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Original Article

Combined immunotherapeutic effect of *Leishmania*-derived recombinant aldolase and Ambisome against experimental visceral leishmaniasis

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Abstract *Background:* Available therapeutics for visceral leishmaniasis (VL), a deadly parasitic infection, are usually associated with inadequate efficacy and adverse aftereffects. Further, the primary site of *Leishmania* parasite are host macrophages resulting in compromised immunity; ensuing marked T-cell immunosuppression. Such settings emphasize the exploration of chemo-immunotherapeutic strategies for improvising the infected person's immune status with better resolution of infection.
Methods: Present work employs the immunization of *Leishmania*-infected hamsters with *Leishmania*-derived recombinant aldolase (rLdAld) and enolase (rLdEno) proteins in consort with the sub-optimal dose of Ambisome (2.5 mg/kg). After the completion of immunization, hamsters were sacrificed on day 60 and 90 post infection and different organ samples were collected to perform immunological assay for evaluating the therapeutic efficacy and modulation in protective cellular immune responses.
Results: Combining these proteins, particularly rLdAld with Ambisome (2.5 mg/kg), has significantly reduced the parasitic load (~80%) with remarkable enhancement in DTH and lymphoproliferative responses compared to the infected control and only Ambisome treated groups. Moreover, cytokine levels at RNA and protein levels were noticed to be inclined towards Th-1 phenotype through up-regulation of IFN-γ and TNF-α with significant down-regulation in IL-10 and TGF-β expression, an indication towards the generation of protective immunity against experimental VL.
Conclusion: Our experimental findings demonstrated that the chemo-immunotherapeutic approach could be an effective way of controlling human VL infection at minimal dosages of

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antileishmanial with reduced side-effects and propensity of drug resistance emergence.

Introduction

Visceral Leishmaniasis (VL), a chronic protozoan infection that severely weakens the immunes system of affecting individuals, is caused by Leishmania donovani complex.[1] VL has been associated with varied impoverishing effects and might leads to mortality if left untreated. India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil nations majorly contributes to most VL cases worldwide.[2] Active VL infection is marked by the suppressed cellular immune responses, displaying an enhanced expression of anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 with a pronounced curtailment in Th1 cytokine levels. Therefore, therapeutic regimes or immunization procedures inducing a Th1 prototype through secretion of pro-inflammatory cytokines like IFN-γ, IL-12, and TNF-α by the mixed population of T-cell subsets is a prerequisite to control VL.[3] Further, individuals residing in endemic regions are usually pre-contacts to the *Leishmania* parasite and are considered asymptomatic VL reservoirs.[4] These individuals are more susceptible for developing a full-fledged VL infection after encountering secondary infections such as tuberculosis, HIV, or because of malnourishment state.[5] Additionally, few VL patients progresses to post-kala-azar dermal leishmaniasis (PKDL) conditions after the completion of the treatment course.[6] Additionally, prevailing antileishmanial are clinically disconcerting on account of allied demerits.[7,8] Henceforth, the entire situation underlines the requirement of alternative therapies that simultaneously boost the immune status of a diseased person with improved resolution of infection.[9,10] In the present circumstances, chemo-immunotherapeutic interventions turn up as an alternative treatment option for VL, an effective way of remedial to different disease forms such as allergies, cancer, tuberculosis and viral infection (hepatitis).[11–14] Chemo-immunotherapies, i.e., administration of available therapeutics with a potent immuno-antigen against any infectious pathogen, resulted in activating the host's repressed immune system and pathogenic clearance even at minimal drug dosages.[15] Hence, the practice of chemo-immunotherapy regimes to augment cellular immunity with a persuasive control against VL gains momentum progressively.[16]

Our prior study suggested that Leishmania-derived recombinant proteins viz. Aldolase (rLdAld) and Enolase (rLdEno), alone exhibited an enhanced immunotherapeutic effect against established Leishmania infection.[17] These proteins noticeably modulated the immune status of infected animals by stimulating the cellular defensive responses such as an enhanced DTH reaction and skewed Th1 cytokine profile. Further, liposomal amphotericin B (Ambisome), a recommended antileishmanial to treat VL patients,[18] reports to be associated with relapses and demands new short course multiple drug therapies.[19] Thus, we thought to investigate rLdAld and rLdEno proteins together with liposomal Amphotericin B (Ambisome) to optimize their immunotherapeutic effect and minimizes the drug dosages with effective clearance of *Leishmania*.[20] In the present work hamster was used as an animal model system to carry out all the experiments because of its close relevance with human VL infection.[21] Herein we showed that administration of a single minimal dose of Ambisome in conjunction with Leishmania-derived recombinant proteins reduces the parasitic load with stimulation of subdued immune responses in Leishmania-infected hamsters.[9]

Materials & methods

Animals and parasite

Syrian golden female hamsters (seven to eight weeks old) were obtained from the Laboratory animal facility of CSIR-CDRI and used in this study. *Leishmania donovani* strain (MHOM/IN/80/Dd8), procured from the American type culture collection (ATCC, Manassas, VA, USA) maintained in vitro at 26 °C in Medium M199 containing 10% FBS.

Ethics statement

Animal experiments were carried out by following the norms of committee for the purpose of control and supervision of experiments on animals (CPCSEA), India under the regulation of the institutional animal ethics committee (IAEC, Approval no. 150/09/Para/IAEC dated 23.10.09).

Purification of recombinant proteins and antigen preparation

Leishmania-derived Th1 stimulatory proteins, i.e., aldolase and enolase purified through Ni-NTA affinity-based chromatography method as described previously.[22] Thereafter, purified proteins tested for the endotoxin content using Limulus amoebocyte lysate test kit (Thermo Fisher, USA) and concentrated to a smaller volume following prior protocols.[17] Soluble *Leishmania* antigen (SLD) was also extracted from the log phase culture of promastigotes as per the protocol of Gupta et al., 2007 and kept at −80 °C until further use.[23]

Immunization schedule

50 female hamsters were divided into five groups of ten hamsters in each group. Animals of all the groups were infected with 5×10^5 purified amastigotes (by percoll gradient method) per animal except the control hamsters

which served as uninfected group. After fifteen days of post-infection (p.i.), hamsters were immunized intradermally (i.d.) with rLdAld and rLdEno at 12.5 μg and 25 μg doses respectively. Subsequently, two booster shots of proteins (rLdAld and rLdEno) were given to these hamsters at fifteen days intervals. While on day 28 p.i., Ambisome was administered intracardially to immunized animals and infected hamsters with a single sub-optimal dose of 2.5 mg/kg/animal. *Leishmania*-infected hamsters which did not receive any protein immunization and Ambisome dosages were served as infected control.

Immunotherapeutic efficacy of these proteins in combination with Ambisome were assessed at different time intervals, i.e., on day 60 and 90 p.i. by sacrificing five hamsters from each group. Mesenteric lymph nodes, splenic tissue, and blood samples were collected at these time points for the assessment of parasitic load and cellular immune responses.[24]

Immunological assays

Determination of delayed-type hypersensitivity (DTH)

For the estimation of DTH response, 50 μg of SLD in 50 μl of sterile PBS was administered intradermally in the left hind footpad of hamster and only 50 μl of PBS solution injected in the right hind footpad of hamster serving as a solvent control. After 24 h of administration, DTH reaction was evaluated through measurement of footpad thickness with a digital Vernier Caliper. DTH response was assessed as footpad thickness in mm units by measuring the difference in footpad swelling between the two footpads (with and without SLD) in each hamster.[17]

Assessment of parasite burden

Splenic tissue dab smears prepared after measuring the weight of the spleen belong to different groups and processed for measuring the parasite burden in terms of Leishman Donovan Units (LDU), as explained in previous report.[17] Briefly, air dried splenic tissue smears were fixed in methanol and stained with 10% Giemsa stain (Sigma–Aldrich, USA). Parasite load represented as LDU through counting the number of amastigotes per 1000 macrophage cell nuclei and calculated using following formula:

$$\text{LDU} = \text{Number of amastigotes per 1000 host cell nuclei} \times \text{spleen weight (in grams)}$$

Measurement of lymphoproliferative response

Lymphoproliferative response evaluated as per the procedure described by Garg et al. [25] Briefly, mesenteric lymph nodes teased out with the help of needles in PBS solution and passed through a cell strainer to remove any traces of tissue debris. Successively, cells centrifuged for 10 min at 900 g, 4 °C and pellet was treated with RBC lysis buffer to remove RBCs if there was any and washed with PBS once. Lastly, a suspension of 2×10^6 cells/ml prepared in complete RPMI media and 0.1 ml per well was plated in 96-well tissue culture plates (Nunc, Denmark) in triplicates. After four to 5 h of adherence, cells stimulated with SLD and known mitogen (served as a positive control) like Concanavalin A (Sigma–Aldrich, USA) at a concentration of 1 μg/ml. Unstimulated cells as well as medium only kept as blank controls and incubated for 72 h in a CO_2 incubator (37 °C with 5% CO_2). After incubation, 100 μl of supernatant pipette out from each well to a fresh plate and 50 μl of XTT (Biological Industries, Israel) was added to the remaining media containing cells and incubated 4 h before the termination of the experiment. Finally, absorbance was measured at 480 nm subtracted with 650 nm as a reference wavelength in a SPECTRAmax PLUS 384 microplate reader (Molecular Devices, USA).

mRNA analysis of cytokines through real time qPCR

Total RNA isolated from the splenic tissue samples of the hamsters belongs to different experimental groups using Trizol (Life Technologies, USA) method as per the manufacturer's instructions. After RNA isolation, samples are treated with DNase to remove the traces of DNA, if any. Purity of RNA determined through NanoDrop 2000 (Thermo Scientific, USA) and samples exceeding absorbance ratios of (A260/280) $\geq$ 1.90 processed further for cDNA preparation. cDNA synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystem) according to the company's protocol. For performing real time qPCR, primers were designed using Beacon Designer software (Bio-Rad) using their gene sequences (in CDS format) and real-time qPCR of the cDNA samples executed as per the reaction conditions described in former published study.[17] All the Ct values normalized with the RPL-18 reference gene and relative fold change for different cytokines estimated by $2^{-\Delta\Delta}$Ct method.[26]

Measurement of Th1 and Th2 cytokines through ELISA

Levels of IFN-γ, TNF-α, IL-12, IL-10, IL-4 and TGF-β (in pg/ml) cytokines estimated in serum samples of different experimental groups by following the manufacturer's protocols mentioned in ELISA kit (YH ELISA kit, China).

Statistical analysis

Statistical analysis done using GraphPad Prism 8.01 tool (GraphPad Software, San Diego, CA, USA). Results represented as mean ± SEM. Data (pooled data of two independent experiments) analyzed by one-way ANOVA test, and comparisons were made with Kruskal–Wallis test to

assessed the significance between treated and infected groups. A p-value of ≤ 0.05 considered as significant.

Results

Immunization with recombinant proteins + Ambisome augments DTH response

DTH response, a primary index of cell-mediated immunity, showed to be elicited significantly in rLdAld + Ambisome ($p = 0.004$, **) and rLdEno + Ambisome ($p = 0.007$, **) treated hamsters compared to the untreated infected control animals on day 60 p.i. Although the response improved initially in proteins + Ambisome treated groups, by day 90 p.i., values were significant for rLdAld + Ambisome ($p = 0.006$, **) treated hamsters only in comparison to the infected ones. Initially, there was an inclined DTH response observed (by Day 60 p.i.) in Ambisome only treated hamsters than infected hamsters, but DTH reaction decreased subsequently at later time points (Fig. 1).

Proteins + Ambisome therapy confers protection against *Leishmania* infection

Assessment of parasite load was done to estimate the therapeutic efficacy of different treatments in the hamster model. Data demonstrated that rLdAld + Ambisome treated hamsters were optimally protected even at day 90 p.i. compared to the other treated groups and infected control. A significant parasitic reduction was observed to the tune of ~75–80% in rLdAld + Ambisome ($p = 0.001$, ***) and rLdEno + Ambisome ($p = 0.004$, **) treated animals on day 60 p.i. which decreased eventually by day 90 p.i (Fig. 2). However, a substantial decline in parasitic burden was noticed in rLdAld + Ambisome ($p = 0.0266$, * on day 90 p.i.) treated hamsters indicating the potentiated host cellular responses due to the immunomodulatory effect of rLdAld protein. Additionally, hamsters treated with only Ambisome (2.5 mg/kg), also showed a remarkable decrease in burden at earlier time points ($p = 0.009$, **) but increased progressively by day 90 p.i. which was comparable to the untreated infected animals, as displayed in Fig. 2. The possible cause of upsurge in parasite number at later time points was might be because of insufficient effectiveness of therapy and could be overcome by optimization of dose schedules.

Induction of *Leishmania*-antigen specific lymphoproliferative responses

Soluble *Leishmania*-derived proteins induces antigen specific lymphoproliferative response, an indicative for activation of host cellular defensive responses. Results displayed that hamster treated with rLdAld + Ambisome and Ambisome only ($p = 0.0001$, **** and $p < 0.0001$, ****) exhibited enhanced lymphoproliferative responses in comparison to the untreated animals at day 60 p.i. However, these responses decreased at later time points in the Ambisome alone treated animals than rLdAld + Ambisome ($p = 0.026$, *) treated ones due to possible additive effect of rLdAld immunogenicity. Nevertheless, lymphocyte proliferation in rLdEno + Ambisome treated animals was comparable (day 60; 0.2830 ± 0.004896, day 90; 0.3672 ± 0.008304) to the infected group which signifies the inefficiency of rLdEno protein in inducing protective cellular responses even at multiple shorts and not relatable with the above experimental findings (Fig. 3).

Combinatorial therapy shifts Th2 transcript profile towards Th1 phenotype

Additional decisive factor for the chronicity of VL infection is cytokine profile of affected individuals. mRNA transcripts of IFN-γ, TNF-α and IL-12 cytokines comparatively up-regulated on day 60 and 90 p.i. in both rLdAld + Ambisome and rLdEno + Ambisome treated hamsters as compared to the infected control. Therein, rLdAld + Ambisome treated hamsters exhibited significant up-regulation of IL-12 and TNF-α cytokines on day 60 ($p = 0.019$, **) and day 90 ($p = 0.035$, *) p.i. compared to the untreated infected groups, respectively. On the contrary, Th2 cytokines, namely IL-4, IL-10 and TGF-β, down-regulated moderately in all the experimental groups compared to infected ones at both the time points of observation. The relative expression of IL-4 and TGF-β declined substantially in rLdAld + Ambisome treated hamsters at day 60 p.i. and both the time intervals, respectively, as depicted in Fig. 4.

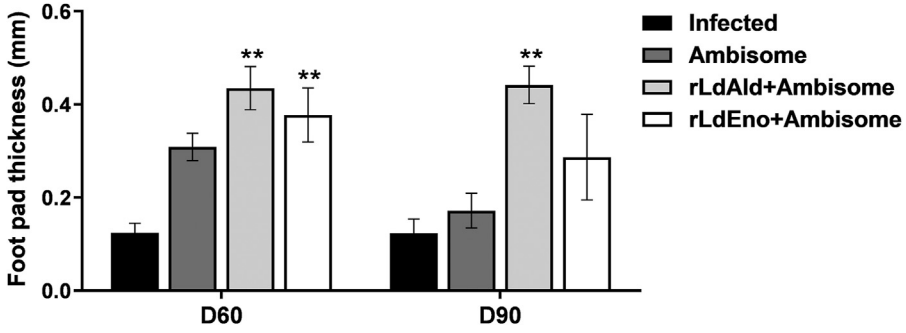

Figure 1. Assessment of DTH reaction (in mm) to SLD via measuring footpad swelling on days 60 and 90 p.i. in different groups. Significant values indicated the difference among the untreated and treated infected groups (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

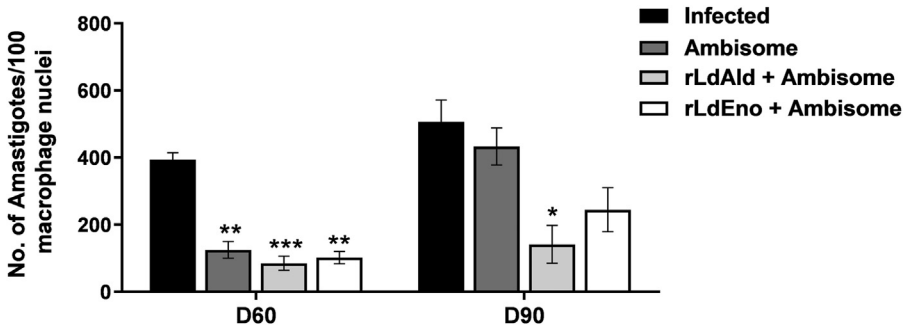

Figure 2. Evaluation of parasitic burden as Leishman Donovan Units in the splenic tissue of treated and untreated infected hamsters on days 60 and 90 p.i. Significant values indicated the difference between the untreated and treated infected groups (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

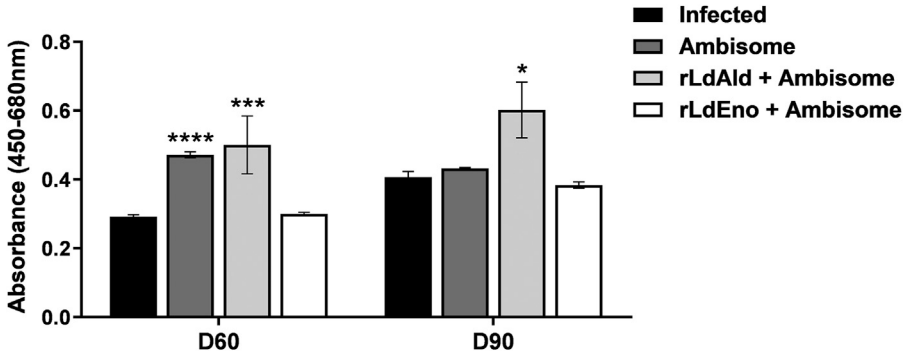

Figure 3. *Leishmania*-antigen specific lymphoproliferative response measured in treated hamsters as well as unimmunized infected hamsters on days 60 and 90 p.i. Significant values indicated the difference between the untreated and treated infected groups (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

Cytokine levels in serum samples signify inclination to protective Th1 prototype

Amongst Th1 cytokines, IFN-γ, TNF-α and IL-12 levels were noticeably elevated in rLdAld + Ambisome treated hamsters at late-stage infection compared to the infected control. However, values were significant for IFN-γ ($p = 0.032$, *) and TNF-α ($p = 0.023$, *) cytokines at day 60 p.i. while IL-12 ($p = 0.007$, **) significant at day 90 p.i. (Fig. 5). Moreover, cytokine levels were initially increased in Ambisome only treated hamsters but subsequently declined as time progressed. Further, the level of IFN-γ in the rLdAld + Ambisome ($p = 0.011$, **) treated group was significantly higher than the only Ambisome treated group at day 90 p.i. Quite the opposite, the amount of Th2 cytokines, i.e., IL-4, IL-10 and TGF-β decreased in all the treated groups in comparison to the untreated infected control at both the time points, but values were significant only for rLdAld + Ambisome treated hamsters on day 90 p.i. (Fig. 5).

Discussion

The existing therapeutics for VL allied with several peripheral effects and exhibited varied efficacies due to regional variation along with recurrences of PKDL and HIV-VL cases.[27] Towards this, chemo-immunotherapy conventions emerged as a possible way of controlling VL by minimizing the adverse aftereffects with a lower risk of appearing drug resistance and simultaneously boosting the compromised immunity of infected personnel.[28] Seifert et al. observed that LEISHDNAVAX vaccine with liposomal amphotericin B had improvised the drug's efficacy even after the shorter treatment procedures against experimental VL.[29] Likewise, the combination of DPPE drug and parasite-derived cysteine proteinase potentiated the leishmanicidal effect of the DPPE along with stimulation of protective cellular responses in *L. amazonensis* infected mice.[30] In line with these reports, our present work displayed that rLdAld/rLdEno Th1-stimulatory proteins with Ambisome conferred optimal reduction in the parasite load, an indication towards the remission of infection. Additionally, *Leishmania* recombinant proteins, particularly rLdAld with Ambisome, triggered a robust DTH response[31] and significant lymphoproliferation[32] in *Leishmania*-infected hamsters, considered as a strong correlate of developing protective immunity against experimental VL.[33] Although, these responses subsided as the infection progresses due to the subversion of host defensive pathways to establish a permissive niche for the unrestricted growth of

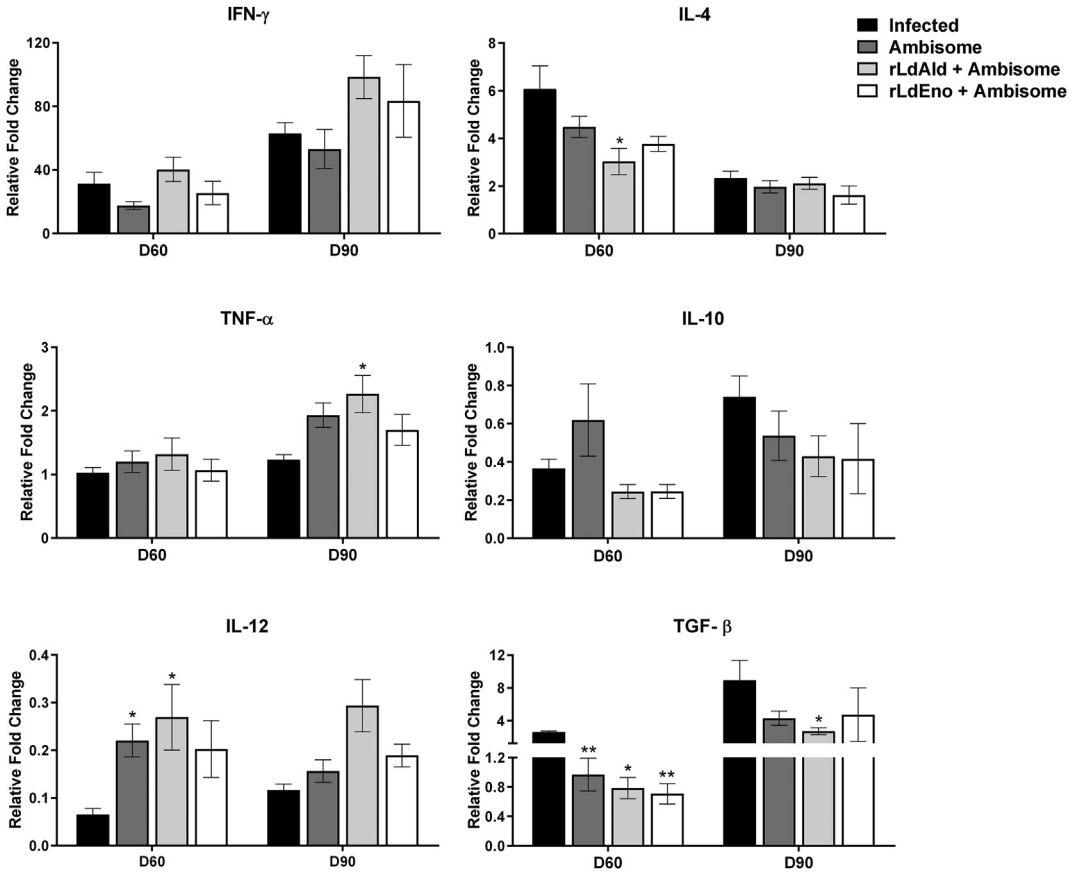

Figure 4. mRNA expression profile of cytokines in the spleen of untreated and treated infected hamsters on days 60 and 90 p.i. by quantitative real-time PCR. Significant values indicated the difference between the untreated and treated infected groups (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$).

parasites. Additionally, Ambisome alone treated group also exhibited improved cellular immune responses than the infected control, perhaps because of Ambisome self-immunomodulatory properties.[24]

Another crucial parameter for determining the state of *Leishmania* infection is the alteration of Th1/Th2 cytokine profile after any immunization course or antileishmanial therapy.[34] Recently published reports from our group anticipated that the development of Th-1 skewed cytokine phenotype over Th2 profile after vaccination with parasite-derived chimeric protein or synthetic peptides facilitated protection to VL infection.[35,36] Interestingly, Silva et al. observed that DPPE 1.2 and cysteine proteinase increases the level of IFN-γ with reduced secretion of active TGF-β as compared to the alone DPPE or protein-treated animals. Such immunization subsequently boosted the host immunity to fight off cutaneous leishmaniasis.[30] Similarly, skewed Th1 phenotype due to the up-regulation of IFN-γ, TNF-α and IL-12 cytokine levels with decreased expression of Th2 cytokines in rLdAld + Ambisome treated hamsters signifies the induction of protective responses against experimental VL. Besides, published reports also suggested the dominant effect of IFN-γ over macrophages microbicidal activities and other effector killing mechanisms contributing to the *Leishmania* clearance.[37] On the other side, TNF-α and IL-12 cytokines are associated with reduced growth of *Leishmania* through activation of phagocytic macrophages[38] and induction of IFN-γ independent antileishmanial effects,[39] respectively.

Conversely, Th2 cytokines, namely IL-10, an essential Th1 suppressive cytokine, were documented to be declined significantly in treated VL patient's samples,[3] in line with our observed experimental findings. On top of that, IL-4 and TGF-β cytokines, which rise typically during the diseased condition and hampers the macrophage activity by lowering nitric oxide and reactive oxygen species production, were also decreased in treated hamsters.[40] Therefore, alteration in cytokine milieu at transcript and protein levels signify towards the induction of a protective Th1 type response constraining *Leishmania* infection. Furthermore, despite being a part of the same metabolic pathway, rLdAld exhibited better immunomodulation than rLdEno recombinant protein. Such discrepancy in protein's immunogenicity might be due to differences in their structure and epitope-based interaction with the host factors.

In a nutshell, rLdAld protein and a suboptimal dose of Ambisome offer an enhanced immunotherapeutic effect well-supported with substantial antigen-specific cellular responses and setting out Th1 prototype. We have yet to determine better treatment regimens and delivery systems to augment the protective responses to restrict VL infection effectively. Henceforth, it reinforces prior studies

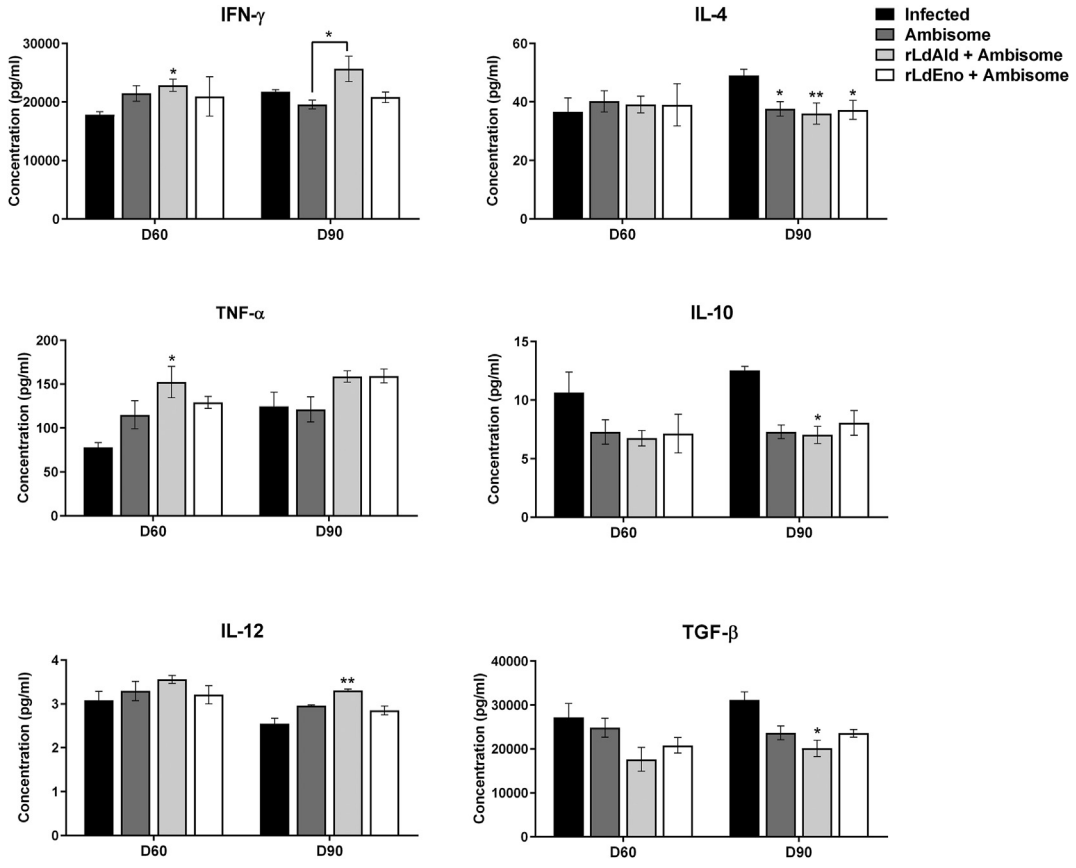

Figure 5. Analysis of Th1 and Th2 cytokine protein levels in the serum samples of treated and untreated infected hamsters on day 60 and 90 p.i. Significant values indicate the difference in cytokine concentration between the untreated and treated infected hamsters (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ and ****, $p \leq 0.0001$).

relevance and warrants investigations to optimize treatment regimens conferring better efficacies with a lesser risk of relapses before translation to human trials.

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Author contributions

K., S.J. and A.D. structured the experimental design. K. and N.K.Y. purified recombinant proteins and performed the experiments. N.K.Y., S.J. and S.R. assisted in doing animal experiments. K. analysed and interpreted the data. K., A.S. and A.D. wrote and reviewed the manuscript. K., A.S., and A.D. conceived the study.

Declaration of competing interest

The authors declare no conflict of interest.

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