates NF-κB promoter activity via TLR9

Previously, we demonstrated that the paromomycin/miltefosine combination stimulates NF-κB promoter activity via TLR4 (Das *et al.*, 2012). Therefore, firstly, we investigated the effect of the drugs on the intracellular expression of TLR9 in control human DCs. Quantitative flowcytometric data revealed that paromomycin and miltefosine, either as a monotreatment or combined, considerably increased TLR9 expression in a concentration-dependent manner in DCs from healthy controls (Figure 2A). Further, to explore whether paromomycin and miltefosine functionally interact with TLR9 expressed intracellularly, we employed an interaction assay with wild-type HEK293 cells (293-null) and HEK293 cells engineered to express eitherTLR9 (293-hTLR9) or TLR4 (293-hTLR4A- MD2-CD14) with the drugs. Firstly, HEK cells (293-null and 293-TLR9) were treated with paromomycin and miltefosine, alone or combined, and then NF-κB promoter activity was measured using a κB-luciferase reporter construct after 12 h incubation at 37°C. Our results showed that monostimulation [paromomycin (30 μM, 21.41 $mg{\cdot}L^{-1}$; $P = 0.0023$), miltefosine (5 μM, 2.03 $mg{\cdot}L^{-1}$; $P = 0.0012$) and combined stimulation (paromomycin 30 μM, 21.41 $mg{\cdot}L^{-1}$; miltefosine 3 μM, 1.22 $mg{\cdot}L^{-1}$; $P = 0.0326$)] significantly increased NF-κB promoter activity in 293-TLR9 cells in a dose-dependent manner (Figure 2B,C). No significant amounts of TLR9-dependent NF-κB activity were detected with neomycin or ilmofosine stimulation of the cells (Figure 2B). No detectable luciferase activity was observed in null-293 cells with the combination drug treatments (Figure 2C). Endotoxin-free ovalbumin protein was used as a negative control and did not up-regulate NF-κB promoter activity significantly above background levels. Interestingly, paromomycin induced more TLR9-dependent NF-κB promoter activity than miltefosine, as a monotreatment on 293-TLR9 cells (Figure 2B). However, induction of TLR9-dependent NF-κB promoter activity was considerably less with the monotreatment of either paromomycin or miltefosine in 293-TLR9 cells, compared with their combined effect (Figure 2B,C). Importantly, it was noted that the TLR4/9-mediated NF-κB promoter activity was MyD88-dependent as no drug-induced luciferase activity was detected in 293-TLR9 or 293-TLR4 cells co-transfected with MyD88DN plasmid (a construct encoding a dominant negative MyD88 with a deletion of its death domain to evaluate roles of MyD88; Figure 2D).

Paromomycin/miltefosine combination-induced DC maturation is dependent on TLR9 and MyD88 in VL patients

To determine the mechanism of DC maturation in VL patients treated with the paromomycin/miltefosine combination, we first examined *ex vivo* phenotypic maturation of cultured DCs in response to the drugs. For this, we generated BM-derived DCs from VL patients ($n = 10$) and introduced MyD88DN construct or RNA interference for TLR9 or TLR4 in indicated cases (see methods/figure legends). Next, we stimulated the DCs with the combination of paromomycin/miltefosine (30:3) for 24 h, followed by flowcytometric assessment of surface expression of CD40, a hallmark of DC maturation. The results demonstrated that the drug combination could successfully up-regulate CD40 surface expression in wild-type DCs, DCs with control siRNA and DCs with TLR4 siRNA (Figure 3A). However, there was no difference in CD40 expression between unstimulated DCs and drug-stimulated DCs with TLR9 siRNA or DCs with MyD88DN plasmid (Figure 3A).

With specialized in the initiation of an adaptive T-cell response (Moser and Murphy, 2000). Firstly, the influence of paromomycin/miltefosine combination-treated or untreated VL-DCs (DCs derived from VL patients) on *in vitro* allogeneic T-cell priming was examined. For that, VL-DCs with control siRNA or TLR9 siRNA or TLR4 siRNA or MyD88DN plasmid were stimulated with or without paromomycin/miltefosine combination (30:3), irradiated and cocultured with allogeneic CD4+ T-cells derived from the same patient. Interestingly, wild-type VL-DCs or VL-DCs with control siRNA or TLR4 siRNA demonstrated an enhanced ability to stimulate allogeneic T-cells in response to paromomycin/miltefosine combination (Figure 3B). However, the paromomycin/miltefosine combination did not augment the ability of VL-DCs with TLR9 siRNA or VL-DCs with MyD88DN plasmid to stimulate allogeneic T-cells from the same patient (Figure 3B). Notably, control CpG2006 treatment was found to trigger the ability of DCs with control siRNA or TLR4 siRNA to stimulate allogeneic T-cells, but not the VL-DCs with TLR9 siRNA or MyD88DN plasmid (Figure 3B). Simultaneously, LPS induced VL-DCs with control siRNA or TLR9 siRNA or MyD88DN plasmid to stimulate allogeneic T-cells, but not those DCs with TLR4 siRNA (Figure 3B).

We further investigated the allostimulatory capacity of VL-DCs induced by the paromomycin/miltefosine combination, by assessing the production of IFN-γ by T-cells in MLR. Surprisingly, our findings demonstrated that T-cells primed with the paromomycin/miltefosine-stimulated VL-DCs, with control siRNA or TLR4 siRNA, produced significantly more IFN-γ than T-cells primed with untreated VL-DCs or drug combination treated VL-DCs with TLR9 siRNA or VL-DCs with MyD88DN plasmid (Figure 3C). Notably, control CpG2006 treatment was found to trigger the ability of DCs with control siRNA or TLR4 siRNA, but not of VL-DCs with TLR9 siRNA or MyD88DN plasmid, to stimulate allogeneic T-cells to produce IFN-γ (Figure 3C). Similarly, LPS evoked VL-DCs with control siRNA or TLR9 siRNA or MyD88DN plasmid, but not DCs with TLR4 siRNA, to stimulate allogeneic T-cells to produce IFN-γ (Figure 3C).

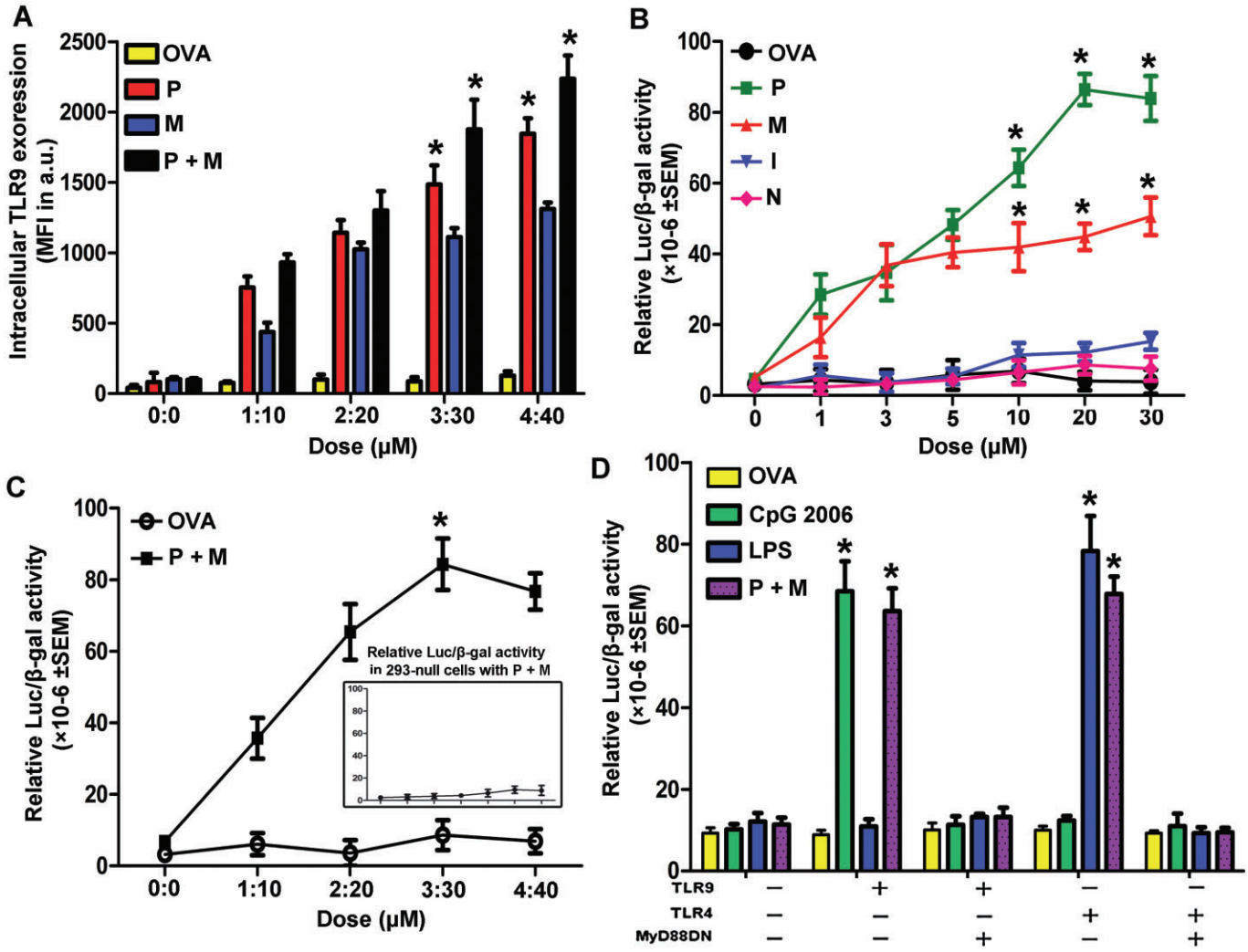

Figure 2

Paromomycin/miltefosine combination interacts with TLR9 *in vitro* and activates NF-κB promoter activity via TLR9. (A) Kinetics of changes in intracellular TLR9 expression in cultured DCs of control healthy subjects ($n = 10$) with stimulations by various concentrations (μM) of paromomycin or miltefosine (alone or combined) or equimolar doses of OVA protein (control) for 12 h at 37°C. Here, 1:10 for example means 1 μM of miltefosine and 10 μM of paromomycin used single or in combination, and so on. Results denote differential modulation of TLR9 expressions with respect to stimulations; quantitative flowcytometric data are expressed as mean fluorescence intensity (MFI; arbitrary units). Asterisks denote $P <$ 0.05(Mann Whitney U test). (B,C) Dose–response of paromomycin and miltefosine [alone (B) or combined (C)] for NF-κB promoter activity. HEK293-TLR9 cells were transfected with NF-κB reporter luciferase plasmid or empty vector (see Methods section). Cells were pre-incubated with or without PMB (10 μg·mL^{-1}) and then stimulated with different doses of paromomycin or miltefosine (1–30 μM; alone or combinational ratios) or 0.1 μg·mL^{-1} OVA and incubated for 12 h. Then cell extracts were prepared for determination of luciferase activity. Results represent the ratio of luciferase (luc) to β-galactosidase (β-gal) and are the mean ± SEM. of three experiments performed in triplicate. Asterisks denote $P < 0.05$ versus protein control, OVA (Mann–Whitney *U*-test). (D) MyD88 is essential for TLR9-mediated induction of NF-κB promoter activity. 293-TLR9 or HEK293-TLR4 cells were transfected with NF-κB reporter luciferase plasmid or with pSV40/LACZ plasmid and co-transfected with MyD88DN or empty vector (see Methods section). Cells stimulated with miltefosine (M) and paromomycin (P) combination (3 and 30 μM respectively), 0.1 mM CpG 2006, 0.01 μg·mL^{-1} LPS or 0.1 μg·mL^{-1} OVA and incubated for 12 h, after which extracts were prepared for determination of luciferase activity. Results represent the ratio of luciferase (Luc) to b-galactosidase (β-gal) and are the mean ± SEM of three independent experiments performed in triplicate. An asterisk denotes $P < 0.05$ versus protein control, OVA (Mann–Whitney *U*-test).

Maturation of parasitized control DCs by combination of paromomycin/miltefosine is TLR9 dependent

Our results suggested TLR9 and MyD88 are critical factors in paromomycin/miltefosine-mediated DC maturation in VL patients. To investigate the capacity of paromomycin/miltefosine to induce maturation of DCs *in vitro*, the expression of CD40, CD80 and CD86 was measured by flow cytometry on monocyte-derived DCs of healthy controls with/without RNA interference of TLR9. The results illustrate paromomycin/miltefosine induced an up-regulation in the expression of the co-stimulatory molecules CD40, CD80 and

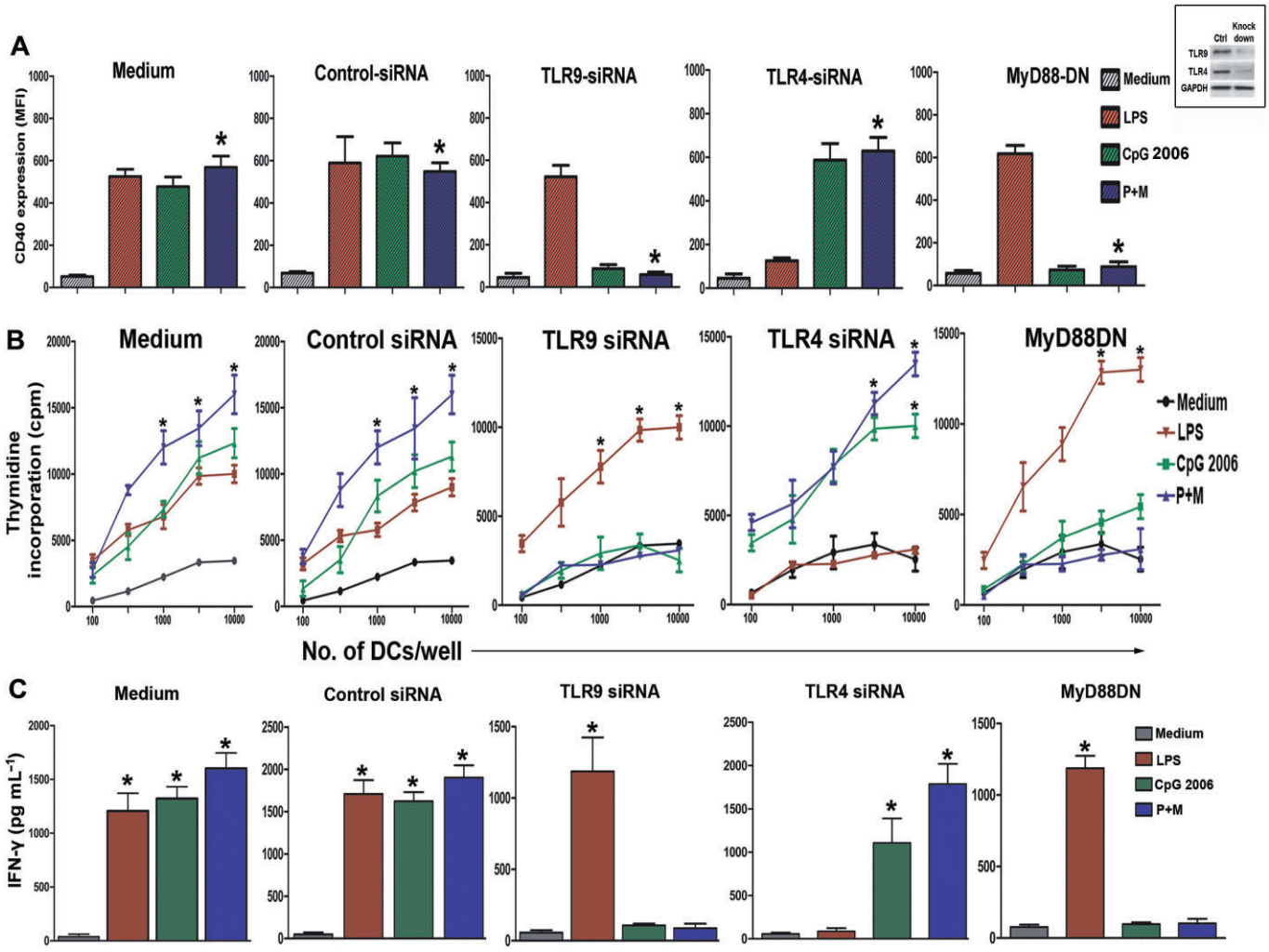

Figure 3

TLR9 and MyD88 are required for optimal stimulation of CD4+ T-cells by paromomycin/miltefosine-stimulated DCs in VL patients. Analysis of paromomycin/miltefosine-stimulated and unstimulated BMDCs from VL patients ($n = 10$). At day 6 of the BMDC culture, cells were pretransfected with/without control siRNA or TLR9 siRNA or TLR4 siRNA or MyD88DN plasmid and later cultured with or without the combination of paromomycin/miltefosine (30 and 3 μM respectively) or 0.01 μg·mL^{-1} LPS or 0.1 mM CpG 2006 in a fresh medium for 2 more days as described in Methods section. (A) Flow cytometric analysis of paromomycin/miltefosine-stimulated and unstimulated BMDCs from VL patients for CD40 surface expression. Then DCs were transfected for RNA interference, stimulated as above, stained with PE-conjugated CD40 mAb and analysed with FACS. The Western blot gel demonstrates total protein levels of TLR9, TLR4 and MyD88 in control and knock-out sets. (B) Allostimulatory activity of BM DCs from VL patients. To measure the allostimulatory activity of BMDCs, differentially transfected and treated DCs (as above) were irradiated and incubated with allogeneic CD4+ T-cells of the same patient. T-cell responses were evaluated by [^{3}H]-thymidine incorporation of CD4+ T-cells. The data indicate mean ± SEM of triplicate samples of one representative experiment. (C) IFN-γ production from restimulated T-cells was measured by ELISA and expressed as pg·mL^{-1}. The data indicate mean ± SEM of triplicate samples of one representative of three independent experiments. An asterisk denotes $P < 0.05$ versus control (Mann–Whitney U-test).

CD86 in parasitized DCs (Figure 4A,B). Interestingly, this paromomycin/miltefosine-induced up-regulation of CD40, CD80 and CD86 in parasitized DCs was reduced in the presence of TLR9 siRNA (Figure 4C). These results suggest that the TLR9 functional deficiency impaired maturation in parasitized DCs induced by paromomycin/miltefosine. However, as monotherapy, paromomycin or miltefosine did not induce significant TLR9-dependent up-regulation of CD40, CD80 and CD86 on parasitized DCs (Figure 4A–C).

Decreased antigen presentation by DCs leading to down-regulation of MHC II is regarded as a hallmark of pathogenesis in leishmaniasis (Chakraborty *et al.*, 2005). Surprisingly, treatment of *L. donovani*-infected DCs with the combination of paromomycin/miltefosine efficiently restored MHC-II expression in a TLR9-dependent manner (Figure 4D). Notably, as a monotherapy, the individual drugs were not able to induce a significant TLR9-mediated maturation of parasitized DCs in the same duration of time (Figure 4).

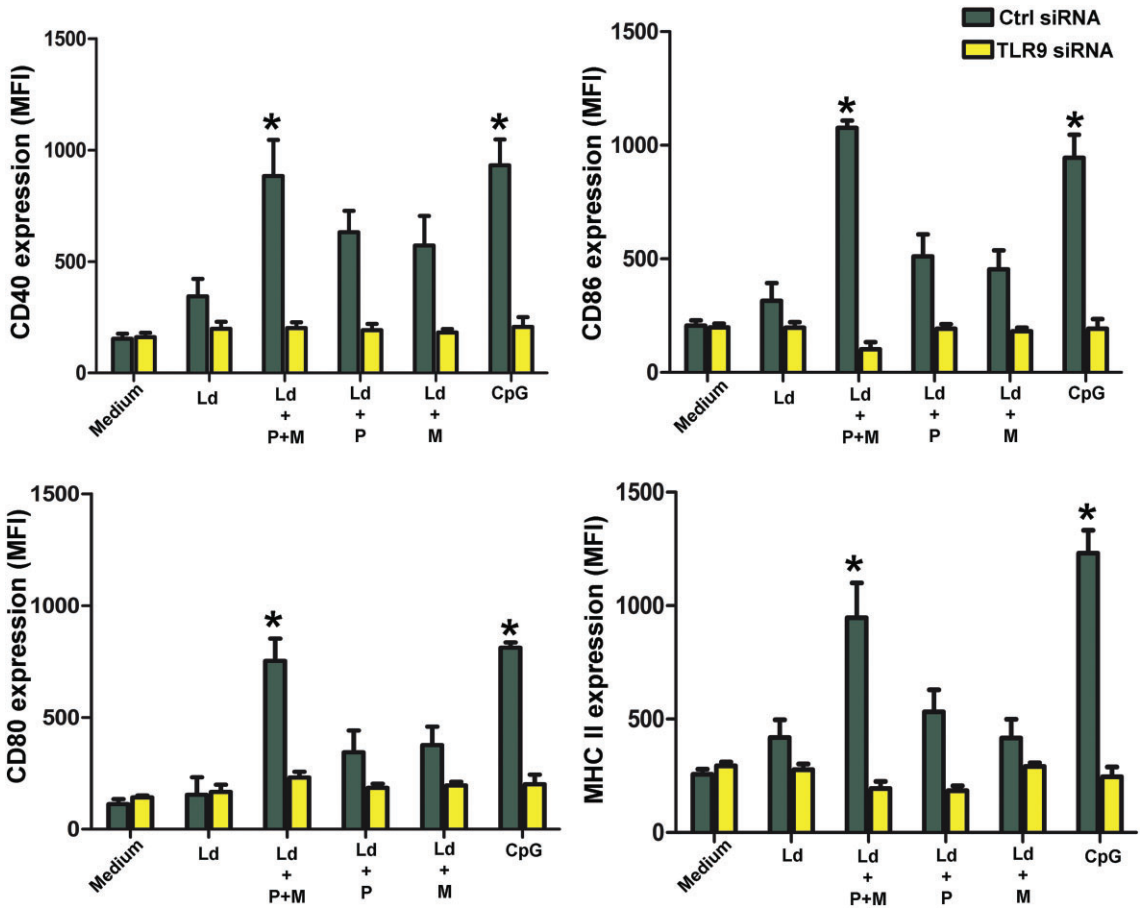

Figure 4

Phenotypic DC maturation by paromomycin/miltefosine combination is impaired in TLR9-deficient DCs. (A–D) Control monocyte-derived DCs (1×10^6 cells mL^{-1}) were either transfected with/without control siRNA or TLR9 siRNA and were then infected with/without *Leishmania donovani* parasites for 24 h. Parasitized DCs were then treated with paromomycin and miltefosine alone/or in combination. After 24 h of incubation treated macrophages were analysed by flow cytometry for CD40 or CD80 or CD86 or MHC-II (FL2-H) expression as described in Methods section. Results are representative of at least three independent experiments of different patients ($n = 7$). An asterisk denotes $P < 0.05$ versus control (Mann–Whitney *U*-test).

Paromomycin/miltefosine combination induces TLR9-dependent modulation of effector functions in DCs

Both paromomycin and miltefosine have been reported to confer protection against leishmaniasis (Sundar *et al.*, 2011). Although a TLR9-mediated cytokine response by miltefosine in macrophages has been reported (Mukherjee *et al.*, 2012), the combined effect of paromomycin/miltefosine on TLR-mediated modulation of effector functions of host DCs has not been explored. For this purpose, we infected control human DCs with *L. donovani* and then treated the parasitized cells with paromomycin and/or miltefosine. The modulation of effector functions in DCs was evaluated in terms of production of IL-12 and IL-10 at the transcript and release level. Our results demonstrated that paromomycin and/or miltefosine up-regulated *L. donovani*-mediated down-regulation of IL-12 in DCs, both at the transcript and release level (Figure 5A,B). Contrastingly, both the drugs, as a monotreatment or combined, down-regulated *L. donovani*-mediated up-regulation of IL-10 in DCs, both at the transcript and release level (Figure 5C,D). Interestingly, we observed that gene silencing of TLR9 in infected DCs reversed the effect of paromomycin and/or miltefosine on IL-12 and IL-10 production (Figure 5A–D). As our results suggested that paromomycin and/or miltefosine protects DCs against *L. donovani* infection *in vitro*, we aimed to probe their TLR9-mediated role in modulating effector responses in VL patients *ex vivo*. It is noteworthy that both the drugs in combination exhibited a Th1 polarized anti-leishmanial immune response *ex vivo* that was reversed by pretreatment of cells with human inhibitory ODN (TLR9 blocker) (Figure 5E,F). However, paromomycin was more potent at inducing TLR9-dependent IL-12 production in uninfected DCs than miltefosine and had very little effect on IL-10 production from these cells (Figure 5A–D).

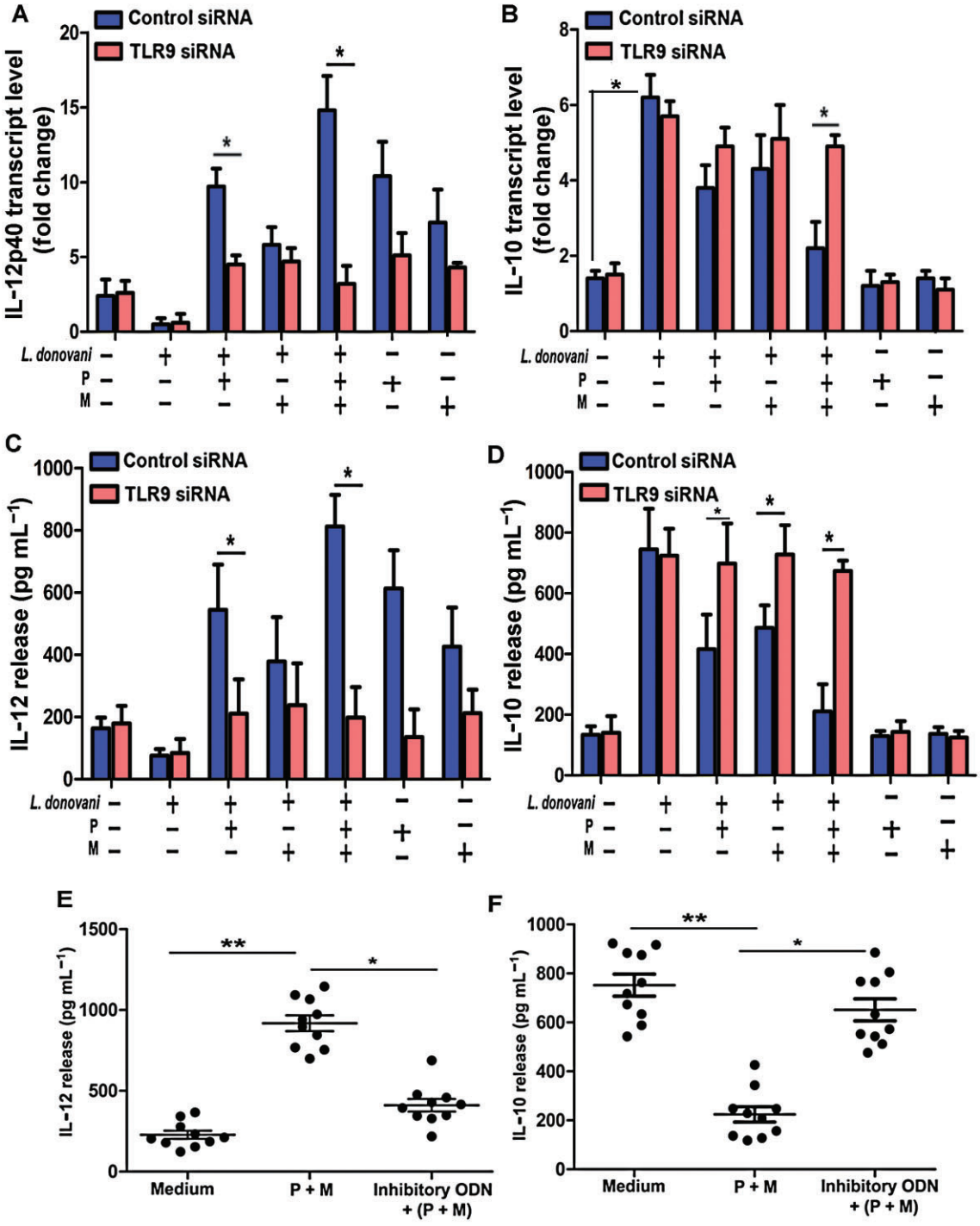

Figure 5

Paromomycin/miltefosine combination induces TLR9-dependent modulation of effector functions in DCs. (A,B) The control DCs ($n = 8$) were either transfected with control siRNA or TLR9 siRNA and were then infected with *Leishmania donovani* parasites for 24 h (see Methods). Parasitized DCs were then treated with paromomycin and miltefosine, alone or in combination. RNA was isolated and levels of mRNA expression for IL-12p40 (A) and IL-10 (B), were determined by quantitative RT-PCR. Results are presented as changes (fold) relative to uninfected control DCs. (C,D) Control DCs, either transfected with control siRNA or TLR9 siRNA, were cultured in a 24-well plate (1×10^6 cells·mL^{-1}) were pretreated and infected as described above. The culture supernatants were assayed for the levels of IL-12 (C) and IL-10 (D) in the ELISA as described in the Methods section. ELISA data are expressed as means of values from triplicate experiments that yielded similar observations. The data represent the mean ± SEM of results from three independent experiments of control subjects ($n = 8$) that yielded similar results. (E,F) Paromomycin and miltefosine promotes TLR9-mediated effector function in AVL patients: BMDCs of VL patients ($n = 10$) were stimulated *ex vivo* with the combination of paromomycin–miltefosine with/without pretreatment with human inhibitory ODN (TLR9 blocker) and the production of IL-12 (E) and IL-10 (F) was estimated at the release level by ELISA. The geometric mean of cytokine production of all populations was determined per donor and compared between the stimulated and non-stimulated groups. Horizontal bars denote the median values. Each dot represents one individual.

TLR9 is involved in the paromomycin–miltefosine-induced histone H3 modifications and binding of the transcription factor at the promoter locus of IL-12 and IL-10 in infected DCs

For studying the molecular mechanism by which the combination of paromomycin–miltefosine regulates DC effector functions, we analysed TLR9-mediated histone modifications (acetylation and phosphorylation) at the IL-10 and IL-12 locus by CHIP assay and RNA interference technique.

It is reported that histone modifications at the IL-12 promoter locus are a crucial step for activation of NF-κB and the production of IL-12 (Saccani *et al.*, 2002). Using the CHIP assay we demonstrated that *L. donovani* infection decreased phosphorylated and acetylated H3 levels of the IL-12p40 promoter in DCs that was effectively up-regulated by the combination of paromomycin–miltefosine (Figure 6A,B). However, it was quite surprising to note that RNA interference of TLR9 completely reversed the effect of paromomycin–miltefosine on H3 modifications, i.e. inhibition of TLR9 activity significantly attenuated paromomycin–miltefosine-mediated histone modification at the IL-12p40 locus in infected DCs compared with drug only-treated parasitized DCs with control siRNA (Figure 6A,B).

In contrast, in similar experiments performed for the IL-10 promoter locus, it was found that infection of DCs with *L. donovani* was associated with a significant rise in H3 phosphorylation and acetylation at the IL-10 promoter locus that was effectively down-regulated by treatment with the combination of paromomycin–miltefosine (Figure 6C,D). Interestingly, inhibition of TLR9 activity by RNA interference before paromomycin–miltefosine treatment showed no effect on the enhanced phosphorylation and acetylation of H3 in parasitized DCs induced by this drug combination (Figure 6C,D).

Notably, when paromomycin and miltefosine was used as a monotherapy, the TLR9-mediated significant effect was mainly observed for IL-12, but IL-10 levels were not altered (Figure 6E,F). Overall, the data suggest that the TLR9-dependent regulation of histone modifications at the IL-12 and IL-10 promoter locus in parasitized DCs are involved in the paromomycin–miltefosine-induced modulation of the Th1-biased immune response, which cures infected individuals.

Discussion

The therapeutic arsenal against VL is limited. Antimonial drugs, the mainstay of treatment, can no longer be used in India, where the incidence of VL is highest, due to resistance (Bryceson, 2001). Traditional second-line drugs (pentamidine and amphotericin B) are more toxic and difficult to administer (Sundar 2001); newer formulations of amphotericin B, liposomal amphotericin B are not affordable in less developed countries (Gradoni *et al.*, 2003; Bern *et al.*, 2006). DCs specialize in capturing, processing, transporting and presenting antigens to T-cells (Banchereau and Steinman, 1998). They initiate primary T-cell responses by presenting antigenic peptides on MHC molecules and providing co-stimulatory signals that are essential for optimal T-cell maturation (Kaisho and Akira, 2001). DC maturation is a critical link between innate and adaptive immunity. The innate immune system triggers host defense mechanisms against microorganisms via TLR-dependent signalling in DCs (Janeway and Medzhitov, 2002). The activation of APC functions is primarily mediated by the TLR, which play a key role in the control of parasitic infection (Gallego *et al.*, 2011). During *Leishmania* infection, the participation of TLR9 and TLR4 are pivotal for mediating proinflammatory cytokine responses (Kropf *et al.*, 2004; Raman *et al.*, 2010; Tuon *et al.*, 2010). Combination of miltefosine with paromomycin was also reported favourable *in vivo* (Seifert *et al.*, 2010). However, the role of combination of paromomycin/miltefosine on DC maturation and activation in host is not well understood.

The present study, for the first time, highlights the importance of TLR9 in combination therapy by paromomycin and miltefosine triggered maturation and activation of DCs through counter-regulatory cytokine response, which might be crucial for the mechanism of action. We have earlier demonstrated that combination of paromomycin/miltefosine induces TLR4-dependent production of TNF-α and NO (Das *et al.*, 2012). TLR9-deficient mice become more susceptible to *L. major* infection because of transient inhibition of the development of curative Th1 response (Abou Fakher *et al.*, 2009). The findings of the present study demonstrate that paromomycin/miltefosine combination also interacts with TLR9 and evokes NF–κB-mediated responses. Here, we show that NF–κB-mediated responses are initiated in 293-TLR9 cells, but not in 293-null cells. Therefore, as therapy directly targeting TLRs for Th-1 biased immunomodulation would be a good option for elimination of VL, it was tempting to investigate which anti-leishmanial drug or combination can target TLR9 for such host cell-modifying functions.

Further reports on DC maturation has revealed that CpG-DNA (ligand for TLR9 activation) fails to up-regulate surface expression of MHC II and other co-stimulatory molecules on MyD88-deficient DCs, whereas LPS (ligand for TLR4 activation) induces normal phenotypic maturation of DCs (Kaisho and Akira, 2001). Kaisho and Akira (2001) demonstrates that LPS-induced cytokine production from DCs was MyD88-dependent, but LPS could induce functional maturation of MyD88-deficient DCs. Conversely, bacterial DNA could not maturate MyD88-deficient DCs, indicating that MyD88 is differentially required for TLR family signalling. We had previously shown that combination of paromomycin/miltefosine targets TLR4-dependent responses in a MyD88-dependent manner (Das *et al.*, 2012). However, the findings of the current work provide evidence that the drug combination promotes DC maturation through TLR9, not by TLR4. Here, we also report that combination of paromomycin/miltefosine mediates MyD88-dependent maturation of DCs in VL patients through TLR9. DC maturation through TLR4 is MyD88-independent; whereas that through TLR9 is MyD88-dependent (Kaisho *et al.*, 2001). We also demonstrated that TLR4 was unable to induce DC maturation by paromomycin/miltefosine, as the DC maturation process by paromomycin/miltefosine was not MyD88-independent. These findings also suggest that the activation of DCs occurs through a MyD88-dependent mechanism for IFN-γ production in VL patients by paromomycin/miltefosine. Therefore, these results provide

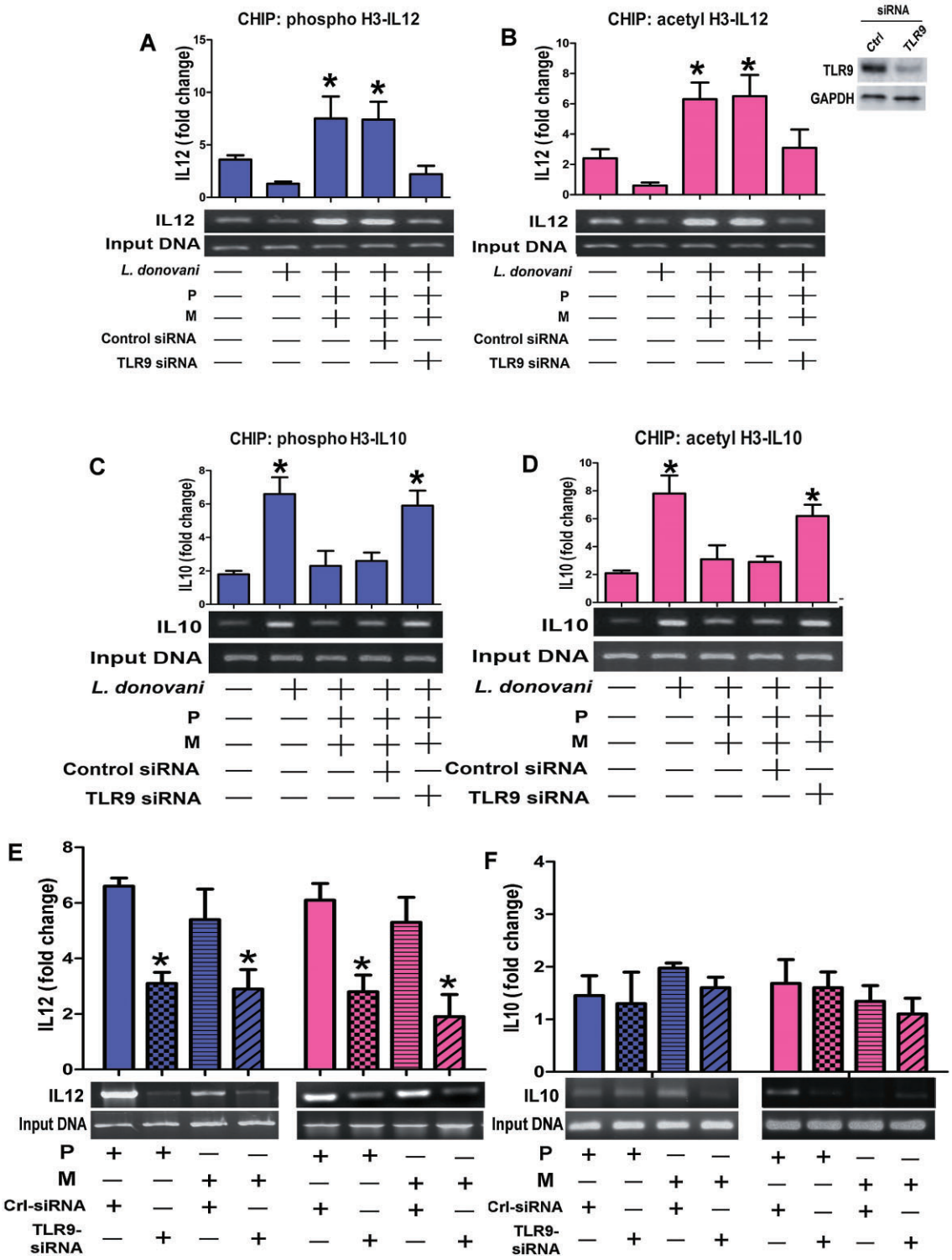

Figure 6

Role of TLR9 in paromomycin/miltefosine-induced histone H3 modifications at the IL-12 and IL-10 promoter in infected DCs. Control DCs (1×10^6 cells per mL) from healthy volunteers ($n = 6$) were either transfected with control siRNA or TLR9 siRNA and were then infected with *Leishmania donovani* parasites for 24 h. Parasitized DCs were then treated with paromomycin and miltefosine; alone or in combination. ChIP assays were conducted as described in the Methods section. Immunoprecipitations were performed using Abs specific to phosphorylated H3 [IP phospho-H3; (A,C)] or acetylated H3 [IP acetyl-H3; (B,D)], and conventional RT-PCR or quantitative real-time PCR was performed using primers specific to the IL-12 (A,B,E) or IL-10 (C,D,F) promoter. *$P < 0.001$ compared with infected DCs without the treatment. (E-F) Changes in phosphorylated/acetylated H3 modifications at IL-12 locus (E) and IL-10 locus (F) with mono treatments of DCs with paromomycin or miltefosine are shown. Fold changes over input DNA are demonstrated.

evidence that TLR9 and MyD88 are crucial factors for DC maturation in VL.

One of the hallmarks of DC activation is the production of inflammatory cytokines, like IL-12, upon TLR stimulation. Indeed, C57BL/6 mice deficient in TLR9 resolved their lesions significantly later than wild-type mice and controlled parasite growth with much lower efficiency (Liese *et al.*, 2007). Because the absence of TLR9 significantly inhibited the development of a protective Th1 response, we particularly studied the importance of combination of paromomycin- and miltefosine-induced TLR9 signalling for the acquisition by DCs of a Th1-inducing DC1 functional phenotype. Results suggested that combination of paromomycin and miltefosine required TLR9 expression to become activated and express inflammatory cytokines through counter-regulation of IL-10 versus IL-12 production. Strong evidence was obtained that paromomycin/miltefosine were the ligand responsible for the TLR9-dependent maturation of DCs to express a DC1 phenotype. Finally, stimulation of DCs with paromomycin/miltefosine strongly enhanced the production of IL-12, but decreased that of IL-10, by specifically modulating histone H3 at the promoter region. Thus paromomycin/miltefosine-mediated gene-specific chromatin modifications are found to be associated with transient silencing of IL-10 genes and priming of the IL-12 gene. Notably, mono treatment of the cells with the drugs produced less IL-12 compared with their combination. Therefore, the observed activation of DCs by TLR9-mediated mechanism will deteriorate intracellular differentiation of the invading amastigotes and promote their capacity to promote protective Th1 cell development as antimicrobial defence mechanisms.

Our observations show not only the mechanism of the immunomodulatory action of miltefosine with paromomycin on DCs for the first time, but also indicate the superiority of the drugs to other anti-leishmanials by directly targeting TLRs for this function. Recent studies show that the synergistic correlation between TLR9 and TLR4 is essential for the release of IL-12 (Theiner *et al.*, 2008). Interplay among TLR ligands synergize through distinct DC pathways to induce specific T-cell responses, providing guidance for design of novel synergestic molecular TLR-targeting drugs and vaccine adjuvants (Zhu *et al.*, 2008). Our findings showed that combination therapy by paromomycin and miltefosine could significantly induce TLR9 in *L. donovani*-infected DCs that might be responsible for triggering a potent anti-leishmanial pro-inflammatory response in synergy with TLR4. This TLR9-mediated promising therapeutic strategy would ensure a resurgence of pro-inflammatory response along with the attenuation of the anti-inflammatory response.

In conclusion, this study for the first time shows that paromomycin/miltefosine renders protection against *Leishmania* infection via TLR9, which can be exploited in future for efficient targeting of drugs. Thus, this study delineates a critical role for TLR9 in both DC maturation and activation and also provides evidence for a MyD88-dependent action of paromomycin and miltefosine in VL. Combination therapies for leishmaniasis symbolize a potential chemotherapeutic ploy that has recently been instigated in different endemic areas. This approach has several rewards over monotherapies, including shortening of the treatment period and diminution of drug resistance. However, *L. donovani* is also reported to develop resistance to drug combinations in a murine model (Garci'a-Herna'ndez *et al.*, 2012). It has been previously demonstrated that miltefosine acts synergistically with other drugs used in combination on *Leishmania* (Seifert and Croft, 2006). In this study, we showed that paromomycin/miltefosine have a TLR9-mediated synergistic effect on host immune responses. Therefore, their combined effect on the parasite and/or host Th-1 response plus opposing resistance makes the paromomycin–miltefosine combination a success.

Acknowledgements

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Conflict of interest

The authors declare no competing conflict of interest.

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