Unresponsiveness of *Mycobacterium w* vaccine in managing acute and chronic *Leishmania donovani* infections in mouse and hamster

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SUMMARY

The role of *Mycobacterium w* (*Mw*) vaccine as an immunomodulator and immunoprophylactant in the treatment of mycobacterial diseases (leprosy and pulmonary tuberculosis) is well established. The fact that it shares common antigens with leishmanial parasites prompted its assessment as an immunostimulant and as an adjunct to known anti-leishmanials that may help in stimulating the suppressed immune status of *Leishmania donovani*-infected individuals. The efficacy of *Mw* vaccine was assessed as an immunomodulator, prophylactically either alone or in combination with anti-leishmanial vaccine, as well as therapeutically as an adjunct to anti-leishmanial treatment in *L. donovani*-infected hamsters, representing a chronic human Visceral Leishmaniasis (VL) model. Similarly, its efficacy was also evaluated in *L. donovani*-infected BALB/c mice, representing an acute VL model. The preliminary studies revealed that *Mw* was ineffective as an immunostimulant and/or immunoprophylactant in hamsters infected with *L. donovani*, as estimated by T-cell immunological responses. However, in the BALB/c mice-VL model it appeared as an effective immunostimulant but a futile prophylactic agent. It is therefore inferred that, contrary to its role in managing tuberculosis and leprosy infections, *Mw* vaccine has not been successful in controlling VL infection, emphasizing the need to find detailed explanations for the failure of this vaccine against the disease.

Key words: *Mycobacterium w* vaccine, immunomodulator, *Leishmania donovani*, T-cell responses, parasite burden, hamster, mouse.

INTRODUCTION

Visceral leishmaniasis (VL), caused by the invasion of the reticulo-endothelial system by the protozoan parasite *Leishmania donovani* (LD), is the most destructive type among a complex of leishmaniasis (Desjeux *et al*. 2001). The characteristic feature of VL is immunosuppression, modulating the T-cell responses towards the Th2 type that helps in disease progression; thereby, the immune system is not always able to play its natural role to control the disease or impair the desired effects of the drugs (Goto and Lindoso, 2004). To circumvent immunosuppression, immunostimulation of the infected host presents a logical and viable alternative (Garg *et al*. 2006).

Mycobacterium spp. are known to potentiate non-specific immune responses of the host besides sharing antigens common to leishmanial parasites (Frommel *et al*. 1988). *M. bovis* (BCG), though effective against human and experimental leishmaniasis, is not recommended as a mass-immunizing agent due to inherent drawbacks (Fortier *et al*. 1987; Frommel and Lagrange, 1989). Among other immunogenic and non-pathogenic strains of *Mycobacterium*, namely *M. habana* and *Mycobacterium w* (*Mw*), the former showed some level of protection against VL (Sharma *et al*. 1998). This emphasizes the need to search for an effective alternative, which renewed the interest in *Mw*, a candidate leprosy vaccine strain – still unexplored in the case of VL. A widely used immunomodulator-*Mw*, a non-pathogenic, quickly growing atypical mycobacterium, isolated from a patient's sputum sample in India, has been proven to be effective and safe as an adjunct to multidrug therapy in multibacillary (MB) leprosy patients (Zaheer *et al*. 1993) and as immunoprophylactant against pulmonary tuberculosis (Gupta *et al*. 2009; Katoch *et al*. 2008). Unlike BCG, it retains immunogenicity even after it is killed (Singh *et al*. 1992; Zaheer *et al*. 1993; Horwitz and Harth, 2003). This led to its assessment, for the first time, against experimental VL as an immunostimulant as well as an adjunct to anti-leishmanials that may help in altering the course and intensity of infection by modulating the immune status of the hosts–LD infected golden hamster (*Mesocricetus auratus*), representing as chronic VL infection model resembling clinicopathological features of human and in inbred

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BALB/c mouse, representative of an active VL infection and a suitable model for immunological studies.

For immunotherapy we have used the *Mw* vaccine as an adjunct to autoclaved *Leishmania donovani* promastigote antigen because, in our earlier studies, we observed that vaccination with ALD in combination with BCG showed excellent prophylactic efficacy against LD infection (Srivastava *et al*. 2003). Miltefosine is the only orally effective drug and is regarded as the second-line of treatment for *L. donovani* infection (Gupta *et al*. 2011). Since, for immunotherapy, removal of the suppression is required together with establishing the *Leishmania*-specific immune responses, we have combined the use of *Mw* and Miltefosine in experimental *L. donovani* infection.

MATERIALS AND METHODS

Animals and infection

Laboratory bred male inbred hamsters (45–50 g) as well as BALB/c mice (18–20 g), from the Institute's animal house facility were used as experimental hosts. They were housed in a climatically controlled room and fed with standard rodent food pellet (Lipton India Ltd., Bombay) and water *ad libitum*.

Parasites

Leishmania donovani (MHOM/IN/80/Dd8), a World Health Organization reference strain, was obtained from Imperial College London and maintained through *in vivo* serial passages in hamsters. For bulk cultivation promastigotes were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM $NaHCO_3$, 20 mM HEPES, along with 10% fetal bovine serum (FBS) (GIBCO, USA) at 26 °C (Garg *et al*. 2006). Parasites were harvested on days 4–5 of culture.

Preparation of soluble L. donovani *promastigote antigen (SLD), autoclaved* L. donovani *promastigote (ALD) and* Mycobacterium w (Mw) *vaccines*

SLD was prepared according to the method described earlier (Gupta *et al*. 2007). Briefly, late log phase promastigotes (10^9) were harvested from 3 to 4 days of culture and washed 4 times in cold phosphate-buffered saline (PBS) and re-suspended in PBS containing a protease inhibitor cocktail (Sigma, USA) and subjected to ultrasonication and centrifugation at 40 000 ***g*** for 30 min. The protein content of the supernatant was estimated and stored at −70 °C.

ALD vaccine was prepared as described previously (Misra *et al*. 2001). In brief, freshly harvested (1×10^{10}) stationary phase promastigotes of *L. donovani*-Dd8 strain were suspended in 5 ml of PBS in a screw-capped glass vial under sterile conditions. The vials were autoclaved (at 15 lb) for 30 min. The protein content was measured and the antigen was stored at 4 °C until use.

Mw vaccine was the kind gift by Cadila Pharmaceuticals Ltd, India, under the DBT collaborative programme 'The *Mycobacterium w* Genome Program: Complete Genome Sequencing and Genomics'. The attenuated $0{\cdot}5 \times 10^9$/ml stock solution of *Mw* bacilli was further suspended in phosphate-buffered saline (PBS) to attain the desired doses for use in different experimental protocols.

Assessment of lymphoproliferative responses (LTT assay)

Mesenteric and inguinal lymph nodes of hamsters were excised aseptically and processed for the isolation of lymphocytes (Kumari *et al*. 2008*a*). The lymphocytes were suspended to 10^6/ml of culture medium and cultured at 10^5 cells/well in 96-well flat bottomed tissue culture plates (Nunc, Denmark). Then 100 μl of concanavalin A (ConA) (10 μg/ml; Sigma) or SLD (10 μg/ml) were added to each well in triplicate. Wells without stimulants served as negative controls. Cultures were incubated at 37 °C in a CO_2 incubator for 3 days when stimulated with Con A and for 5 days when stimulated with SLD. At 18 h prior to termination of culture, 0·5 μCi of [^{3}H] thymidine (BARC, India) was added to each well and cells were harvested on glass-fibre mats (Whatman); radioactivity was counted in a liquid scintillation counter. Results were expressed as stimulation index (SI) which was calculated as mean counts per minute (cpm) of stimulated culture/mean cpm of unstimulated control. SI values of >2·5 were considered as a positive response. An alternative to the above described method, the lymphproliferative response, was also assayed by XTT kit (Roche) according to the manufacturer's instruction.

Assessment of the level of NO activity

The presence of nitrite (NO_2^-) content was assessed using the Griess reagent in the culture supernatants of naïve hamster peritoneal macrophages after exposure with the supernatant of lipopolysaccharide (LPS) or SLD-stimulated lymphocyte cultures (Kumari *et al*. 2008*a*). Briefly, isolated peritoneal macrophages were suspended in culture medium and plated at 10^5 cells/well and exposed to the supernatants of 3-day-old (stimulated with LPS) and 5-day-old (stimulated with SLD) lymphocyte cultures from all the study groups. The supernatants (100 μl) collected from macrophage cultures 24 h after incubation were mixed with an equal volume of Griess reagent (Sigma, USA) and left for 10 min at

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room temperature. The absorbance of the reaction was measured at 540 nm.

Estimation of expression of mRNA cytokines by real-time PCR

Real-time PCR was performed to evaluate the expression of mRNAs for various cytokines and inducible nitric oxide synthase (iNOS) in splenic cells as described previously (Rama Iniguez *et al*. 2006; Samant *et al*. 2009). Briefly, total RNA from splenic tissues of all groups was isolated using Tri-reagent (Sigma-Aldrich) and quantified by using Gene-quant (Bio-Rad). One microgram of total RNA was used for the synthesis of cDNA using a first-strand cDNA synthesis kit (Fermentas). Real-time quantitative PCR was done with 12·5 μl of SYBR green PCR master mix (Bio-Rad), 1 μg of cDNA, and primers at a final concentration of 300 nM in a final volume of 25 μl. PCR was carried out under the following conditions: initial denaturation at 95 °C for 2 min followed by 40 cycles, each consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 40 s per cycle using the iQ5 multicolor real-time PCR system (Bio-Rad). cDNAs from the infected control group were used as 'comparator samples' for quantification of those corresponding to test samples. All quantifications were normalized to the housekeeping gene HGPRT. A no-template control cDNA was included to eliminate contamination or non-specific reactions. The cycle threshold (C_T) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). Differences in gene expression were calculated by the comparative C_T method. This method compares test samples to a comparator sample and uses results obtained with a uniformly expressed control gene, hypoxanthine phosphoribosyl transferase (HPRT), to correct for differences in the amounts of RNA present in the 2 samples being compared to generate a ΔC_T value. Results are expressed as the degrees of difference between ΔC_T values of test and comparator samples. Higher value of ΔC_T indicates lesser expression and vice versa.

Production of reactive oxygen species (ROS) by peritoneal macrophage (PM) cells

ROS in PM cells of mice was determined through a fluorometric assay using 2′,7′-dichlorofluorescin diacetate (DCF-DA) (Sigma, USA) on FACS according to the previously described protocol (Zurgil *et al*. 2006) with minor modification, as described earlier (Singh *et al*. 2009). The ROS level in an individual living cell was determined by measuring the fluorescence intensity on FACS Calibur. Briefly, freshly harvested PM of both treated and untreated groups were adjusted to a concentration of 1×10^6 cells/ml in PBS, washed thrice with PBS and transferred to FACS tubes (1×10^6 cells/tube). For probe loading, cells were incubated with the DCF-DA for 15 min at 37 °C at a final concentration of 1 μM, washed twice in PBS and the ROS level in individual living cells was determined by measuring the fluorescence intensity on FACS Calibur. Data were analysed by CellQuest Software (Becton Dickinson, USA) and mean ROS values were evaluated for the cell population of an individual animal.

Measurement of $CD4^+$/$CD8^+$ T lymphocyte population

Cell surface staining was carried out in the mouse spleen cells to assess the lymphocyte population by FACS using fluorochrome-conjugated monoclonal antibodies (BD, San Diego, CA, USA) directed against different CD antigens, namely, CD4 (fluorescein isothiocyanate or FITC) and CD8 (phycoerythrin or PE) following the manufacturer's protocol as described earlier (Vedi *et al*. 2008). Single-cell suspensions of splenocytes (1×10^6 cells) were initially blocked with Mouse Seroblock FcR at room temperature (RT) for 10 min, washed and labelled with FITC-Rat anti-mouse antibodies directed against CD4 for 10 min at RT and finally incubated with PE-rat anti-mouse antibodies directed against CD8 for another 10 min. Another tube served as control with no labelling. The cell pellet was suspended in sheath fluid and analysed on FACS using CellQuest analysis software (Becton-Dickinson, San Diego, CA, USA) after gating the forward- and side-scatter settings to exclude debris. For each determination 10 000 cells were analysed and the results expressed as a percentage for each cell population.

Measurement of intracellular Th1 and Th2 cytokines

The measurement of intracellular cytokines in the mouse splenocytes was done according to the manufacturer's instructions using antibodies and reagents from BD (San Diego, CA, USA). Briefly, splenocytes (2×10^6/ml) were incubated with Brefeldin A (10 μg/ml) in the dark for 6 h in a CO_2 incubator at 37 °C, re-incubated with mouse Seroblock FcR for another 10 min and washed in PBS to further add FITC-Rat anti-mouse CD4 antibody. Leucoperm A and Leucoperm B (Serotec, UK) were added at RT for 15 min each and cells were dispensed in 2 tubes each containing 1×10^6 cells/100 μl of PBS. PE-rat anti-mouse monoclonal antibodies to cytokines IL-10 and IFN-γ were added individually into 2 separate tubes, cells were washed and finally suspended in 250 μl of PBS containing 0·5% paraformaldehyde for FACS readings (Soni *et al*. 2011).

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Assessment of the immunomodulatory activity of Mw *vaccine*

(A) In peritoneal macrophages of naïve hamsters in vitro *by NO estimation.* The presence of nitrite (NO_2^-) content was assessed using Griess reagent in the culture supernatants of naive hamster PM after incubation with *Mw* bacilli in the ratio of *Mw*: macrophages 10:1, 25:1, 50:1, 100:1, 150:1, 200:1, and 250:1. Briefly, isolated PM (Garg *et al*. 2005) were suspended in RPMI-1640 medium supplemented with 10% FBS and plated at 10^5 cells/well and incubated with *Mw* in the above described ratio of *Mw* to macrophages. The supernatants were collected from macrophage cultures at 24 h and 48 h for estimation of NO production (Kumari *et al*. 2008*a*).

(B) In vivo *immunomodulatory activity of* Mw *in naive hamsters.* To assess the immunomodulatory activity of attenuated *Mw* vaccine, the bacilli were injected intradermally (i.d.) at doses of 1×10^3, 1×10^4 and 1×10^5 to 3 groups of 5 hamsters, while an unvaccinated fourth group served as control. Animals were euthanized on day 30 post-vaccination (p.v.) to dissect out lymph nodes as well as spleen aseptically for the isolation of lymphocytes, and RNA to perform LTT, NO production assays and qRT-PCR.

Assessment of Mw *vaccine in adjunct with Miltefosine in infected hamsters*

To evaluate an adjunct efficacy of *Mw* vaccine (1×10^4 bacilli, intradermally) with the standard anti-leishmanial drug Miltefosine (ED_{50} dose: 20 mg/kg per dose × 5 days), the following 7 experimental groups were assigned. The 1st group: normal uninfected hamster; 2nd group: infected hamster; 3rd group: infected hamster with *Mw* weekly; 4th group: infected hamster with *Mw* weekly + miltefosine; 5th group: infected hamster with *Mw* biweekly; 6th group: infected hamster with *Mw* biweekly + miltefosine and the 7th group: infected hamster with miltefosine only. Infected hamsters were treated with miltefosine and *Mw* on day 25 post-infection (p.i.) using the aforementioned schedules. The assessment of parasitic burden and immune responses (LTT and NO production) in all groups was done on day 60 p.i.

Adjunct efficacy of Mw *vaccine with autoclaved leishmania antigen (ALD) vaccine*

Mw vaccine was further evaluated for its adjunct efficacy with ALD vaccine against LD challenge in hamsters. Vaccination with the optimal dose of *Mw* (1×10^4 bacilli), ALD (1 mg/animal) and BCG (0·1 ml/animal) was initiated in 5 groups of 5 hamsters each as follows: Animals of groups 1 and 2 were injected i.d with *Mw* + ALD and ALD + BCG respectively and those of groups 3, 4 and 5 were given *Mw*, ALD and BCG alone. Hamsters belonging to groups 1–5 were challenged intracardially (i.c.) on day 30 p.v. with 1×10^7 amastigotes and euthanized 30 days later to assess parasite burden and immunological responses generated thereof.

Assessment of immunomodulatory and prophylactic efficacy of Mw *in BALB/c mice*

In addition to hamsters, immunomodulatory and prophylactic studies were also carried out in inbred BALB/c mice. Mice were randomly distributed in 4 groups each carrying 6 animals, namely, untreated control, LD infected, *Mw* vaccinated and *Mw* vaccinated + LD challenged (*Mw* + LD). For the optimal dose, 9×10^4 *Mw* bacilli were suspended in 100 μl of PBS and administered intradermally to BALB/c mice of *Mw*-vaccinated and *Mw* + LD groups on day 0 and subsequently on day 7 (1st booster) post-initiation of the experiment. LD-infected and the *Mw* + LD group were challenged intravenously with 10^7LD amastigotes on day 10 while untreated control animals received 100 μl of PBS as vehicle. On day 30 all BALB/c were euthanized humanely to determine parasite burden as well as their cellular immune responses and intracellular content of Th1 and Th2 cytokines (IL-2 and IL-4, respectively).

Ethics statement

Experiments on the animals (hamsters and BALB/c mice) were performed following the approval of the protocol and the guidelines of the Institutional Animal Ethics Committee of Central Drug Research Institute (127/08/Para/IAEC dated 27.08.2008) which follows the guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals under the Ministry of Forest and Environment, Govt. of India.

Statistical analysis

Results were analysed by one-way ANOVA followed by Dunnet's or Tukey's post-test wherever appropriate using GraphPad Prism (version 3.03) software and are expressed as mean ± S.D. of 5 hamsters/6 mice per group at designated time-points.

RESULTS

In vitro *assessment of immunomodulatory activity of* Mw

The effect of *Mw* vaccine, when assessed *in vitro* at different concentrations in peritoneal macrophages by NO estimation, was observed to have increased

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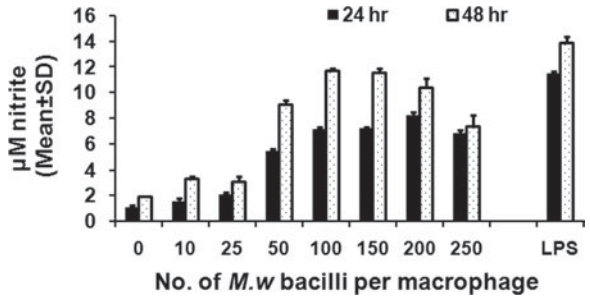

Fig. 1. *In vitro* assessment of immunomodulatory efficacy of *Mw* by estimation of NO production. Peritoneal macrophages of naïve hamsters were co-incubated with *Mw* at a ratio of *Mw*: macrophages 10:1, 25:1, 50:1, 100:1, 150:1, 200:1, and 250:1. Supernatant was collected from each well at 24 h and 48 h and NO production was estimated.

(in nitrite level) at a ratio of 50:1 of *Mw* to macrophages. Maximum nitrite level was found at 100:1 ratio which was, however, inhibited when the ratio was increased up to 250:1 (Fig. 1).

Immunomodulatory efficacy of Mw *vaccine in hamsters*

The efficacy of *Mw* vaccine was also assessed in naïve hamsters at 3 doses of *Mw* vaccine, namely, 1×10^3, 1×10^4 and 1×10^5 bacilli. Only the unvaccinated normal group of hamsters showed higher proliferative responses as assessed by LTT in comparison to all the *Mw* vaccinated groups ($P<0{\cdot}01$) when stimulated with mitogen Con A. However, all the experimental as well as control groups exhibited negligible responses to SLD (Fig. 2A). There was no change in NO content in PM of naïve hamsters co-incubated with the supernatant of lymphocytes isolated from *Mw*-vaccinated hamsters and stimulated with LPS or SLD ($P>0{\cdot}05$) (Fig. 2B).

The expression of iNOS transcripts, Th1 cytokines (TNF-α, IFN-γ and IL-12) and Th2 cytokines (IL-4 and IL-10) in *Mw*-vaccinated groups was found to be expressed at the same level as was observed in the control group ($P>0{\cdot}05$). A noteworthy up-regulation was observed in TGF-β expression only in *Mw*-treated hamsters at all doses over the normal group of hamsters (Fig. 2C and D).

Efficacy of Mw *vaccine as an adjuvant*

The effectiveness of *Mw* vaccine as an adjunct to multidrug therapy was previously worked out in MB leprosy patients (Zaheer *et al*. 1993). On the basis of these findings, *Mw* vaccine was further tested for its adjunct efficacy with standard anti-leishmanial drug (Miltefosine) as well as immunogen (ALD) against experimental VL.

(A) With anti-leishmanial drug-Miltefosine. There was no improvement in therapeutic efficacy of miltefosine when given along with *Mw* vaccine in hamsters as a high parasite count was observed as compared to the infected control. ($P>0{\cdot}05$) (Fig. 3A). In addition, there was no proliferative response and NO production in the cells of *Mw* and *Mw* + Miltefosine treated groups against mitogens and SLD (Fig. 3B and C).

It was observed that *Mw* was unable to improve the chemotherapeutic efficacy of Miltefosine as a high parasite count in the animals treated with *Mw* + Miltefosine comparable to the infected control ($P<0{\cdot}05$) was obtained (Fig. 4A). All the other parameters, namely, NO production and LTT response in the *Mw* + Miltefosine group were in tune with the infected group.

(B) With autoclaved leishmania antigen (ALD). Similarly, no adjunct efficacy was observed when *Mw* was given along with ALD as a high parasite count was observed in the splenic dab smears of all the groups, namely, *Mw*, ALD, ALD + *Mw*, BCG and the infected control on day 30 post-challenge ($P>0{\cdot}05$) except for the ALD + BCG group of animals which showed a significant decrease in the number of parasites ($P<0{\cdot}01$) (Fig. 4A). Further, the *Mw* + ALD-vaccinated group showed poor proliferative responses as well as nitrite production while the ALD + BCG group exhibited impressive responses to mitogens (Con A and LPS) and SLD when compared with the unvaccinated infected control (Fig. 4B and C). Moreover, the *Mw* + ALD group exhibited a decreased level of Th1 cytokines (TNF-α, IFN-γ and IL-12) and an enhanced level of Th2 cytokines (TGF-β, IL-4 and IL-10) that was comparable to the infected control (Fig. 4D, E and F).

Immunomodulatory efficacy of Mw *vaccine in BALB/c mice*

(A) Mw *induces production of ROS by PM of naïve animals.* Vaccination of *Mw* bacilli to naïve animals activated the PM resulting in an amplified oxidative burst. However, the LD-infected group showed a remarkable decrease in ROS production, while the *Mw* + LD group marginally restored the generation of ROS over unvaccinated LD-infected animals ($P<0{\cdot}001$) (Fig. 5A).

(B) Mw *induces proliferation of the CD4+/CD8+ T lymphocyte population.* Vaccination with *Mw* bacilli marginally increased the proliferation of $CD4^+$ T_H cells in the *Mw*-treated group animals which was augmented further ($P<0{\cdot}001$) after LD infection in the *Mw* + LD group animals (Fig. 5B). A significant ($P<0{\cdot}001$) increase in the splenic population of CD8 + Tc lymphocytes in the *Mw* vaccinated and *Mw* + LD group was observed.

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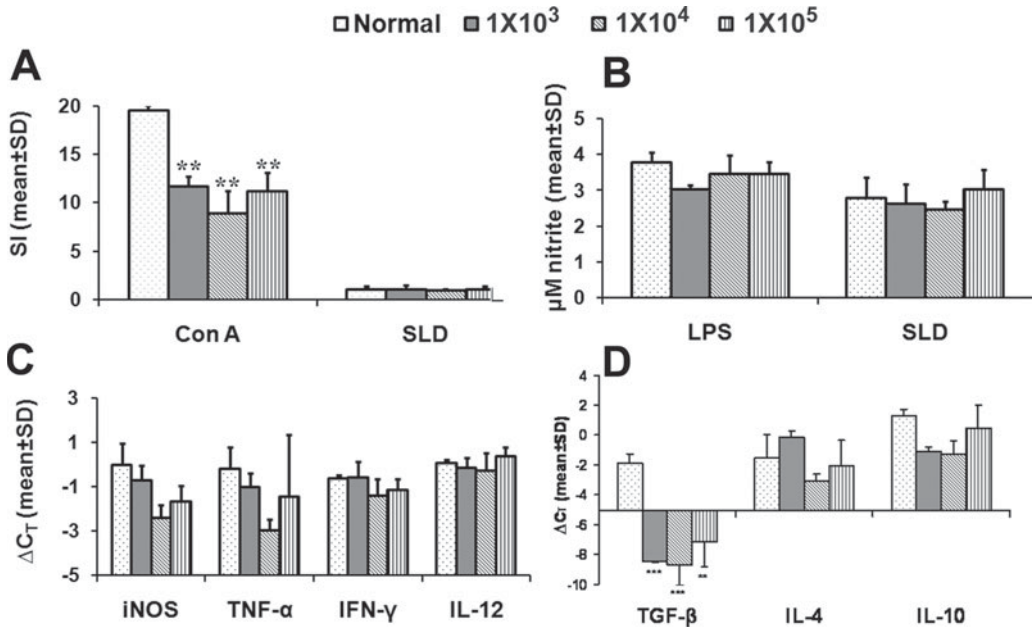

Fig. 2. Assessment of immunomodulatory activity of *Mw in vivo* in naive hamsters on day 30 p.v. at different doses, namely, 1×10^3, 1×10^4 and 1×10^5. The cellular response was measured by LTT in lymphocytes isolated from lymph nodes stimulated with SLD and Con A (A), and by NO estimation in lymphocytes stimulated with SLD and LPS (B). Splenic iNOS and Th1/Th2 cytokines estimation was done by quantitative real-time RT-PCR (C and D). Each bar in the graphs represents mean ± S.D. Significance values indicate the difference between the different experimental and normal group (*$P<0{\cdot}05$, **$P<0{\cdot}05$, ***$P<0{\cdot}001$).

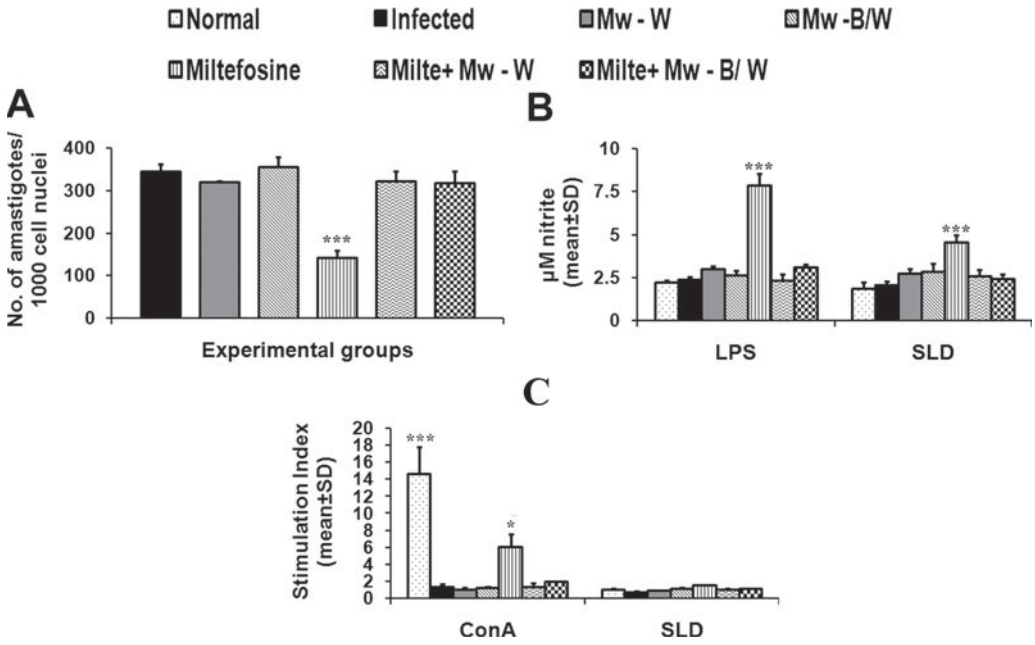

Fig. 3. Therapeutic efficacy of *Mw* vaccine in adjunct with standard drug Miltefosine administered to hamsters with established *Leishmania* infection was assessed on day 60 p.i. The animals were treated with *Mw* (i.d.)/week × 4 doses (*Mw*-W), *Mw* biweekly × 4 doses (*Mw*-B/W), *Mw*/week × 4 doses + Miltefosine (Milte + *Mw*-W) and *Mw* biweekly (4 doses) + Miltefosine (Milte + *Mw*- B/W) respectively. Animals of a fifth group were given miltefosine alone. Parasite load was counted in spleen of all experimental groups on day 60 p.i. (A), while cellular response was estimated through NO production (B) and LTT (C). Each bar in the graphs represents mean ± S.D. Significance values indicate the difference between the different experimental infected group (*$P<0{\cdot}05$, **$P<0{\cdot}05$, ***$P<0{\cdot}001$).

The LD-infected mice, however, did not reveal any significant change over untreated controls (Fig. 5B and C).

(C) Mw *induces a mixed Th1/Th2 type of immune response*. Vaccination with *Mw* significantly ($P<0{\cdot}001$) increased the production Th1/ Th2

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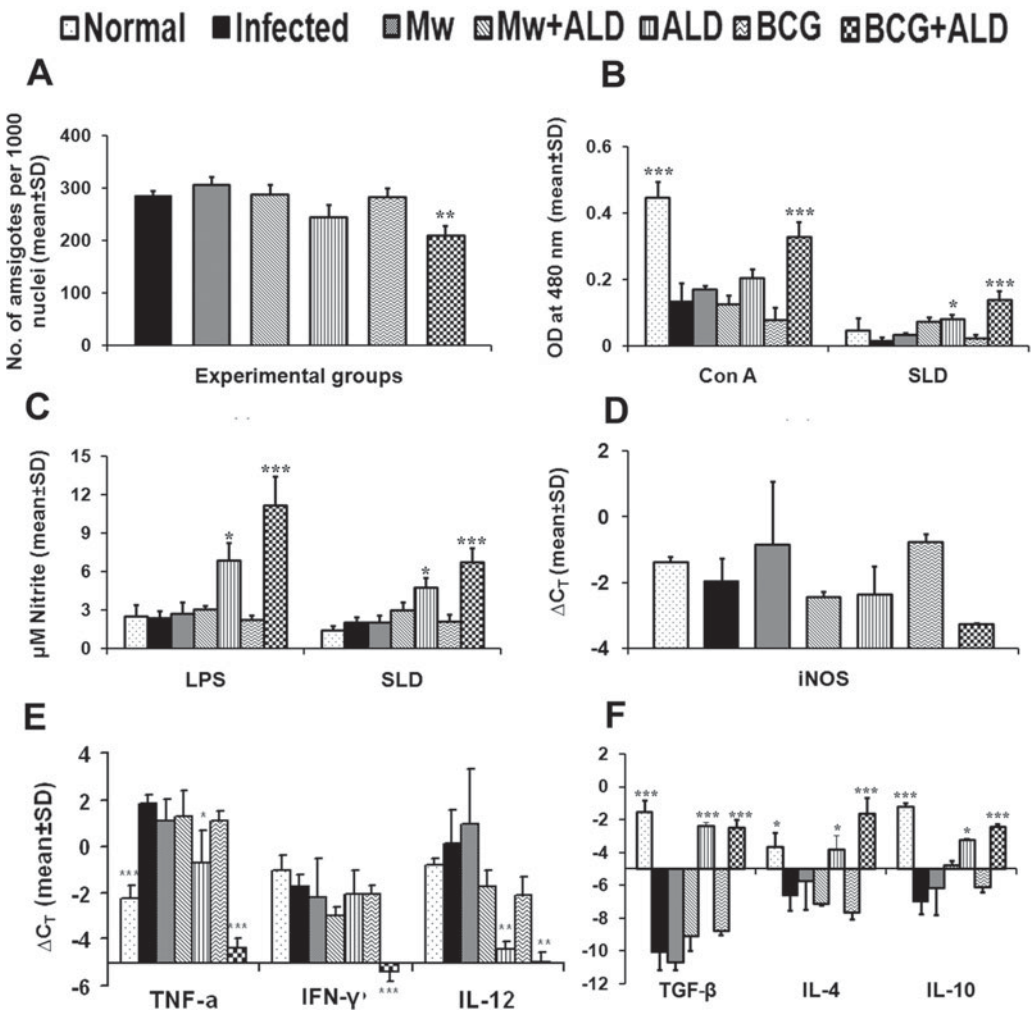

Fig. 4. Evaluation of prophylactic efficacy of *Mw* when used alone or in adjunct with ALD vaccine. Hamsters of all groups were autopsied on day 30 p.c. Parasite burden was assessed in spleen (A), lymphoproliferative response by XTT (B) and NO production (C) was estimated in lymphocytes. The cytokine profile of the spleen was measured through quantitative real-time RT-PCR (D, E and F). Each bar in the graphs represents mean ± S.D. Significance values indicate the difference between the different experimental and infected group (*$P<0{\cdot}05$, **$P<0{\cdot}05$, ***$P<0{\cdot}001$).

cytokines-IL-2 and IL-4 in the naïve mice, respectively. The exposure of *Mw*-vaccinated mice to LD infection, however, led to a profound decrease in the production of the above cytokines, favouring establishment of LD infection (Fig. 5D and E) as a high parasite load (315 ± 45 amastigotes/ 100 macrophages) was observed in the spleens of the *Mw* + LD group which was comparable to the infected controls (327 ± 54 amastigotes/100 macrophages).

DISCUSSION

A key restraining factor for the advancement of vaccines against *Leishmania* is the appropriate adjuvant to enhance and modify the effective and long-lasting immune response. BCG, a known immunomodulator stimulating several Toll-like receptors (TLRs) that can potentiate a Th1-biased immune response (Heldwein *et al*. 2003; von Meyenn *et al*. 2006; Villarreal-Ramos, 2009), can alone protect mice against leishmaniasis (Smrkovski and Larson, 1977; Weintraub and Weinbaum, 1977) and has also long been used as an adjuvant in pre-clinical and clinical efficacy trials of candidate vaccines against leishmaniasis (Garg *et al*. 2006; Kumari *et al*. 2008*b*; Noazin *et al*. 2008). In spite of the fact that it is the most effective adjuvant, in the present scenario of prevailing disease conditions, BCG, alongwith few inherent drawbacks, of which most the important is its variable efficacy found in different clinical trials that appears to depend on geography, needs to be supported by a better alternative which can overcome its drawbacks (Hart and Sutherland, 1977; Colditz *et al*. 1994; Fine, 1995; Aronson *et al*. 2004).

The present study has been done to evaluate the role of *Mw* as an adjuvant against leishmaniasis using, for the first time, the hamster, a stringent model of VL, as well as BALB/c mice as animal models.

Administration of *Mw* was initiated at high (recommended) doses (5×10^8 and $2{\cdot}5\times10^8$) to hamsters which resulted in a higher parasitic burden than that of the infected control (data not shown).

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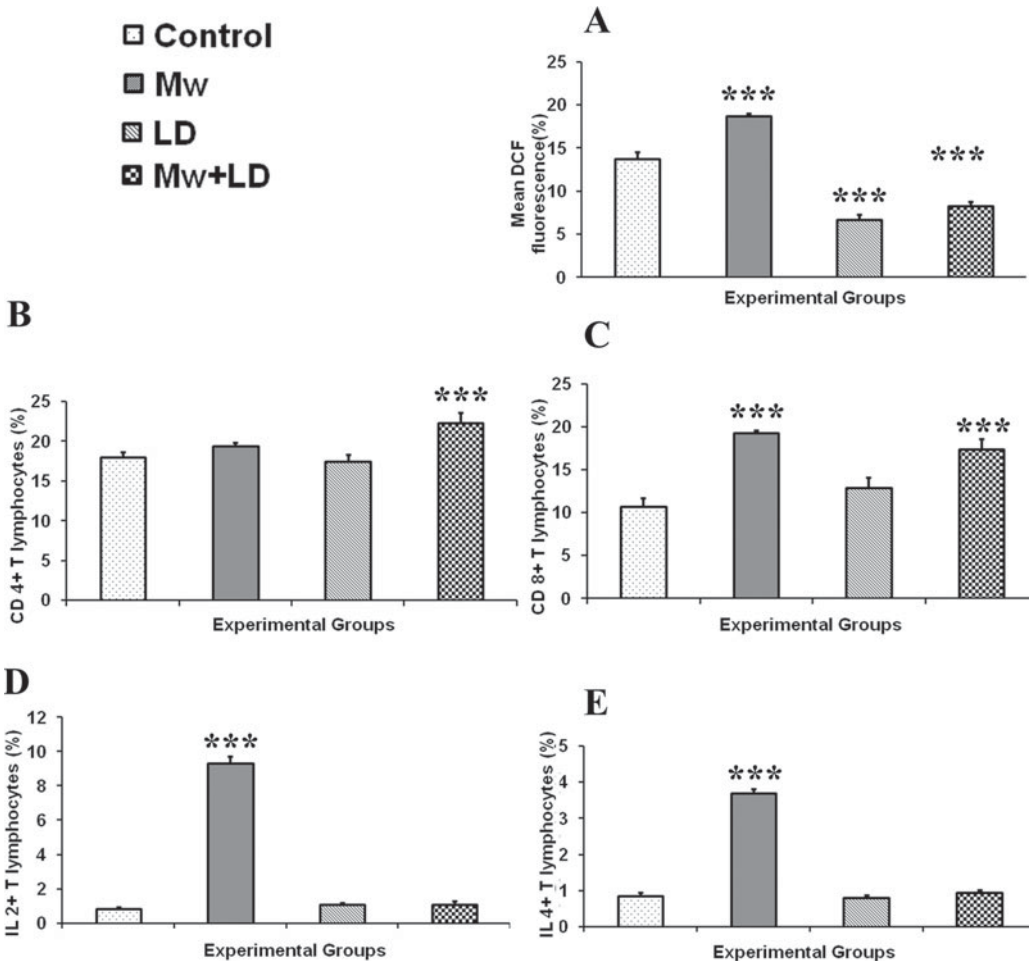

Fig. 5. Macrophages were collected from the peritoneal cavity of *Mw* and/or LD-treated and untreated BALB/c mice, washed with phosphate-buffered saline (PBS) and adjusted to a concentration of 1×10^6 cells/ml. ROS levels were determined by using the fluorescent probe 2′,7′- dichlorofluorescein diacetate (DCF-DA) and fluorescence intensity measured on FACS Calibur (A). Flow cytometric measurements of splenic CD4+ (B) and CD8+ (C) T cell subpopulations in mice treated with *Mw* (9×10^4 *Mw* bacilli/mouse), LD (10^7 amastigotes/mouse), LD + *Mw* (9×10^4 Mw bacilli $+10^7$ amastigotes/mouse) and untreated controls given equal amount of vehicle only. Flow cytometric detection of intracellular type 1 cytokine IL-2 (D), and type 2 cytokine IL-4 (E) in splenocytes of mice treated with *Mw* (9×10^4 *Mw* bacilli/mouse), LD (10^7 amastigotes/mouse), LD + *Mw* (9×10^4 *Mw* bacilli + 10^7 amastigotes/ mouse) and untreated controls given an equal amount of vehicle only. Bars represent mean ± S.D.

The observations after reassessment of immunomodulatory efficacy of the *Mw* vaccine *in vitro* indicated effectiveness of lower doses as a higher ratio of *Mw:* macrophages resulted in suppression in NO production.

On the basis of the above-stated findings the assessment of the immunomodulatory activity of *Mw* was initiated in hamsters at lower doses. No significant change in immunological responses was observed, except for the enhanced expression level of TGF-β in *Mw* treated hamsters which was significantly higher than that of normal controls. TGF-β, a pleiotropic cytokine, can intervene in immunosuppression by inhibiting IL-2-dependent T- and B-cell proliferation and IL-2-dependent antibody production by B-cells (Kehrl *et al*. 1986) and macrophage activation (Ding *et al*. 1990) and its role in maintaining or exacerbating the characteristic immunosuppression observed in leishmaniasis have been suggested (Rodrigues *et al*. 1998).

Further, it was reported earlier that ALD, in combination with BCG, has shown excellent efficacy against LD infection (Srivastava *et al*. 2003). However, in this study the combination of ALD + *Mw* vaccine did not confer any protection against LD challenge as was evident by the high parasitic burden and negligible proliferative response or NO production in the vaccinated animals. Moreover, the levels of mRNA expression of all the cytokines in this group were analogous to infected controls. *Mw* vaccine in combination with Miltefosine even was unable to decrease the parasitic load, or induce proliferative responses or NO production. It is established that Miltefosine promotes death of *Leishmania* by its ability to induce IFN-γ from macrophages and to enhance IFN-γ

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responsiveness by inducing IFN-γ receptor in macrophages. The IFN-γ-dominated response further helps to clear the parasite in a susceptible host (Wadhone *et al*. 2009). But the observations in this study have suggested that *Mw* is demolishing the protective responses induced by Miltefosine as the immune responses in the Miltefosine + *Mw*-treated groups were observed to be parallel with the infected control group. The results in hamsters therefore revealed that the *Mw* vaccine failed to exhibit any protective or therapeutic efficacy when used alone or in adjunct with anti-leishmanials. However, contradictory to our results, Adhikari *et al*. (2012) demonstrated *Mw* vaccine to be effective when used with Amphotericin B in infected BALB/c mice. This may be attributed to the usage of different experimental animals i.e. mice and hamsters in both the studies. It is known that they show different clinico-pathological features upon infection. Although, BALB/c mice infected with *L. donovani* or *L. chagasi* is the most widely studied model of VL, this is considered to be susceptible whereby the infection progresses during the first 2 weeks, and is then controlled by the host immune response (Murray *et al*. 1987; Wilson *et al*. 2005; Paciello *et al*. 2010). On the other hand, the better model to study the progressive disease is hamsters infected with *Leishmania* (*L. donovani* or *L. chagasi*) that develop a disease similar to human progressive VL with hepatosplenomegaly, hypoalbuminaemia, hypergammaglobulinaemia, and pancytopenia (Garg and Dube, 2006).

We have assessed the role of the *Mw* vaccine in BALB/c mice also wherein the increased production of ROS by macrophages and T lymphocyte proliferation supports the immunostimulatory characteristics of the vaccine. On further exposure to LD amastigotes, *Mw*-vaccinated mice exhibited a profound decrease in ROS production although the level was marginally higher than in the infected controls. These findings suggest that LD neutralizes the immunostimulatory effects of *Mw* in BALB/c mice by down-regulating the Th1 responses in the form of ROS contents and also by activating the cytotoxic T cells.

Thus, the present findings provide ample evidence that although the *Mw* vaccine stimulated a mixed Th1/ Th2 type of immune response in naive mice, it generated Th2 response in naïve hamsters displayed by a high expression of TGF-β. We observed contrasting results particularly with regard to the immunomodulatory effects of *Mw* in the two animal models used. Whereas in the hamster *Mw* was ineffective as an immunostimulant or immunoprophylactant, it triggered an effective immune response in BALB/c mice which was, however, not adequate to protect animals against LD infection. Hence, it is inferred that the *Mw* vaccine does not play any significant role in the management of experimental VL and also could not augment the efficacy of anti-leishmanial drug or vaccine, emphasizing the need to find explanations for the failure of this vaccine against the disease.

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