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ORIGINAL ARTICLE

Leishmania recombinant antigen modulates macrophage effector function facilitating early clearance of intracellular parasites

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Background: Immunmodulation combined with chemotherapy has emerged as an alternative to treat infections. The study evaluates immunomodulatory properties of a *Leishmania* recombinant protein (rA6) in activating macrophages and clearing intracellular parasites.

Methods: The rA6 from a previously identified cDNA clone was analyzed for inducing the production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages, post and prior to infection with promastigotes by Griess method and flow cytometry. Phagocytosis and killing by treated macrophages was evaluated using *Staphylococcus aureus* as an index organism. Intracellular clearance of PKH67-labeled parasites from treated macrophages was assessed flowcytometrically. Combined effect of rA6 with miltefosine/AmBisome in reducing intracellular amastigotes was examined microscopically.

Results: Treatment with rA6 post infection caused increased production of NO with increased number of macrophages producing NO and ROS coupled with enhanced phagocytic and killing capacity. Antigen stimulated macrophages expressed high level of iNOS and TNF-α mRNA. It synergized with miltefosine and AmBisome and facilitated early clearance of intracellular amastigotes at sub-optimal drug doses.

Conclusion: The study demonstrates immunomodulatory potential of rA6 and presents first evidence on synergism between rA6 and anti-leishmanial drugs, thus placing it as a promising candidate for adjunct therapy.

Keywords: Immunmodulation, *Leishmania donovani*, Macrophages, Recombinant antigen

Introduction

Leishmania are dimorphic organisms shuttling as promastigotes in insect vector and as amastigotes within the host macrophages. Leishmaniasis remains one of the world's most devastating neglected tropical diseases, with an estimated 350 million people in 88 countries worldwide living at a risk of developing one of the many forms of the disease.[1] There is no vaccine yet available for this disease. Although the drugs are available yet the treatment is hampered by partial clearance of parasites, drug toxicity, emerging resistance, and prolonged treatment regimens, thereby endorsing the need for the development of new alternatives.[2] Limited success of anti-leishmanial drugs and vaccines under trial justifies for a novel approach including combination of immune-modulation along with chemotherapy for the effective treatment of leishmaniasis.[3]

Over the last two decades, immunomodulators have evolved as a viable adjunct, acting synergistically to the established therapeutic modalities in infectious diseases.[4–7] Hence the identification and evaluation of such immune-modulators, which induce a specific Th1 response and preferentially activate the macrophages leading to clearance of intracellular parasites, may be a vital strategy for effective control of leishmaniasis. The immunomodulatory properties of a number of immune-reactive targets in the *Leishmania* parasite have been tried alone or combined with chemotherapy, but with limited success.[8–12]

Previously our laboratory reported the identification of a novel gene (A2/1 [Accession No. AY377788]) from an expression cDNA library screened using immune sera and indigenously established *Leishmania*-specific cell line.[13] The BLAST analysis of cDNA (A2/1) did not show similarity to any existing sequence in the GenBank database, indicating it to be a novel gene. The gene encodes an antigen, which induced the release of IFN-γ from human peripheral blood mononuclear cells in vitro.[14] The expressed recombinant protein (~44kD), termed rA6, when used as prophylactic vaccine along with the adjuvants, monophosphoryl lipid A (MPL-A) and CpG-ODN, although induced macrophage activation and IL-12 production, but conferred only

partial protection in a hamster model of experimentally induced visceral leishmaniasis.[15] With the aim to achieve complete clearance of parasites from the host tissues, in the current study we have tried to explore the immunomodulatory/immunotherapeutic potential of rA6 in terms of strong macrophage activation and efficient parasite clearance, alone as well as in combination with standard anti-leishmanial drugs, miltefosine and liposomal amphotericin B (AmBisome) using an ex vivo infection model. The results indicate that when used alone on peritoneal macrophages, the rA6 induced a strong oxidative burst in these cells leading to a very efficient intracellular clearance of parasites. Interestingly the recombinant protein when used in combination with the known chemo-agents miltefosine or AmBisome, synergized with the anti-leishmanial capacity and facilitated an early clearance of intracellular parasites at even sub-optimal doses of the drugs, thus reducing the duration of drug exposure to the host macrophages. The findings may lead to development of a biopharmaceutical against leishmaniasis, which will have great clinical implications in terms of better management of this disease.

Materials and methods

Parasites and animals

Promastigotes of *Leishmania donovani* strain (MHOM/IN/80/Dd8) from in vitro axenic cultures and virulence maintained though serial passages in Syrian golden hamsters *(Mesocricetus auratus)* in our laboratory were used in the experiments.[16] Inbred strains of BALB/c mice of either sex were used for obtaining peritoneal macrophages. The study was approved by the Institutional Animal Ethics Committee (IAEC Reg no. 47/1999/CPCSEA) of the Post Graduate Institute of Medical Education & Research, Chandigarh, India.

Expression and purification of recombinant proteins

In order to get bulk recombinant A6 protein, the *Escherichia coli* M15 bacteria was transformed with pQE plasmid containing A6 cDNA insert and expressed after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). Histidine-tagged rA6 was purified using nickel-nitriloaceticacid (Ni-NTA) metal affinity chromatography (Qiagen, Valencia, CA, USA) under denaturing condition (excluding the use of toxic imidazole) and later refolded by dialyzing against decreasing concentration of urea following the methods as described earlier. The purified protein fractions were analyzed over 12% SDS-PAGE.[15]

Isolation and treatment of murine peritoneal macrophages

Macrophage monolayers were prepared as described previously.[17] Briefly, peritoneal exudate cells were harvested from BALB/c mice using chilled serum-free RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO, USA). The collected cell suspension was washed and pellet was resuspended in RPMI-1640 supplemented with: 2 mM L-glutamine, 5 mM HEPES buffer, 100 IU/ml penicillin and 100 μg/ml streptomycin, 10% fetal calf serum (Sigma, USA). Cells (1×10^5 cells/ml) were seeded in 24-well culture plate (Nunc, Roskilde, Denmark) and incubated at 37°C in a 5% CO_2 atmosphere (Thermo Fisher Scientific, Waltham, MA, USA). The non-adherent cells were removed and the macrophage monolayer or J774A.1 murine macrophage like cell line (obtained from National Center for Cell Studies, Pune, India) were treated with either bacterial lipopolysaccharide (LPS) (5 μg/ml) or recombinant antigen (rAg) rA6 (2 μg/ml). Macrophages were separately infected with stationary phase *L. donovani* Dd8 promastigotes with multiplicity of infection 1:10. The cells were washed after 5–6 h to remove non-internalized parasites. Some of the infected cells were stimulated with rA6 for an additional 24 h either prior or post infection. The unstimulated cultures which served as control were set up in parallel to obtain the background data in the absence of any particular stimulation.

Dose optimization for rA6

Estimation of nitric oxide activity in peritoneal macrophage and cell line

To optimize the concentration of rA6 causing maximum macrophage activation, nitric oxide (NO) was estimated in culture supernatant after stimulation with different doses of rA6 for different time intervals, by Griess reaction.[18] Bacterial LPS from a commercial source was used as positive control stimulator in all experiments. Equal volumes of culture supernatant were mixed with Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine, both from Sigma, USA, in 5% phosphoric acid) for 10 min at room temperature. The absorbance was measured in an ELISA reader (Perkin Elmer, Waltham, MA, USA) at 540 nm.

In order to quantify the cells releasing NO and reactive oxygen species (ROS), intracellular production was measured in various treated groups using 5, 6 diaminofluorescein-2diacetate (DAF-2DA) and 2′7′-dichlorodihydrofluorescein diacetate (H2DCFDA) respectively (both from Sigma, USA).[19,20] Briefly, macrophages (2×10^5 cells/ml) were infected with *L. donovani* promastigotes (Dd8) and some infected cells were stimulated with rA6 as mentioned previously. After 24 h, cells were de-adhered using accutase (HiMedia, Mumbai, India), washed and labeled with DAF-2DA (2 μM) and H2DCFDA (20 μM) separately for 30 min at 37°C. Cells were subsequently acquired on a flow cytometer (FACS Calibur; Becton Dickinson, San Diego, CA, USA) and analyzed using CellQuest™ software. For all the experiments results were obtained by calculating mean from three independent experiments.

Cytotoxicity assay

To find the concentration of rA6 that causes least cytotoxicity for specific time interval, antigen was titrated in an MTT (3-[4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazoliumbromide]) assay.[21] Briefly, macrophages (1×10^5 cells/well) were treated with different doses of rA6 for different time intervals. MTT (HiMedia, Mumbai, India) solution (5 mg/ml) was added and incubated for 4 h at 37°C. Then 0.04 N HCl (hydrochloric acid) in isopropanol was added and MTT formazan formed was measured spectrophotometrically at 540 nm. The percent cytotoxicity was calculated. For all subsequent experiments, the cell viability was checked by trypan blue exclusion method after completion of each treatment and effector functions were then evaluated as described in the following sections.

Semi-quantitative estimation of mRNA of *iNOS* and *TNF-α* gene

The effect of rA6 on the expression of *iNOS* and *TNF-α* was determined by RT-PCR. Total RNA was extracted from peritoneal macrophages stimulated with rA6 or LPS using Trizol reagent (Sigma, USA). RNA was reverse transcribed to make cDNA copies (MBI Fermentas, now Thermo Fisher Scientific, Waltham, MA, USA), which were then used for the amplification of *iNOS* and *TNF-α* genes by PCR using their specific primers. Primer sequences used were: *iNOS* Forward 5′-ACATCGACCCGTCCACAGTAT-3′, and Reverse 5′-CAGAGGGGTAGGCTTGTCTC-3′; *TNF-α* Forward 5′-CCTCCTGGCC AACGGCATGG-3′, and Reverse 5′-GCAGGGGCTCTTGACGGCAG-3′; *GAPDH* Forward 5′-TGACCACAGTCCATGCCATC-3′, and Reverse 5′-GACGGACACATTGGGGGTAG-3′. The intensities of the product bands were quantified by densitometric scanning of gels (Syngene, Frederick, MD, USA). The mRNA values were normalized to *GAPDH* expression levels.

Evaluation of phagocytic and killing activity of activated macrophages

Staphylococcus aureus count was adjusted (1×10^5 cfu/ml) by OD measurements (OD 600=0.2–0.3). Peritoneal macrophages (unstimulated, infected and antigen treated post infection) were mixed with bacteria in a 1:1 ratio and incubated at 37°C. After 2 h, chilled Hanks' Balanced Salt Solution (HBSS) was added to stop phagocytosis further and centrifuged at 110×g for 4 min in cold. Supernatant containing non-phagocytosed bacteria were diluted, plated onto nutrient agar (NA) plates and viable bacterial colonies were counted after 24 h incubation. Remaining cells with phagocytosed bacteria were incubated in HBSS for 2 h at 37°C, lysed and plated onto NA plates to count viable bacterial colonies. Results were expressed as phagocytic index (PI) and killing index (KI).[22]

$$\text{PI or KI} = \frac{\text{Viable bacterial count at 0 min} - \text{Viable bacterial count at n time}}{\text{Viable bacterial count at 0 min}} \times 100$$

Intracellular parasite clearance study

Flowcytometric analysis

The promastigotes (2×10^7) were labeled with PKH-67dye (2×10^{-6} M) (Sigma, USA) for 5 min and dye quenched with equal volume of fetal calf serum. Parasites were washed and co-incubated with peritoneal macrophages at a ratio of 10:1 for 24 h. The macrophages were stimulated with rA6 post and prior to infection with *Leishmania* promastigotes for an additional 24 h (37°C, 5% CO2). The un-internalized parasites were removed and the cells were acquired on flow cytometer to measure the fluorescence.

Microscopic analysis

Macrophage monolayer was cultured on glass coverslips (2×10^5 cells/18 mm coverslip) in complete RPMI medium and infected with the promastigotes as mentioned earlier. Infected macrophages were further incubated in the presence of rA6 for 1-3 days. Cells were fixed with methanol, stained with Giemsa stain (HiMedia, India), and number of amastigotes per macrophage was calculated.

Assessment of combined effect of rA6 and miltefosine/AmBisome on intracellular

Leishmania *amastigotes*

The range of concentrations of miltefosine and AmBisome spanning the therapeutic levels in clinical use, was used in the in vitro experiments. Mouse peritoneal macrophages infected in vitro with *L. donovani* promastigotes were treated with rA6 (2 μg/ml) either alone or in combination with miltefosine (5–20 μM) or AmBisome (0.2–0.4 μg/ml) for 1–3 days and clearance of intracellular amastigotes was evaluated microscopically.

Endotoxin assay

In order to rule out the presence of endotoxin in the antigen preparation and confirm that macrophage stimulating activities are specifically due to rA6, endotoxin contamination in rA6 was assayed by limulus amebocyte lysate (LAL) method using the Toxin Sensor chromogenic endotoxin LAL assay kit (GenScript, Piscataway Township, NJ, USA). A standard curve was plotted using endotoxin standard concentrations ranging between 0.01 EU/mL and 0.1 EU/mL and endotoxin level in rA6 preparation was determined.

Statistical analysis

Analyses were performed with GraphPad Prism 5 software (GraphPad Inc., San Diego, CA, USA). A two tailed Student's *t*-test was performed and multiple comparisons between different groups were done using ANOVA with Bonferroni correction. The $p<0.05$ was considered significant.

Results

The rA6 stimulated murine peritoneal macrophages produced higher nitric oxide

After stimulation of macrophages with different doses of rA6 (1–10 μg/ml), it was observed that maximum NO was produced when cells were stimulated with 2 μg/ml of rA6 (42.41 μM ±1.48 μM) followed by a decline at doses of 5 μg/ml and above (Supplementary Figure 1A). The NO production from peritoneal macrophages on stimulation for different time intervals with either LPS (5 μg/ml) or rA6 (2 μg/ml) peaked at 24 h after stimulation (64.24±3.92 μM and 46.56±3.22 μM) (Supplementary Figure 1B).

Dose and time kinetics of rA6 induced cytotoxicity of macrophages in vitro

To ascertain the dose-dependent response of recombinant antigens rA6 on macrophages the percentage viability was quantitated after treatment with different doses of rA6 by MTT assay (Supplementary Figure 2A and 2B). The percentage of live cells

was 85.7% and 74.1% at 2 μg/ml of rA6 and 5 μg/ml of LPS respectively. Toxicity was dose-dependent which increased with the increasing dose of rA6 i.e. above 2 μg/ml. It was observed that the dose below 2 μg/ml did not show marked decrease in toxic effects instead started showing a decline in NO level. Lower doses of rA6 (below 2 μg/ml) possessed less stimulatory activity in our experimental set up. Since 2 μg/ml of rA6 induced maximum activation in terms of NO production along with maximum cell viability at 24 h; macrophages were treated with this dose in all subsequent experiments.

Recombinant antigen rA6 augmented NO and ROS production in infected macrophages

Activation of peritoneal macrophages with 2 μg/ml of rA6 post infection induced significantly increased production of nitrites in the culture supernatant (40.12±1.82 μM) as compared to their respective infected-unstimulated cells (15.95±3.33 μM) and marginal but insignificant increase when treated with rA6 prior to infection (37.63±1.52 μM) (Figure 1A). Treatment of J774A.1 cell line with rA6 prior infection (33.12±0.88 μM) and post infection (36.63±1.68 μM) caused increment in NO level which was statistically not significant as compared to control (23.38 ±1.79 μM). An increase in the percentage of peritoneal cells producing intracellular NO (15.25±1.77) was observed when treated with rA6 after infection (Figure 2A and 2C) and this increase was highly significant in case of ROS production (30.6±3.51%) (Figure 3A and 3C). A similar trend was seen in J774A.1 cells with NO (25.22±2.1%) (Figure 2B and 2D) and ROS (33.46 ±3.54%) (Figure 3B and 3D) production when treated with rA6. However, in all the above cases treatment with rA6 prior to infection failed to cause significant increase in the percentage of cells.

Increased expression of *iNOS* and *TNF-α* genes upon antigen stimulation

The mRNA expression analysis of unstimulated and rA6 stimulated peritoneal cells showed significantly higher *iNOS* and *TNF-α* expressions ($p<0.05$) (ethidium bromide stained gel picture in Supplementary Figure 3) after stimulation with rA6 in both uninfected (3.51±2.18 vs 34.24±2.98 and 13.65±3.09 vs 46.820±4.957) and infected conditions (1.33±0.733 vs 31.47 ±3.45 and 8.617±3.309 vs 29.37±5.26), respectively (Figure 4A and 4B).

The rA6 treatment enhanced the phagocytosing and killing abilities of macrophages

The PI and KI of rA6 treated macrophages exposed to *Staphylococcus aureus* bacteria was significantly higher than the non-treated cells (uninfected [59.00±5.29] or infected cells [47.00±3.46]) (Figure 5A) and (uninfected [40.04±2.02] or infected cells [33.97±2.48]) (Figure 5B) respectively which indicated a significant improvement in the macrophage microbicidal functions post Ag-treatment.

The rA6 antigen stimulation leads to intracellular parasite clearance

On exposure to rA6 prior to or after infection with PKH-67-labeled parasites, the macrophages showed a significant ($p<0.01$) decrease in mean fluorescence intensity (MFI) (MFI=475.6±73.11 and MFI=427.33±62.61, respectively) as compared to fluorescence emitted by untreated-infected cells (MFI=828.65±95.28) (Figure 6A), indicating a facilitated clearance of intracellular parasites after rA6 exposure. There was a concomitant significant decrease in the percentage of cells positive for PKH-67 fluorescence after treatment with rA6 before (56.57±4.25) and after infection (44.47±5.52) (Figure 6B), as compared to untreated cells.

The rA6 synergizes with miltefosine and AmBisome facilitating early clearance of intracellular amastigotes

Since the doses above 10 μM of miltefosine and 0.3 μg/ml of AmBisome showed significant cytotoxicity towards macrophages (Supplementary Figure 4A and 4B), we thought of

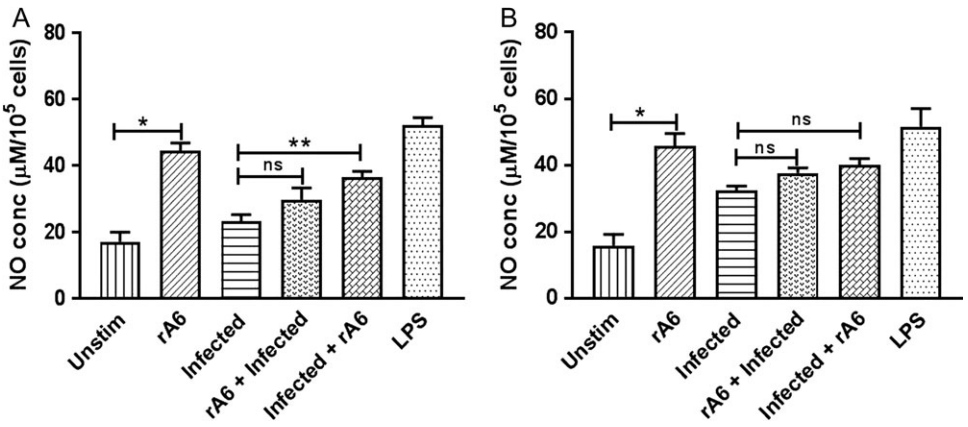

Figure 1. Nitric oxide (NO) production in peritoneal macrophages and J774A.1 cell line. Histograms showing production of NO in culture supernatant of (A) peritoneal macrophages and (B) J774A.1 cell line when stimulated with rA6 before and after infection. Data are shown as mean±SEM (n=3). *p<0.05; **p<0.01; LPS: lipopolysaccharide; ns: not significant.

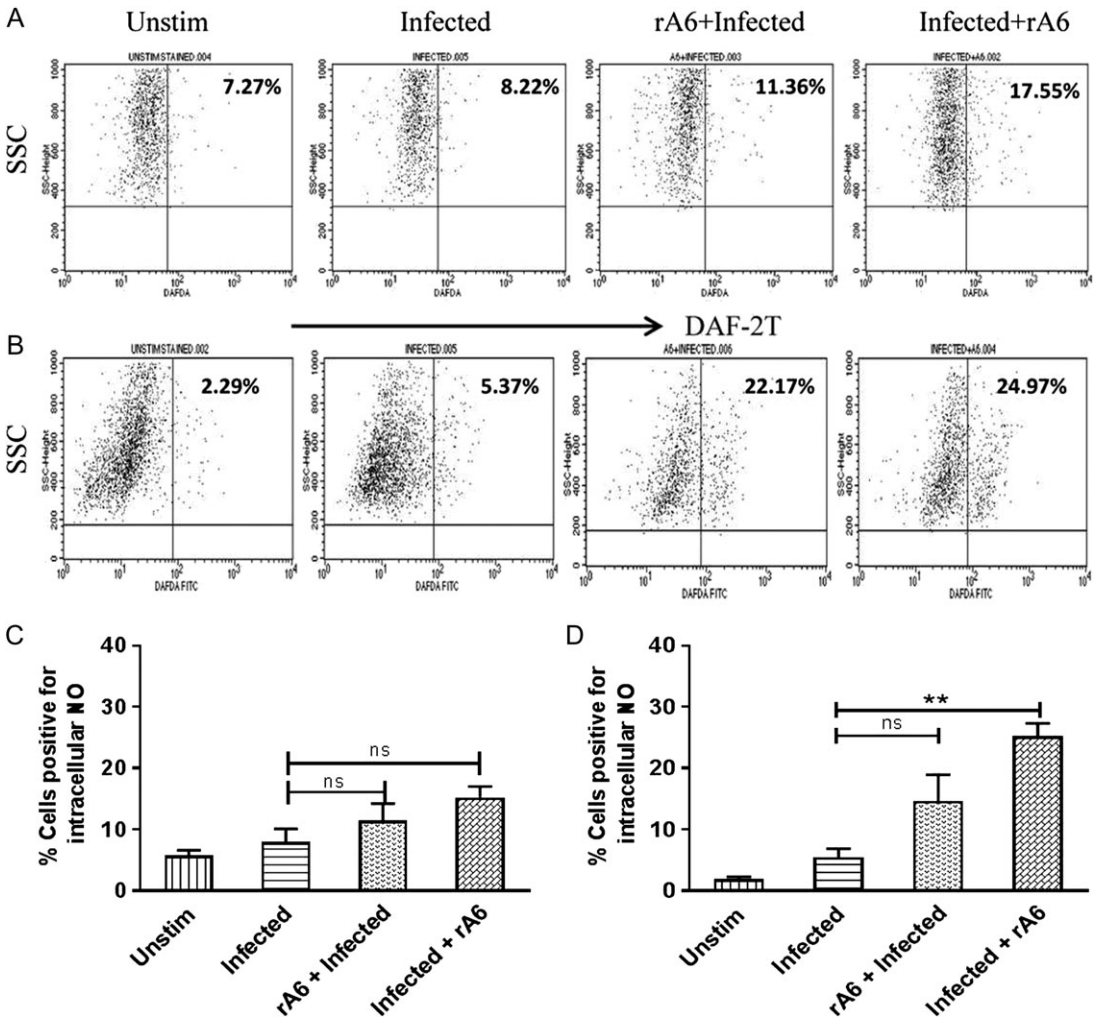

Figure 2. Generation of intracellular nitric oxide (NO). Representative Dot Plots for (A) peritoneal macrophages and (B) J774A.1 cell line showing increase in the percent positive cells for intracellular NO when treated with rA6 before and after infection. Graph showing percent positive cells for intracellular NO in (C) peritoneal cells and (D) J774A.1 cell line under different treatment conditions. Data are shown as mean±SEM (n=3). **p<0.01; ns: not significant; SSC side scatter.

evaluating the combinatorial effect of immune-modulation by antigen in addition to anti-leishmanial effects of these drugs on intracellular amastigotes. Microscopic analysis of Giemsa stained infected macrophages after incubation with 10 μM miltefosine (Figure 7) and 0.4 μg/ml AmBisome (data not shown) caused only about 90–95% reduction in intracellular parasite burden after 72 h treatment. However, the combination of miltefosine/AmBisome at different concentrations along with rA6 was significantly ($p<0.01$) more potent in clearance of intracellular parasites than alone (Figure 8A and 8B). But the most striking finding was that the rA6 caused almost complete clearance of amastigotes at even sub-optimal doses of miltefosine (10 μM at 48 h) ($p<0.05$) and AmBisome (0.3 μg/ml at 72 h) ($p<0.01$) without affecting the cell viability. Microscopically, the macrophages showed empty parasitophorous vacuoles indicative of intracellular killing of the parasites. Overall, the findings indicate that rA6 treatment caused an early clearance of parasites from macrophages at even sub-optimal dose of anti-leishmanial drugs.

The stimulatory potential of rA6 was not due to endotoxin contamination

Endotoxin concentration in rA6 preparation was found to be 0.0028 EU/mL. This level was below the lowest detection limit (0.005 EU/mL) indicating that the stimulatory activities of rA6 in the present study were not due to endotoxin contamination.

Discussion

The combination of chemotherapy with immunomodulators may improve the treatment by increasing the efficacy of anti-leishmanial drugs through activating the immune cells along with decreasing the toxicity of drugs by achieving almost complete parasite clearance with relatively low doses of drug.[23] The present work describes the immunomodulatory and anti-leishmanial activity of rA6 and its role in activating macrophages to clear the infection.

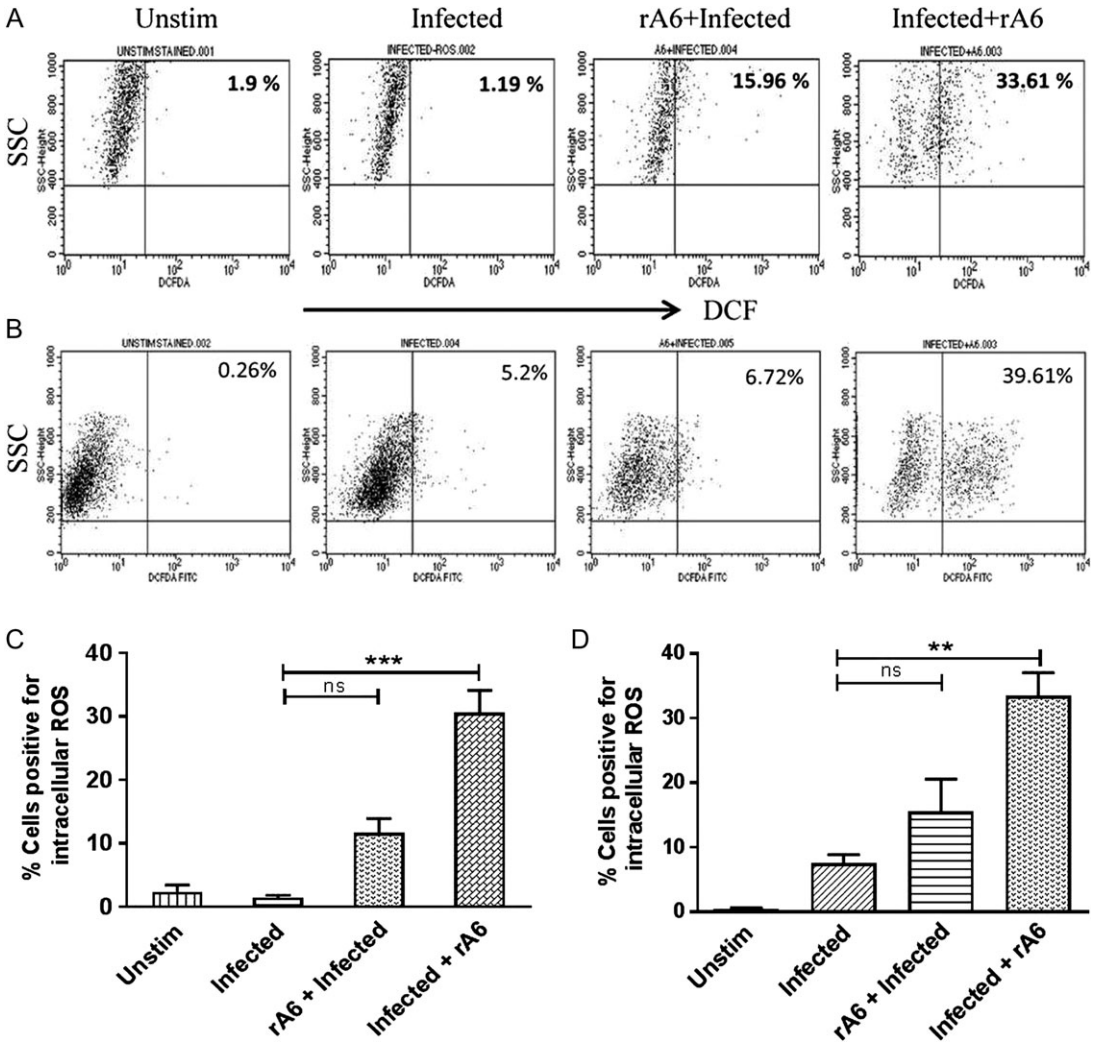

Figure 3. Generation of intracellular reactive oxygen species (ROS). Representative dot plots for (A) peritoneal cells and (B) J774A.1 cell line show significant increase in the percentage of positive cells for intracellular ROS when treated with rA6 after infection. Graph showing percent positive cells for intracellular ROS in (C) peritoneal cells and (D) J774A.1 cell line under different treatment conditions. Data are shown as mean±SEM (n=3). **p<0.01; ns: not significant; SSC side scatter.

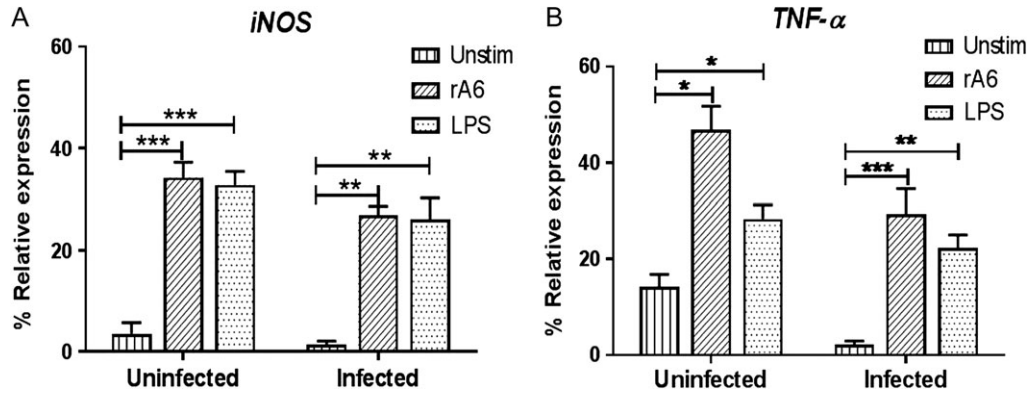

Figure 4. Semi-quantitative RT-PCR analysis of *iNOS* and *TNF-α* mRNA expression. Murine peritoneal macrophages were stimulated with rA6 (2 μg/ml) and LPS (5 μg/ml) under uninfected and infected conditions and RNA was isolated at 24 h. The percent relative expression of (A) *iNOS* and (B) *TNF-α* was calculated after normalizing to *GAPDH* gene. Data are shown as mean±SEM (n=3). *p<0.05; **p<0.01; ***p<0.001. LPS: lipopolysaccharide.

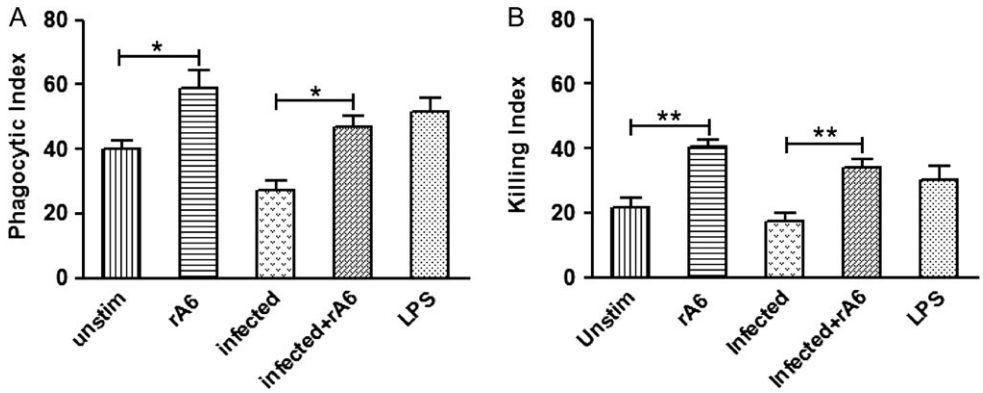

Figure 5. Bacterial phagocytosing and bacterial killing activity of rA6 treated macrophages. The unstimulated, rA6 stimulated, promastigote infected and promastigote infected+rA6 treated macrophages were incubated with *Staphylococcus aureus*. Supernatant containing unphagocytosed bacteria and cell pellet with phagocytosed bacteria were plated onto nutrient agar plates and viable bacterial colonies were counted to calculate (A) Phagocytic index and (B) Killing index. Data are shown as mean±SEM (n=3). LPS: lipopolysaccharide; *p<0.05; **p<0.01.

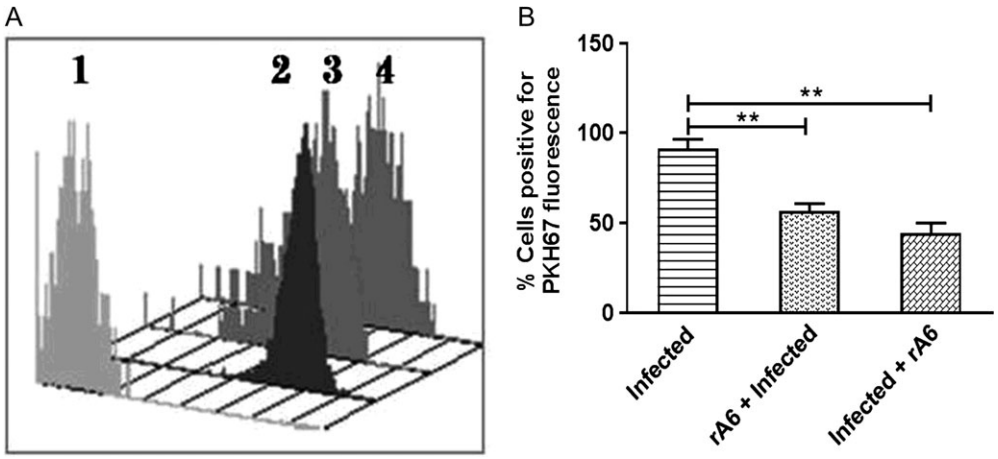

Figure 6. Intracellular parasite clearance. (A) A representative histogram showing fluorescence intensity of differently treated peritoneal macrophages. PKH-67 fluorescence was measured on a log scale in FL1 channel. Peak 1-unstained cells, 2-cells treated with rA6 post-infection, 3-cells treated with rA6 prior to infection, 4-untreated infected cells. (B) Data shows percentage of macrophages positive for PKH-67 fluorescence when treated with rA6. Data are shown as mean±SEM (n=3) from three independent experiments. **p<0.01.

Leishmania infection is known to cause downmodulation of NO and ROS production in macrophages, which helps the parasite to survive in the hostile intracellular environment.[24,25] Any agent that potentiates the oxidative burst would help the macrophage to clear the infection. The immunomodulatory role of a novel antigen rA6, cloned in our laboratory, was evaluated in terms of inducing oxidative burst in an *ex vivo* infection model using mouse peritoneal exudate macrophages as well as J774A.1 human macrophage like cell line. The results clearly indicate that it could induce the production of NO and ROS in a dose and time-dependent manner with a maximal production at 24 h after stimulation with a selected dose of 2 μg/ml. These findings suggest that a dose of 2 μg/ml of rA6, in spite of causing 15% toxicity, is potent enough to enhance the effector functions of the remaining 85% of viable macrophages, including infected as well as bystander cells. Few earlier studies have also reported maximal nitrite accumulation in supernatant at 24 to 48 h after stimulation of bone marrow-derived macrophages or monocyte-derived macrophages with *Salmonella Dublin* or with LPS.[26,27] Our experiments demonstrated an accumulation of intracellular NO and ROS under infected conditions when stimulated with rA6, suggesting that the antigen activates more number of infected as well as bystander macrophages to produce large quantities of NO and ROS. These findings clearly demonstrate that rAg is capable of modulating the effector functions of host macrophages to produce two most potent leishmanicidal molecules, leading to clearance of intracellular parasites.

The increased production of NO by macrophages on stimulation with rA6 was found to be coupled with increased expression of iNOS along with marked upregulation of TNF-α gene, which has earlier been proposed to function in an autocrine manner to induce *iNOS* gene expression by macrophages and induces NO production.[27] The antigen stimulation augmented the macrophage microbicidal functions as evidenced by increased PI and KI after in vitro exposure of *S. aureus* coupled with an increased ability to clear the intracellular *Leishmania*. These results suggest that rA6 can activate the macrophages to induce high

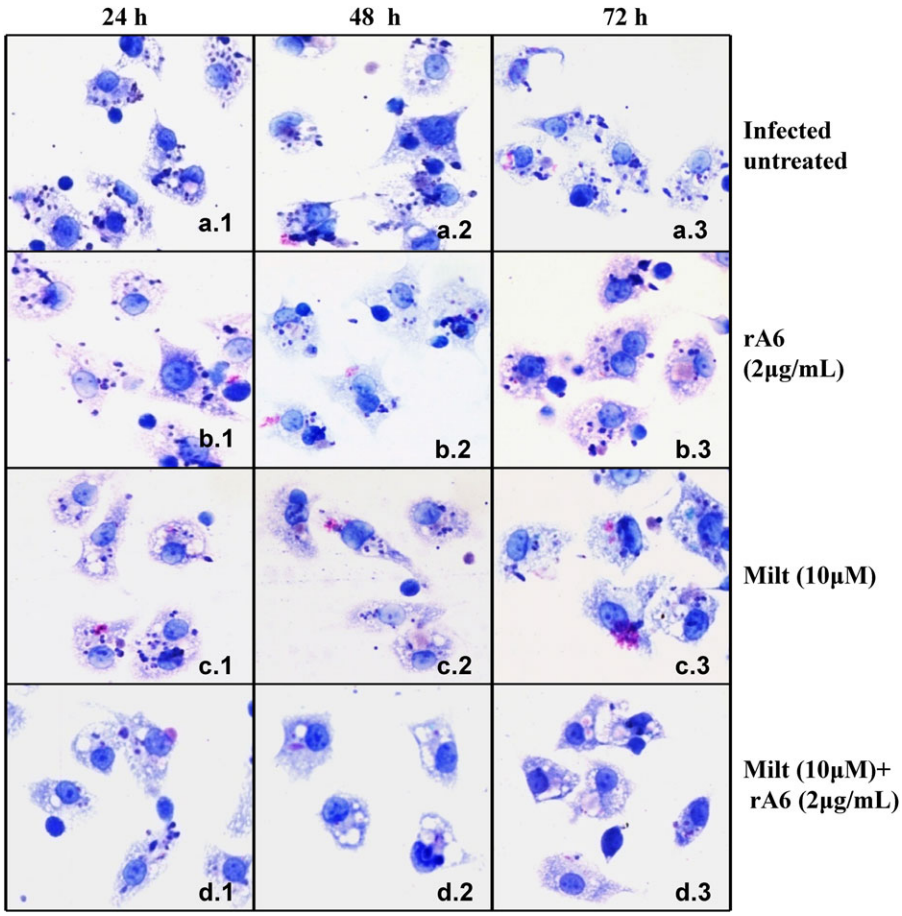

Figure 7. Synergistic effects of rA6 and miltefosine against intracellular amastigotes. The micrograph shows representative images from bright field optical microscopy (magnification 1000X) of Giemsa stained macrophages at 24 h, 48 h and 72 h of treatment; a: infected and untreated control cells; b: treated with rA6 (2 µg/ml); c: treated with miltefosine (10 µM); d: treated with miltefosine (10 µM) and rA6 (2 µg/ml). Please note early parasite clearance (after 48 h) in cells treated with miltefosine and rA6 (panel d.2) which could not be achieved as effectively even after 72 h of treatment with either rA6 or miltefosine alone (panel b.3 and c.3).

production of important mediators contributing to the clearance of intracellular amastigotes.

The most remarkable finding of this study was that the rA6 stimulation synergizes with anti-leishmanial killing abilities of chemo-agents like miltefosine and AmBisome, by facilitating faster clearance of intracellular amastigotes even at their sub-optimal doses. These results highlight the synergistic effects of rA6 together with anti-leishmanial drugs in not only early clearance of intracellular parasites via enhanced effector functions of macrophages, but also in reducing the toxicity of drugs due to shorter treatment regimens with lower effective doses. This may have very significant clinical implication for effective management of this infection and decreasing the chance of drug-resistance development.

Limitations of the study

Although the study has clearly demonstrated the immunomodulatory potential of rA6 in an ex vivo infection model, yet an in vivo validation of the current findings in experimental animal model is lacking. The in vivo experiments coupled with the development of a suitable systemic delivery system for rA6 to reach infected lymphoid organ would be interesting. The effects of rA6 on T cell functions and macrophage activating cytokines secreted by activated T cells have not been explored which might be an interesting aspect to study. Despite these limitations, the present study provides compelling evidence that rA6 possess host cell activating properties and may act as a potent therapeutic agent.

Conclusions

Our report on rA6 with a revelation that it plays an immunomodulatory role in activating the effector functions of parasitized macrophages and reduces the intracellular parasite burden, places it as a promising candidate for an adjunct therapy. Further studies are being undertaken to develop a biopharmaceutical translation out of this protein encouraging trials of this potentially superior therapeutic approach against leishmaniasis.

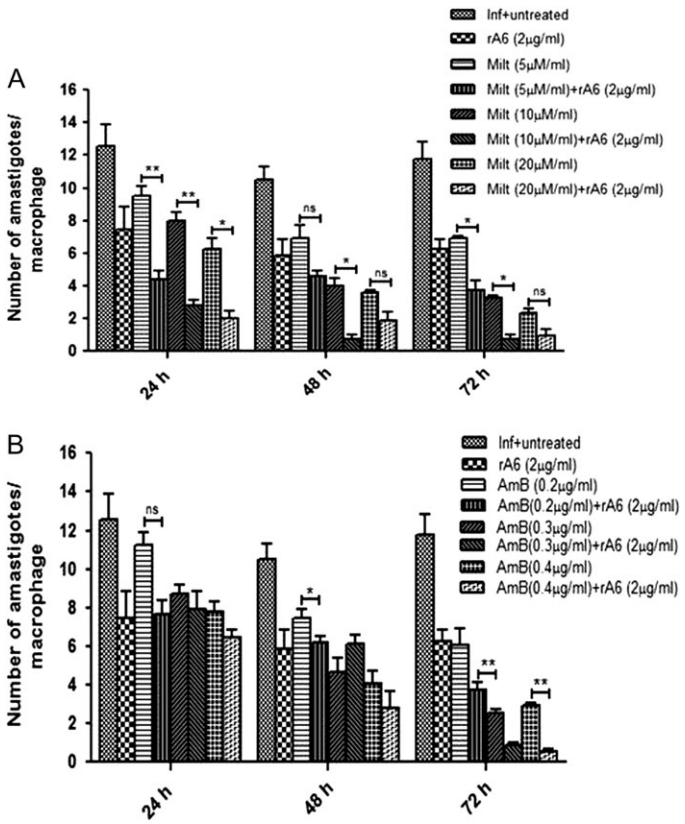

Figure 8. Effect of rA6 treatment, alone or in combination with (A) miltefosine or (B) AmBisome, on infected macrophages. Data shows the mean number of amastigotes per macrophage determined by counting under a light microscope after treatment with different combinations of rA6 with miltefosine or AmBisome. The effect of rA6 in combination with miltefosine (10 μM) after 48 h and AmBisome (0.3 μg/ml) after 72 h time point in clearing intracellular parasites is highly notable. Each time point represents mean±SEM (n=3).

Supplementary data

Supplementary data are available at Transactions online (http://trstmh.oxfordjournals.org/).

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