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ORIGINAL PAPER

Leishmania donovani reduces the levels of retinoic acid–synthesizing enzymes in infected macrophages and favoring its own survival

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Abstract

People suffering from malnutrition become susceptible to the infection like *Leishmania* sp., as it results in a compromised immune response. Retinoic acid (RA), an important constituent of nutrition, shows an immune-modulatory activity. However, its role in the containment of infection is not yet ascertained, particularly in case of visceral leishmaniasis (VL). VL patients ($n = 10$) and healthy endemic controls ($n = 9$) were recruited to measure the serum levels of RA. An in vitro model of *Leishmania* infection using the murine mφ cell line J774.1 was used to investigate the RA-synthesizing enzymes (RALDH-1 and RALDH-2). Parasite loads among infected mφ were measured by quantitative expression of *kDNA* in the presence of an inhibitor of the RALDH-2 enzyme. We found a significant decrease in the serum levels of RA in VL cases. Importantly, we observed decreased levels of *RALDH-1* and *RALDH-2* among *L. donovani*–infected mφ along with simultaneous decrease as well as increase in the Th-1 and Th-2-associated factors, respectively. Furthermore, the pretreatment of mφ with an RALDH-2 inhibitor improved parasite in vitro infection. Our findings show impaired RA pathway among infected mφ and indicate that an intact RA pathway is critical for anti-*Leishmania* immune response.

Keywords Visceral leishmaniasis · *Leishmania* · Retinoic acid · Immune suppression · Retinoic acid–synthesizing enzymes

Abbreviations

RA	Retinoic acid
VitA	Vitamin A
RALDH	Retinal dehydrogenase
VL	Visceral leishmaniasis
ATRA	All trans-RA

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Introduction

Leishmaniasis is a protozoan disease caused by the *Leishmnia* species. Visceral leishmaniasis (VL) or kala-azar, a fatal form if not treated on time, is mainly found in the Indian subcontinent and predominantly in Bihar and adjoining districts of Eastern Uttar Pradesh as well as West Bengal and neighboring countries of India (Clem 2012; Malaviya et al. 2011). VL affects the visceral organs of the body, including the liver, spleen, bone marrow, and lymph nodes. It has been observed that people suffering from malnutrition are more susceptible to infection from *L. donovani.* Importantly, it has already been demonstrated that malnutrition is a major problem in developing countries and favors the early visceralization of *L. donovani* parasites (Ibrahim et al. 2013). Malnutrition, due to protein and/or any micronutrient deficiency, results in a compromised immune response in the host (Anstead et al. 2001) and increases the rate of mortality and morbidity in younger children (less than 5 years of age), particularly those suffering from Zinc, Folate, Iron, and vitamin A (VitA) deficiencies.

Retinoic acid (RA), a common form of vitamin A (VitA), is a key micronutrient that also plays a role in immune modulation (García 2012). Mostly, esters or retinol are the common form of retinoids in the body. RA is synthesized in a two-step process. In the first step, retinol is converted into retinal by the enzyme Retinal dehydrogenase-1 (RALDH-1) and later, it is converted into retinoic acid by Retinal dehydrogenase-2 (RALDH-2) (Canete et al. 2017). RA plays an important role in Th-1 cell differentiation and regulation. A decrease in the levels of RA supports the secretion of IL-10 cytokine and modulates the immune system towards Th-2-mediated pathway (Bono et al. 2016). It was reported that supplementation with VitA reduces re-infection rates with *Ascaris lumbricoides* in children from Mexico (Payne et al. 2007). Furthermore, supplementation with VitA along with Zinc was found to be helpful in recovery from malaria infection among children aged between 6 and 12 months (Owusu-Agyei et al. 2013). Deficiency of VitA has also been reported in tuberculosis patients and has been linked to the disease pathology (Mathur 2007). In a mouse model study, it has been demonstrated that a decrease in the levels of VitA supports the secretion of IL-10- and Th-2-mediated immune response in cutaneous leishmaniasis (Stephensen et al. 2004). However, when monocytes isolated from healthy children were exposed with *Leishmania* antigen and RA, an increase in the level of IL-10 was observed.

Retinoic acid is thought to play a dual modulatory role and requires detailed study to further elucidate its mechanism of action in modulating the immune system (Maciel et al. 2014). As RA has a significant role in the immune regulation, pathogens try to impair RA pathways and/or its concentration. A decrease in the serum levels of vitamin A has been observed in several infectious diseases such as malaria and measles (Nabakwe et al. 2005; Rosales et al. 2002). As a significant study on the role of RA in VL disease has not been published, the present study aimed to understand the levels of RA in serum of VL patients and to elucidate the levels of RALDH-1 and RALDH-2 enzymes among infected macrophages (mφ).

Methods

Recruitment of patients and healthy controls

Patients suffering from VL were recruited from Balaji Utthan Sansthan Patna, Bihar, after their informed consent. These freshly diagnosed cases ($n = 10$) were rk39-positive [Age (Mean ± S.D.), 25.95 ± 13.05; all male cases; all rk39-positive; parasite load + 1 in splenic aspirates of all cases]. rk39 is a screening test and preferably used to identify the suspected cases of VL. Their presumptive diagnosis was confirmed by demonstration of *Leishmania* parasite in splenic aspirate (Chunge et al. 1989). Healthy controls, sharing the same endemic environment, were included in the study after informed consent and proper evaluation. The study was approved by the Institute Ethics Committee (Ref. No.: MNNIT/IEC/2013-14/02). The blood samples were collected into plain vacutainer tube (red color top) and the coagulated blood was centrifuged to separate serum. These serum samples were then transported to All India Institute of Medical Sciences (A.I.I.M.S.), New Delhi, in 2–8 °C under dark conditions for further analytical analysis.

Culture of *Leishmania donovani* promastigote

Promastigotes form of *L. donovani* parasites (DD8 strain) was obtained from Central Drug Research Institute (C.D.R.I.), Lucknow, and maintained in M199 media (Cat. No.-31100035, Gibco Life Technologies, USA) containing kanamycin antibiotic (80 μg/ml) with 10% fetal bovine serum (FBS; Cat. No.-10270106, Gibco Life Technologies, USA) at 25 °C (Dey et al. 2002; Tiwari et al. 2016). The same is used for establishing infection in macrophage cell line J774A.1.

Culture of J774A.1 mouse macrophage cell line

J774A.1, a mouse macrophage cell line, was obtained from Central Drug Research Institute (C.D.R.I.), Lucknow, and maintained in RPMI-1640 media (Cat. No.-31800-022, Gibco Life Technologies, USA) supplemented with 10% FBS (Cat. No.-10270106, Gibco Life Technologies, USA) at 37 °C with 5% CO_2 incubator at central facility in M.N.N.I.T. Allahabad (Forero et al. 1999). For sub-culturing, cells were first trypsinized, washed (2×), and then re-suspended in fresh complete RPMI-1640 media. To establish infection with the promastigotes form of parasite, 2×10^6 million J774A.1 cells were counted with the help of Neubauer chamber and seeded in the six well plates. After flattening of cell for 3 h, the non-adherent cells were aspirated out. The adherent J774A.1 cells were incubated with the promastigotes form of parasite (MOI; 1:10). After 12 h of incubation, the non-infecting parasites were removed by washing with incomplete RPMI-1640 (3×). The infection with *Leishmania* parasites was quantitatively measured by gene expression of *kDNA* among infected macrophages.

Inhibitor assay

N,*N*′-octamethylenebisdichloroacetamide (Cat. No.-0215805083, MP Biomedicals, USA) is an inhibitor of RALDH-2 enzymes (Amory et al. 2011; Mey et al. 2003). The inhibitor was reconstituted in cell culture grade

DMSO. The macrophage cell lines were treated with a 40 μg/ml dose of an RALDH-2 inhibitor for 12 h and then incubated with the promastigotes form of *Leishmania donovani* (MOI; 1:10) for another 12 h. After removal of non-infecting *L. donovani* parasites from the media, J774A.1 macrophage cells were again treated with an RALDH-2 inhibitor for 12 h. DNA and/or mRNA were isolated and analyzed *kDNA* expression and various other pro-inflammatory and anti-inflammatory cytokines/enzymes using qPCR.

Quantitative expression of genes

Total RNA were isolated by the Trizol (Cat. No.-T9424, Sigma Aldrich, USA) method as suggested by the manufacturer. One microgram of total RNA was used for the cDNA preparation by random hexamer (Cat. No.-S1230S, New England Biolabs, USA) as well as oligo (dT) (Cat. No.-S0131, Thermo Fisher Scientific, USA) primers using Reverse Transcriptase (RT) enzyme (Cat. No.-M0253S, New England Biolabs, USA). Expression analysis was performed using SYBR green master mix (Cat. No.-F416L, Thermo Fisher Scientific, USA) in real-time PCR (qPCR) (Cat. No.-TCR0096, PikoReal real-time PCR System, Thermo Fisher Scientific, USA). cT values were normalized by expression of *HGPRT* gene in control and experimental tubes. The data is represented as fold increase considering control tube (only mφ) as reference (Ovalle-Bracho et al. 2015; Rai et al. 2012). Primer sequences for the gene expression analysis are as follows: ***IFN-γ*** 5′-ATCT GGAGGAACTGGCAAAA-3′(F), 5′-TTCA AGACTTCAAAGAGTCTGAGGTA-3′(R); ***Arginase-1*** 5′-CACTGGGAATTTGCATGGGC-3′(F), 5′-GGTC TACATCTCGCAAGCCA-3′(R); ***iNOS*** 5′ -CTTTGCCA CGGACGAGAC-3′(F), 5′-TCATTGTACTCTGAGGGCTG AC-3′ (R); ***TNF-α-*** 5′-TCTCATGCACCACCATCAAG GACT-3′(F), 5′-ACCACTCTCCCTTTGCAGAACTCA-3′(R); ***IL-10*** 5′-ACCTGCAGTGTGTATTGAGTCTG-3′(F), 5′- CCCTGGATCAGATTTAGAGAGC-3′(R); ***HGPRT*** 5′-GTTGGGCT TACCTCACTGCT-3′(F), 5′-TAAT CACGACGCTGGGACTG-3′(R); ***RALDH-1*** 5′-CACA TCTCCTATAGTAACCAGCA-3′(F), 5′-GCTT GCGCTTATATCTATCCTTC-3′(R) and ***RALDH-2*** 5′-GTGGTCCCCTTGTCCATTCA-3′(F), 5′-CTTC GATTTGGCAGCTCAGG-3′(R).

HPLC-based estimation of retinoic acid in blood serum

The C-18 column was used for the measurement of retinoic acid (RA) in blood serum. The serum samples were first deproteinized with 500 μl methanol (HPLC grade, 1:1 ratio; Cat. No.-67561, Merck, Germany). The tubes were centrifuged at 10,000 rpm for 10 min to remove the precipitates. The clear supernatant was subjected to HPLC for estimation of RA. The total run time was 12 min and injecting volume was 20 μl. The peaks were characterized using absorbance at 344 nm. RA concentrations were measured by area under the curve or peak in the chromatogram (Barua 2003; Jones et al. 2015).

Quantification of parasite load

Leishmania donovani specific primers for *kDNA* 5′-CTTT TCTGGTCCTCCGGGTAGG-3′ (F), and 5′-CCAC CCGGCCCTATTTTACACCAA-3′ (R) were used for estimation of parasitic load in infected macrophages (Verma et al. 2010). Primers were commercially synthesized from Eurofins genomics, India. PikoReal™ real-time PCR System (Thermo Fisher Scientific, USA) was used for amplification and detection of *kDNA* using SYBR Green I (Cat. No.-F415L, Thermo Fisher Scientific, USA). A 10 μl of PCR reaction was performed, having SYBR Green I PCR master mix, 5 pmol of forward and reverse primers, and 1 μl of DNA from the macrophages (isolated using phenol-chloroform-isoamyl alcohol method) along with nuclease free water. Cycling parameters were 95 °C for 10 min and 40 cycles at 95 °C for 60 s and 61 °C for 15 s. Each reaction was carried out in duplicates. Standards, test samples, and negative controls were analyzed for each run. DNA of *L. donovani* corresponding to different numbers of parasites (10×10^6 to 100 parasites) was used for the construction of the standard curve ($p < 0.0001$, $r^2 = 0.9858$) (data not shown).

Statistical analysis

The normal continuous variables were presented in Mean ± S.D. However, non-normal co-variants should be shown in the median and interquartile range. For the comparison of all continuous variables between the studied groups, paired/unpaired *t* test and Mann-Whitney test/Wilcoxon signed rank test were used, as and when required. *p* value less than 5% level of significance was regarded as significant results. The analysis is done using SPSS 15.0. Graphs and figures were made in Graph Pad Prism 5 version.

Results

Decreased levels of retinoic acid (RA) in serum samples of VL patients

RA levels were estimated among freshly diagnosed VL patients. These patients were first screened and then confirmed by the demonstration of *Leishmania* parasites in their splenic aspirate. All cases were shown to have + 1 parasite load. The clear supernatant from deproteinized

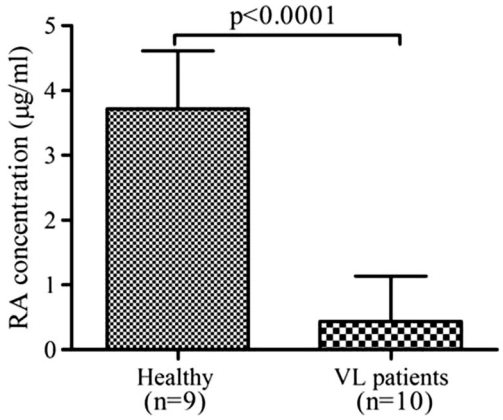

Fig. 1 Levels of retinoic acid (RA) in serum samples of visceral leishmaniasis (VL) patients: bar diagram shows the level of RA (μg/ml) among healthy endemic controls ($n = 9$; Mean ± S.D., 3.717 ± 0.8938) and VL patients ($n = 10$; Mean ± S.D., 0.4335 ± 0.7030) using HPLC. Significance is shown as p value (< 0.05) using paired t test

serum samples (with HPLC grade methanol, 1:1 ratio) were subjected to HPLC-based estimation of RA. Findings indicate decreased levels of RA among VL patients (Fig. 1; $n = 10$; Mean ± S.D., 0.4335 ± 0.7030) as compared to healthy subjects ($n = 9$; Mean ± S.D., 3.717 ± 0.8938, $p < 0.0001$, unpaired t test).

Downregulation in the expression of RA-synthesizing enzymes in *L. donovani*–infected macrophages

Interested by the finding, we were inclined to measure the levels of mRNA expression of enzymes, involved in the endogenous synthesis of RA (RALDH-1 and RALDH-2), among infected mφ. RALDH-1 (retinol → retinal) and RALDH-2 (retinal → RA) are the enzymes involved in the conversion of retinol to RA. Our finding shows significant decrease in the level of gene expression of *RALDH-1*and *RALDH-2* enzymes in mφ (J744A.1) infected with *L. donovani*, as shown in the form of relative fold change using real-time PCR (Fig. 2a, $p = 0.004$ and Fig. 2b, $p = 0.035$, paired t test). In order to confirm the infection in J774A.1 mφ with the promastigotes form of *L. donovani* parasite, we found the presence of *kDNA* in infected mφ (Mean ± S.D., 0.4791 ± 0.2751) as compared to uninfected mφ, (Mean ± S.D., 0.02179 ± 0.01142) as shown in the form of absolute expression ($2^{-\Delta cT}$) using real-time PCR (Fig. 2c, $p = 0.0159$, paired t test). The amplification curve [relative fluorescence unit (RFU) vs cT value] showed a doubling of PCR products with each cycle in exponential phase and thus confirms the specificity of *kDNA* expression in the experimental well (curve b, Fig. 2d).

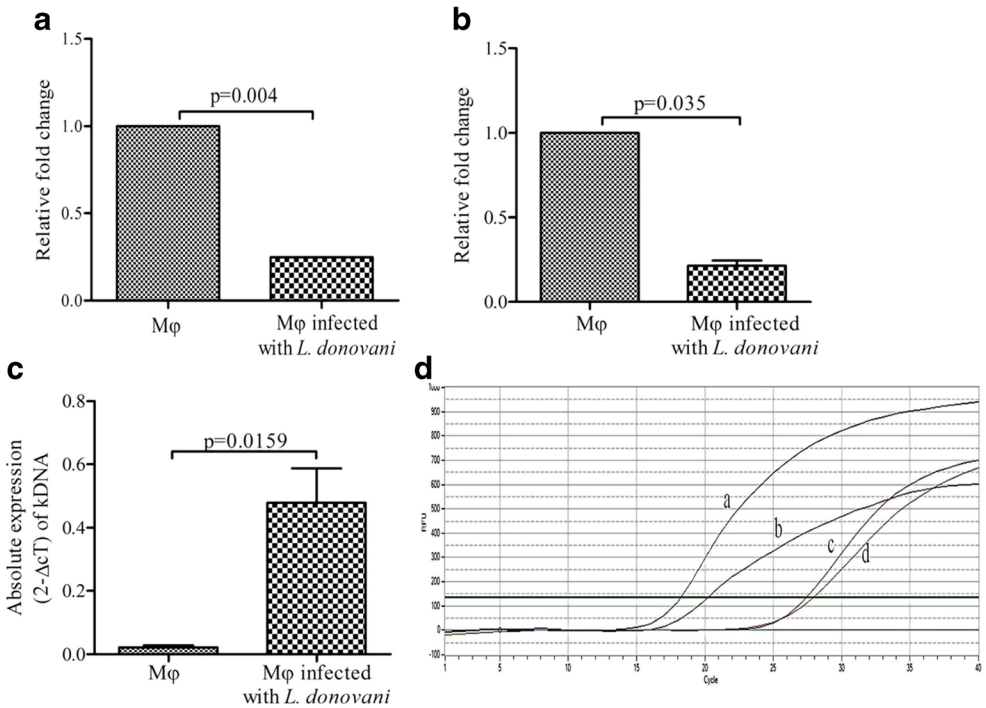

Fig. 2 Levels of gene expression of *RALDH-1* and *RALDH-2* enzymes in macrophages infected with *L. donovani*: **a** Bar diagram shows the relative fold change in the gene expression of *RALDH-1* among uninfected and *L. donovani*–infected mφ (J774A.1 cell lines) ($2^{-\Delta\Delta cT}$ value, $p = 0.004$, paired t test). **b** The relative fold change in the level of gene expression of *RALDH-2* among uninfected and *L. donovani*–infected mφ (J774A.1 cell lines) is shown ($2^{-\Delta\Delta cT}$ value, $p = 0.035$, paired t test). **c** The figure shows the absolute level of expression of *kDNA* in *L. donovani*–infected mφ (J774A.1 cell lines) compared to uninfected mφ. **d** RFU/cT of positive control i.e., *L. donovani* only (**a**), *L. donovani*–infected macrophages (**b**), negative control (**c**), and non-template control (**d**). All the experiments were performed in triplicate and repeated five times independently. Significance is shown as p value (< 0.05) using appropriate statistical tests. Data of five representative experiments are shown herewith. RALDH, retinal dehydrogenase; RFU, relative fluorescence unit

Inhibitor of RALDH-2 enzyme impairs the expression of protective cytokines/enzymes

Intrigued by the decreased levels of RALDH enzymes in infected mφ, we tried to understand the significance of RALDH pathway in mounting Th-1 and/or Th-2 types of immune response. In this regard, we measured the expressions of certain signature molecules/enzymes at the mRNA level using real-time PCR upon treatment with an RALDH-2 enzyme inhibitor. These are known to be associated with either Th-1 (iNOS, IFN-γ, and TNF-α) or Th-2 (Arginase-1 and IL-10) pathway. Findings show decreased expression of *iNOS* (Fig. 3c, $p = 0.005$, paired *t* test), *IFN-γ* (Fig. 3d, $p = 0.0001$, paired *t* test) and *TNF-α* (Fig. 3e, $p = 0.001$, paired *t* test), and simultaneous increase in the levels of expression of *Arginase*-1 (Fig. 3a, $p = 0.009$, paired *t* test) and *IL-10* (Fig. 3b, *NS*) in infected mφ with *L. donovani*. In order to understand the linkage between RALDH pathway and the effector immune response, we incubated the mφ with an RALDH-2 enzyme inhibitor (*N*,*N*′-octamethylenebisdichloroacetamide), prior to infection with *L. donovani*. It is interesting to observe further downregulation of Th-1-associated molecules/enzyme [Fig. 3c, *iNOS* ($p = 0.0001$, paired *t* test), Fig. 3d, *IFN-γ* ($p = 0.0001$, paired *t* test), and Fig. 3e, *TNF-α* ($p = 0.001$, paired *t* test)] in infected mφ treated with an inhibitor. Contrary to Th-1 pathway, we observed significant upregulation of expression of *Arginase-1* (a Th-2-associated enzyme) in infected mφ, which is further elevated upon pretreatment with an RALDH-2 inhibitor (Fig. 3a, $p = 0.031$, paired *t* test). Besides this, we also observed increased levels of *IL-10* among infected mφ upon prior treatment with an RALDH-2 inhibitor (Fig. 3b, *NS*).

Inhibitor of RALDH2 enzyme favors the increase in parasitic load

To find out the association between RALDH pathway and the growth of *Leishmania* parasite inside the mφ, we incubated the mφ with an RALDH-2 inhibitor, prior to infection with *L. donovani*. Interestingly, we observed an increased parasite load after treatment with an RALDH-2 inhibitor, which was measured by qPCR in terms of cT value (Fig. 4a, $p = 0.0020$, paired *t* test) and absolute expression (Fig. 4b, $p = 0.0019$, paired *t* test) of *kDNA*. The expression of *kDNA* was more than two times higher in infected macrophages, which were

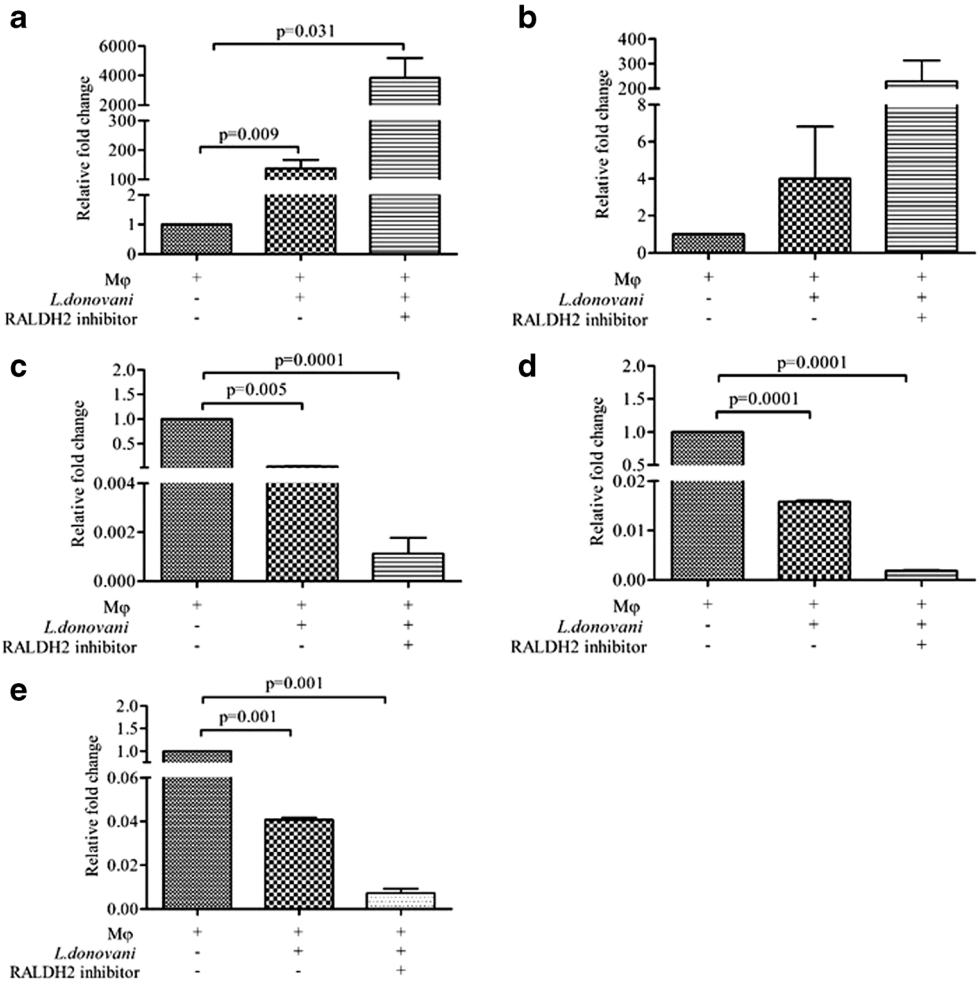

Fig. 3 Expression of Th-1- and Th-2-associated enzymes/molecules in macrophages infected with *L. donovani*: levels of expression of various genes, i.e., *Arginase-1* (**a**), *IL-10* (**b**), *iNOS* (**c**), *IFN-γ* (**d**), and *TNF-α* (**e**), are measured in mφ under different conditions using qPCR. Each bar diagram shows relative fold change in the expression of respective genes under three different conditions (i. Mφ alone, ii. Mφ infected with *L. donovani* promastigotes, and iii. infected mφ pre-treated with an inhibitor of RALDH-2 enzyme, i.e., *N*,*N*′-octamethylenebisdichloroacetamide for 12 h). All the experiments were performed in triplicate and repeated five times independently. Significance is shown as *p* value (< 0.05) using appropriate statistical tests. Data of five representative experiments are shown as Mean ± S.D. IL-10, interleukin-10; iNOS, induced nitric oxide synthase; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor

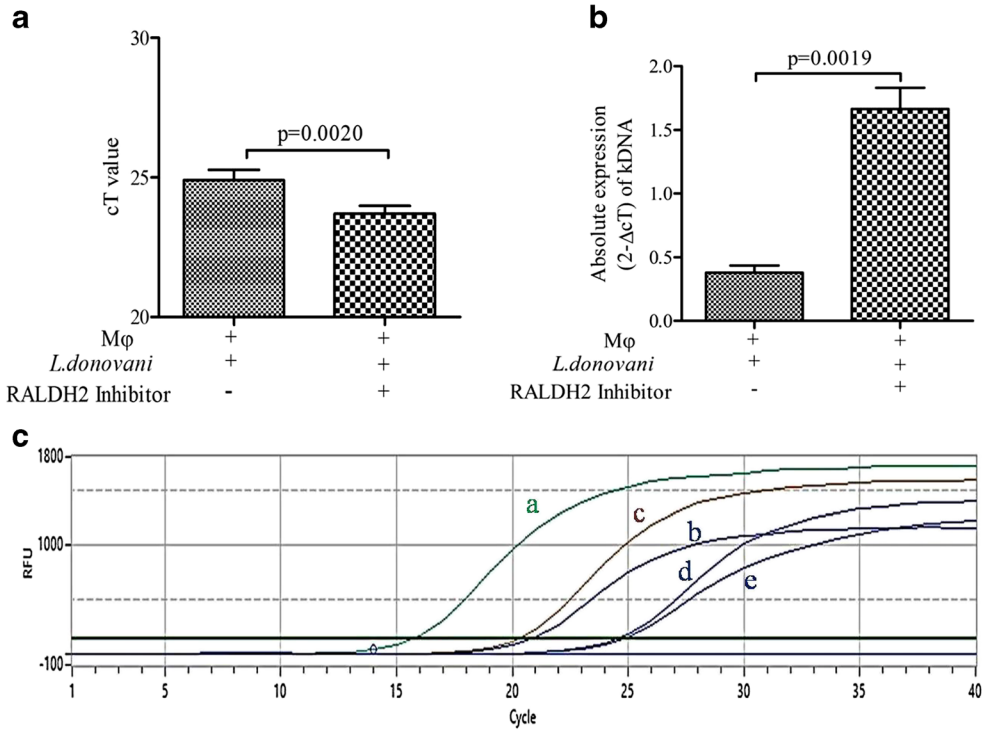

Fig. 4 Increased parasite load upon treatment with inhibitor of RALDH-2 enzyme. **a** Bar diagram shows significant decrease in expression (cT value) of *kDNA* ($p = 0.0020$, paired *t* test) in infected macrophages (Mean ± S.D., 24.90 ± 0.3663) and infected macrophages pre-treated with inhibitor of RALDH-2 (Mean ± S.D., 23.69 ± 0.2887). **b** Significant changes in the absolute expression ($2^{-\Delta cT}$) of *kDNA* ($p = 0.0019$, paired *t* test) were observed between infected macrophages (Mean ± S.D., 0.3790 ± 0.09886) and infected macrophages treated with an RALDH-2 inhibitor (Mean ± S.D., 1.665 ± 0.2904). **c** RFU/cT of positive control, i.e., *L. donovani* only (**a**), *L. donovani*–infected macrophages (**b**), infected macrophages treated with inhibitor (**c**), negative control (**d**), and non-template control (**e**). All the experiments were performed in triplicate and repeated five times independently. Significance is shown as *p* value (< 0.05) using appropriate statistical tests. Data of five representative experiments are shown as Mean ± S.D. RALDH, retinal dehydrogenase; RFU, relative fluorescence unit

previously treated with an RALDH-2 inhibitor in comparison with only infected but untreated mφ. The amplification curve (RFU vs cT) confirmed the increased parasite load in infected mφ upon treatment with an RALDH-2 inhibitor (Fig. 4c as shown in Fig. 2d).

Discussion

Individuals suffering from malnutrition show low levels of RA, which is linked with a poor immune outcome (Hall et al. 2011). Malnutrition is frequently observed among VL patients, as this disease inflicts primarily the socioeconomic-deprived community (Dye and Williams 1993). Our findings showed decreased levels of serum RA in VL patients (Fig. 1), suggesting an impaired and/or biased immune response possibly driven by the absence of RA. However, the role of RA in the development of anti-*Leishmania* immune response is still not clear. All trans-RA (ATRA) has been shown to support the development of induced regulatory T cell (iTreg) subset of the immune system, which suppresses the generation of Th-1 and Th-17 (Liu et al. 2015). In other words, ATRA stabilizes as well as induces the IL-10 producing $FOXP3^+$ iTreg cells (Kim 2008). Our earlier findings showed an increased frequency of $IL\text{-}10^+$ $FOXP3^+$ Treg cells in blood as well as at the disease site (bone marrow) among VL patients (Rai et al. 2012). The increased Treg cells, can be named induced as it responded to the *L. donovani* antigen, were shown to be linked to the poor immune response (Rai et al. 2012). Furthermore, ATRA skews the proportional balance of Treg and Th-17 cells by favoring the generation of Treg cells. However, our data of low levels of RA and increased frequencies of iTreg in VL patients are difficult to explain in the light of available literature. This must also be noted that the RA levels were measured in serum, but not at the disease site. Furthermore, reports suggest that the IL-17 possibly confers protection against *L. donovani* infection and its levels were shown to be decreased in the serum samples of VL patients (Pitta et al. 2009). However, the role of RA is not yet established in the cellular immune response against *L. donovani*.

Precisely, two RALDH enzymes (RALDH-1 and RALDH-2) convert the intracellular retinol to RA. Our findings showed significant decrease in the mRNA expressions of *RALDH-1* and *RALDH-2* in infected macrophages (Fig. 2a, b, respectively). Therefore, the decreased level of RALDHs and possibly the unavailability of cellular RA may be linked

to the consequences of mφ infection with *L. donovani*. Furthermore, the infected mφ were shown to have decreased levels of cholesterol, resulting in impaired interaction with the T cells (Roy et al. 2016). In a separate study, uptake of cholesterol was linked to an increased expression of RALDH-1 enzyme (Huq et al. 2006). Importantly, the infected mφ, having low levels of cholesterol, showed poor activation and impaired antigen presentation (Ghosh et al. 2014). We were also interested to see the association of RALDH pathway with the functional dichotomous outcome of mφ, particularly upon *L. donovani* infection. It was interesting to observe that the pretreatment of mφ with an RALDH-2 inhibitor decreased the mRNA expression of *iNOS*, a Th-1-associated enzyme. Oppositely, we observed an increased expression of *Arginase-1*, a Th-2-associated enzyme, under similar conditions. This suggests that an impaired expression of RALDHs may be linked to an overall biased phenotype of mφ towards the Th-2 pathway, which is certainly parasite-driven. Moreover, all these lead to an environment that favors the parasite growth. This must also be noted that the treatment of uninfected mφ with an RALDH-2 inhibitors showed decreased levels of *iNOS* and *Arginase-1* both and we did not observe any skewed phenotype among treated uninfected mφ (data not shown).

Though the significance of RA has not been reported in VL patients, but it is known to trigger an antimicrobial response against *M. tuberculosis* (Wheelwright et al. 2014). Our reports show that the levels of RALDH pathway and thus the cellular RA may have a protective role in the containment of *L. donovani* parasite. The significance of this finding was further augmented by the low levels of serum RA in VL patients (Fig. 1). Low levels of RA have also been reported in other intracellular infections, including malaria and tuberculosis (Nabakwe et al. 2005). RA is known to regulate the expression of pro-inflammatory cytokines (Pino-Lagos et al. 2010). Association of malnutrition with the occurrence of VL disease (Cerf et al. 1987) and low levels of RA in malnourished individuals (Combs and McClung 2016) are well reported. An overall unavailability of RA to the infected mφ skews their functional phenotype towards M2 type. Furthermore, an interesting report shows modulation of immune response by parasitic infection among multiple sclerosis patients in an RA-dependent manner (Correale and Farez 2013). In the light of above-mentioned finding, our data conclusively demonstrate parasite-driven downregulation of *RALDH-1* and *RALDH-2* in infected mφ (Fig. 2).

RA plays a significant role in the regulation of pro-inflammatory cytokines/enzymes (Hurst and Else 2012; Ross 2012). In this context, our finding showed increased expressions of anti-inflammatory cytokines/enzymes (*Arginase-1* and *IL-10*) and decreased expressions of pro-inflammatory cytokines/enzymes (*iNOS*, *IFN-γ*, and *TNF-α*) among infected mφ, which are associated with Th-2 and Th-1 pathways respectively. Interestingly, upon treatment with an RALDH-2 inhibitor, the expressions of these Th-2 and Th-1 cytokines/enzymes were further upregulated and downregulated, respectively. The findings clearly indicate the protective role of the RALDH pathway, thus the level of RA, in the containment of infection. Similarly, decreased expression of *IFN-γ* and *TNF-α* in infected mφ treated with an RALDH-2 inhibitor suggests the potential role of RA in mounting the protective response against *L. donovani* (Kima and Soong 2013). Therefore, depletion of intracellular RA by an inhibitor of the RALDH-2 enzyme skews the immune response towards the Th-2 pathway, which supports *Leishmania* infection. Our findings conclusively confirm that RA plays vital role in the immune response against *L. donovani* infection. Parasite-driven inhibition of RALDH pathway leads to skewed immune response and makes the mφ susceptible to the infection. Thus, decreased concentration of RA creates favorable conditions for *L. donovani* infection and a reverse situation may restore the immune response and thus the containment of parasite.

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Authors' contributions PV, AKK, SS1, and SP, executed all the experiments and helped in the interpretation as well as analysis of the data. They also prepared the first draft of the manuscript. SK maintained the macrophage and parasite culture. SKK, SKS, and MB executed the standardization of HPLC-based estimation of retinol and RA. This part of work was conceptualized and monitored by AKD. CPT recruited the kala-azar patients and provided samples. SS2 helped in lab facilities and manuscript preparation. AKR conceptualized, attracted funding, sought collaboration, monitored the experiment, and edited the manuscript.

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Data availability All the data and materials are available with us.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval and consent to participate The study was approved by the Institute Ethics Committee (Ref. No.: MNNIT/IEC/2013-14/02).

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