Brugia malayi abundant larval transcript 2 protein treatment attenuates experimentally-induced colitis in mice

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Helminths are known to modulate host’s immunity by suppressing host protective pro-inflammatory responses. Such immunomodulatory effects have been experimentally shown to have therapeutic implications in immune mediated disorders. In the present study, we have explored a filarial protein i.e. Brugia malayi recombinant abundant larval transcript 2 (rBmALT2) for its therapeutic effect in dextran sodium sulfate (DSS) induced colitis in mouse model. The immunomodulatory activity of rBmALT-2 was initially confirmed by demonstrating that it suppressed the lipopolysaccharide (LPS) induced nitric oxide synthesis and down-regulated the expression of pro-inflammatory cytokines in vitro by peritoneal exudate cells of mice. Treatment with rBmALT2 reduced severity of colitis associated with significant reduction in weight loss, disease activity, colon damage, mucosal edema and histopathological score including myeloperoxidase activity in colon tissues. rBmALT2 was comparatively more effective in attenuation of colitis when used in the preventive mode than when used for curative purpose. The therapeutic effect of rBmALT2 was found to be associated with downregulation of IFN-γ, IL-6, IL-17 and upregulation of IL-10 cytokines. These results provide strong experimental evidence that BmALT2 could be a potential alternative therapeutic agent in colitis.

Keywords: rBmALT2, Dextran sodium sulfate (DSS), Helminths, Nematodes, Ulcerative colitis

Helminths are highly adaptive parasites surviving several years in their host1. They grow, develop and reproduce within the host and manipulate the host’s immune system by suppressing immune responses initiated against them. Such immunomodulation strategies of helminths also confer a concomitant benefit to the hosts by protecting them from several immune-functional disorders. Number of experimental and clinical studies have been carried out to effectively of the use of helmints or helmint-derived products as therapeutic agents to treat immune mediated disorders1. Ulcerative colitis (UC), caused due to the aggressive T cell responses towards intestinal microflora, is one of the disorders targeted for exploring potential of helminth therapy2. At present, the treatment modalities for UC include treatment with anti-inflammatory and immunosuppressive drugs or surgical interventions in severe cases. Not all patients respond to these drugs and there may also be serious side effects. Moreover, the curative effect of these drugs in UC is only for short-term and there are no other alternative medications available3. Recent works have demonstrated the curative role of natural compounds from plants, Aegle marmelos, Bombax malabaricum, Dillenia indica and Manuka honey in amelioration of experimentally induced UC4-7. As an alternative approach, helminths have also been explored in the treatment of UC by administering experimental animals with whole worms or their eggs or by using their excretory-secretory (ES) or somatic products. Treatment with Schistosoma mansoni or its products has resulted in the attenuation of severity of colitis in different types of murine models8-12. This therapeutic effect is associated with pronounced Th2 immune response along with increased regulatory T cells (Tregs) directed IL-10 and down-regulated Th1 response812. Infection with Heligmosomoides polygyrus larvae has

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Abbreviations: rAs-MIF, Anisakis simplex recombinant macrophage inhibitory factor; rBmALT2, Brugia malayi recombinant abundant larval transcript 2; DSS, Dextran sodium sulfate; EDTA, ethylenediaminetetraacetic acid; MPO, myeloperoxidase; ES, excretory-secretory; LPS, lipopolysaccharide; SmSWP, Schistosoma mansoni soluble worm protein; UC, ulcerative colitis; TNBS, 2,4,6-trinitrobenzene sulphonic acid.
been shown to abrogate intestinal inflammation induced in mice along with suppression of IL-17 release by T cell and upregulation of Th2 type of immune response along with induction of Foxp3+ Treg cells13-16.

Clinical trials in humans have also shown that the exposure to helminth parasites could reduce the severity of disease symptoms in the patients suffering with Crohn’s disease (CD) and ulcerative colitis17. However, helminth therapy using whole worms or eggs has several limitations. The parasites used in the treatment must elicit minimal pathology with nil chance of their aberrant migration or establishment of chronic infection. Moreover, worm therapy is not suitable for immuno-compromised individuals. Hence, treatment with helminth-derived products instead of whole worm is more appropriate.

There are no reports available on exploring the filarial-derived immunomodulators in the treatment of UC. Filarial abundant larval transcript (ALT) proteins, expressed abundantly in infective larval stage may play a significant role in immune evasion as these proteins are critical for initial entry of parasite in to the host. Experimental evidence is also available suggesting that filarial BmALT proteins are involved in up-regulating Th2 responses which may help in the parasite survival18. In another study, T-cell epitopes of BmALT2 are also shown to induce high levels of IL-10 secretions along with suppression of IFN-γ and IL-2 cytokines suggesting its possible role in modulation of host’s immune response and its potential use in the treatment of autoimmune diseases19. Hence, in the present study, we assessed both the curative and preventive effects of Brugia malayi recombinant ALT2 (rBmALT2) in dextran sodium sulfate (DSS)-induced colitis using murine model.

**Methods and Materials:**

*Brugia malayi abundant larval transcript 2 (rBmALT2)—* rBmALT2 was expressed as His-tag protein in Escherichia coli by transforming pSET-A-BmALT2 plasmid into BL21 (DE3) pLysS E. coli cells and was purified using nickel affinity chromatography column (Thermo Fisher Scientific, Mumbai). Purified protein was checked on SDS-PAGE using 15% gel and confirmed by western-blot analysis using monoclonal anti-his G-HRP antibody (Invitrogen, Mumbai). The protein was quantified and endotoxin content was measured by LAL chromogenic endotoxin quantitation kit (Thermo Fisher Scientific, Mumbai).

**Experimental animals—** Female BALB/c mice, 6-8 wk old were inbred and kept under the suitable environment, fed on pelleted animal diet and on drinking water ad libitum in a CPCSEA registered Central Animal House. Approval from Institutional Animal Ethics Committee was obtained for this study.

**In vitro assessment of anti-inflammatory activity of rBmALT2—** BALB/c mice were injected with 1% thioglycollate medium (Sigma-Aldrich, Mumbai) into the peritoneal cavity and on the fourth day PEC were harvested by lavage with cold and sterile Phosphate buffered saline (0.1M, pH 7.2) containing 20 mM EDTA (Sigma-Aldrich, Mumbai). The cells were re-suspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (Gibco, Thermo Fisher Scientific, Mumbai) and plated in triplicates (2×10⁵ cells/100 µl/well) in 96-well flat-bottom plates (Thermo Fisher Scientific, Mumbai). After 4 h of incubation (37°C in 5% CO₂), non-adherent cells were washed off and adherent cells in wells were stimulated with lipopolysaccharide (LPS) from E. coli (10 µg/100 µl/well; Sigma-Aldrich, Mumbai) in the presence or absence of rBmALT2 (25 µg/100 µl/well). The PEC incubated with medium alone represented as controls in the assay. After 24 h of incubation at 37°C in 5% CO₂, the culture contents from triplicate wells were pooled in separate centrifugate tubes and centrifuged. Nitrite levels were estimated in supernatants using Griess reagent20 and mRNA levels of cytokines (IL-6, TGF-β and TNF-α) were assayed in cells using Real-Time PCR analysis.

**Induction of colitis in BALB/c mice—** Colitis was induced in mice by feeding autoclaved drinking water with 5% w/v DSS for 7 days (30-50 kD, MP Biomedicals, India) ad libitum. The control group of mice received normal autoclaved drinking water only. Animals were checked daily for body weight, stools consistency and bleeding.

**rBmALT2 treatment—** In the curative treatment protocol, mice were divided into different groups (n=5-8 per group) as follows: Control-PBS group (normal mice treated with PBS (0.1M, pH 7.2) only); Control-rBmALT2 group (normal mice treated with rBmALT2); DSS-PBS group (Colitis mice treated with PBS); and DSS-rBmALT2 group (Colitis mice treated with rBmALT2). Treatment with rBmALT2 was by i.p. administration of mice with rBmALT2 in
PBS (25 µg/dose/200 µl) on day 1, 3 and 5 of DSS administration. Mice were monitored and sacrificed when all the DSS-PBS group of mice developed colitis.

Similarly, in the preventive treatment protocol, mice were divided into different groups (n=8 per group) as follows: Control-Alum group (mice treated with alum alone); Control-rBmALT2 group (mice treated with rBmALT2 in alum); Alum-DSS group (mice treated with alum followed by induction of colitis); and rBmALT2-DSS group (mice treated with rBmALT2 followed by induction of colitis). Treatment with rBmALT2 consisted of i.p. administration of mice, each with 4 doses of rBmALT2 in alum adjuvant (25 µg/dose/200 µl) at the intervals of 15 days. The colitis induction was done 10 days after the last dose of rBmALT2/Alum. Mice were monitored and sacrificed when all the mice in Alum-DSS group developed colitis.

From sacrificed mice, colons were isolated, scored for mucosal edema and changes in colon length were noted. Distal colon samples were used for determination of myeloperoxidase activity and histopathological examination. Splenocytes were also collected aseptically from sacrificed mice for in vitro splenocytes culture for the estimation of mRNA levels of cytokines.

**Assessment of disease activity index (DAI)—** Disease activity was indexed using scoring of the parameters viz., weight loss (none, 0; 1, 1-5%; 2, 5-10%; 3, 10-15%; 4, >15%), fecal character (0, pellet; 2, loose; 4, watery/diarrhea) and fecal occult blood (0, absent; 2, positive; 4, gross bleeding). The results were expressed as mean of the summative scores.

**Macroscopic and histopathological scoring of colon—** Macroscopic scoring of colon was done for degree of mucosal edema; 0 (normal) to 3 (severe) and change in colon length. For histopathological scoring, colonic segments were fixed in 10% formal saline followed by embedding in paraffin and 5 µm cross sections were cut and stained with hematoxylin & eosin. Sections were graded based on the parameters: Inflammation extent, 0-3; Inflammation severity, 0-3; Crypt damage, 0-4; Colon wall thickness, 0-3; Leucocyte infiltration, 0-3; and Lamina propria mononuclear cells, 0-3.

**Assessment of myeloperoxidase (MPO) activity—** Colonic segments were blotted dry, weighed and transferred to PBS (0.05 M, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Mumbai) at 5 g of tissue per 100 mL buffer. The samples were homogenized on ice for 30 s, subjected to two cycles of sonication (30 s each) and freeze-thawing and centrifuged at 15000g for 15 min at 4°C. The supernatant (0.1 mL) was added with o-dianisidine solution (2.9 mL; Sigma-Aldrich, Mumbai) and change in absorbance was read at 460 nm over 5 min. The MPO activity was presented as units per gram of tissue with a unit being equivalent to the amount of MPO necessary to degrade 1 µmol of H2O2 to H2O per min at 25°C.

**In vitro culture of splenocytes for estimation of cytokines—** Aseptically harvested spleens were minced in RPMI 1640 medium, pelleted and suspended in erythrocyte lysis buffer (Invitrogen, Mumbai). Cells were washed 3 times with RPMI 1640 medium and plated in duplicates (2×106 cells/well/ml) in 24-well flat-bottom plates (Thermo Fisher Scientific, Mumbai) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES buffer and 10% heat inactivated fetal calf serum. Cells were stimulated with rBmALT2 (10 µg/ml), Concanavalin A (2 µg/ml; Sigma-Aldrich, Mumbai; positive control) or medium alone (negative control) and kept at 37°C in 5% CO2 atmosphere for 72 h. Pelleted cells were preserved in RNAzol (Sigma-Aldrich, Mumbai) and stored at −80°C till further use in cytokine mRNA expression assays.

**Assessment of relative mRNA expression by Real-time PCR analysis—** Total RNA was extracted from cells using RNAzol reagent (Sigma-Aldrich, Mumbai) as per the manufacturer’s instructions. One µg of total RNA was reverse transcribed to cDNA using Superscript VILO cDNA synthesis kit (Invitrogen, Mumbai) using random hexamers. cDNA was quantified and gene expression was assessed using TaqMan gene expression assays (Applied Biosystems, Mumbai) for mouse IFN-γ, IL-6, IL-17, IL-10, TGF-β and TNF-α (Gene Ids on request). GAPDH was used as an endogenous control. Final reaction volume was 20 µl including TaqMan Universal Master Mix II (Applied Biosystems, Mumbai). Reaction was performed on Stepone Plus machine (Applied Biosystems, Mumbai) and relative mRNA expression was analyzed using Stepone analysis software (Version: 2.2.2, Applied Biosystems, Mumbai).

**Statistical analysis—** The data was checked for normality assumption and which did not follow normality assumption was analyzed by suitable
non-parametric test. Normally distributed data was analyzed by suitable test for comparison of means. \( P \)-values \( \leq 0.05 \) were considered to be significant. SPSS 21.0 (IBM, India) and XLSTAT ver. 2014.6.04 (Addinsoft) software were used.

**Results**

**Suppression of LPS induced inflammatory effect by rBmALT2**— The nitrite levels were significantly decreased in the culture supernatants of PEC co-stimulated with rBmALT2 and LPS, which indicated the possible macrophage modulation by rBmALT2 against only LPS treated PEC \( (P =0.007, \text{Fig. 1A}) \). In addition, there was significant downregulation of IL-6 and TNF-\( \alpha \) cytokine expression in PEC co-stimulated with rBmALT2 and LPS \( (P <0.05, \text{Fig. 1B}) \).

**Curative effect of rBmALT2 on colitis**— Untreated colitis mice showed increased weight loss during DSS administration with progressive increase in disease activity (Fig. 2 A and B). In the colitis mice treated with rBmALT2, weight loss and disease activity was less severe compared to untreated colitis mice, though the difference was not significant (Fig. 2 A and B). Untreated colitis mice showed signs of colon damage with 31% decrease in colon length compared to that of normal control mice and increased mucosal edema (Fig. 2 C and D). Treatment with rBmALT2 significantly protected colitis mice from colon damage with only 14% decrease in colon length and reduced mucosal edema (Fig. 2 C and D).

Sections of colons from untreated colitis mice showed severe inflammation extended up to sub-mucosa (which was in some cases even trans-mural) with loss of crypt & surface epithelial lining (Fig. 3). There was also marked increase in neutrophil infiltration as evident by increased MPO activity (Table 1, Fig. 3). Administration of rBmALT2 to colitis mice resulted in a decrease in all the above parameters to 68% compared to untreated colitis mice and was also associated with decreased MPO activity (Table 1, Fig. 3).

The splenocytes of untreated colitis mice after in vitro stimulation with rBmALT2 showed upregulated mRNA expression of IFN-\( \gamma \), IL-6, TGF-\( \beta \) and IL-17; in contrast the expression of mRNAs of these cytokines was down-regulated in splenocytes of mice treated with rBmALT2 (Fig. 4A). Further, there

![Fig. 1—Suppression of LPS induced inflammatory effect by rBmALT2. (A) Nitrite concentration in the PEC culture supernatants; (B) Relative mRNA expression of cytokines. [Each bar represents Mean±SEM; *\( P \leq 0.005 \) in comparison with all the other groups analyzed by One-way ANOVA with HSD post-hoc test.]

![Fig. 2—Curative effect of rBmALT2 on colitis mice. (A) Weight change (%); (B) Disease activity index (DAI); (C) Change in colon length (%); and (D) Degree of mucosal edema. [Each bar represents Mean±SEM; \( n=5 \) in Control-PBS, DSS-PBS & Control-rBmALT2 groups; \( n=8 \) in DSS-rBmALT2 group; *\( P \leq 0.05 \) in comparison with DSS-PBS group analyzed by student’s \( t \) test.]
was increased expression of mRNAs of IL-4 and IL-10 in splenocytes of rBmALT2 treated colitis mice compared to untreated colitis mice (Fig. 4A). However, the observed differences in these two groups of mice were found to be statistically non-significant.

Preventive effect of rBmALT2 on colitis mice—
Severe weight loss with increased disease activity was seen in colitis mice pre-treated with alum alone (Fig. 5A and B). However, pre-treatment with rBmALT2 imparted protection from colitis as observed from significantly reduced trend in weight loss and disease activity (Fig. 5A and B). From 5th day onwards after DSS administration the colitis mice pre-treated with rBmALT2 regained their weight (Fig. 5A). The colon length was reduced to the extent of 46% in colitis mice pre-treated with alum alone (Fig. 5C). In contrast, there was only 5% reduction in colon length in rBmALT2 pre-treated colitis mice (P = 0.002, Fig. 5C). Further, preventive treatment with rBmALT2 significantly reduced mucosal edema compared to that in mice which received alum only (P = 0.005; Fig. 5D).

The protection from colon damage imparted by rBmALT2 was also reflected in significant reduction of histopathological scoring and MPO activity in colon tissues in rBmALT2 pre-treated mice as compared to the control group of mice (Table 1, Fig. 4—Changes in mRNA expression levels of cytokines. (A) Curative treatment; and (B) Preventive treatment. [Bar represents Mean±SEM; n=4-8 mice per group, statistical significance among the groups was analyzed by student’s t test.]

![Histopathological score (%) and MPO activity in colon tissues](image)

Distal colon samples were labelled, fixed, paraffin embedded, H&E stained and scored for histopathological changes and presented as % change in score compared to DSS-PBS or Alum-DSS group (for curative protocol or preventive protocol respectively). MPO activity was measured in the colon tissue samples and presented as Mean ± SEM. *P ≤0.05 in comparison with Alum-DSS group as analyzed by student’s t test, ¢P ≤0.05 in comparison with Alum-DSS group as analyzed by Mann-Whitney U test.

Fig. 3—Effect on colon damage. Representative figures for changes in colon length and histopathological sections of H&E stained distal colon tissues (magnification 40X). [Arrow mark shows neutrophil infiltration and loss of crypt and surface epithelial lining of colon.]

Fig. 4—Changes in mRNA expression levels of cytokines. (A) Curative treatment; and (B) Preventive treatment. [Bar represents Mean±SEM; n=4-8 mice per group, statistical significance among the groups was analyzed by student’s t test.]

Table 1—Histopathological score (%) and MPO activity in colon tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>Histopathological score (%)</th>
<th>MPO activity (U/g tissue sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS-PBS (n=5)</td>
<td>100</td>
<td>55.42 ± 11.26</td>
</tr>
<tr>
<td>DSS-rBmALT2 (n=8)</td>
<td>68.25</td>
<td>39.21 ± 11.39</td>
</tr>
<tr>
<td>Alum-DSS (n=6)</td>
<td>100</td>
<td>23.19 ± 2.34</td>
</tr>
<tr>
<td>rBmALT2-DSS (n=6)</td>
<td>5.29 *</td>
<td>6.37 ± 0.48 #</td>
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</tbody>
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Fig. 3). The protection imparted by rBmALT2 was found to be associated with downregulation of cytokines IFN-γ, IL-6 and IL-17 and up-regulation in expression of mRNAs of IL-10 and TGF-β (Fig. 4B).

Discussion

The immunomodulatory effect of BmALT2 was initially assessed in vitro before conducting the experiments on its therapeutic effect in DSS-induced colitis. Significantly suppressed LPS induced nitric oxide synthesis and downregulation of expression of pro-inflammatory cytokines IL-6 and TNF-α by PEC of mice validated immunoregulatory effect of this protein.

Colitis is aggravated due to the abnormal permeability of mucosal barrier allowing bacterial translocation resulting into non-specific and uncontrolled inflammatory conditions. DSS leads to disruption of the mucosal barrier leading to bacterial penetration and involves T cell accumulation and Treg direction towards the site of inflammation. Hence, DSS-induced colitis is a clinically relevant model of interest.

The curative treatment of colitis mice with rBmALT2 resulted in decreased disease activity index, decreased colon length reduction and mucosal edema and also lesser histopathological damage as well as reduced MPO activity in colon segments. The treated mice also showed lesser weight loss compared to the control group of mice, though the difference was not significant. However, the improvement in all the other disease parameters in treated animals suggested overall amelioration of colitis pathology.

This attenuation effect was much more effective when rBmALT2 was used in the prophylactic mode. The mice pre-treated with rBmALT2 regained whatever weight that was lost initially and demonstrated lower disease severity. Colon damage is an important factor to assess the disease severity. There was minimal colon damage in rBmALT2 pre-treated mice as evidenced from significant prevention of shortening of colon and associated decrease in mucosal edema. Neutrophils play an important role in mediating tissue injury in colitis. Pre-treatment with rBmALT2 significantly diminished MPO activity in colon tissues, which was also associated with the decreased histopathological score suggesting the attenuation of the detrimental effect of DSS.

Prior administration of Anisakis simplex recombinant MIF protein (rAs-MIF) was shown to cause significant decrease in severity of DSS-induced colitis associated with lower degree of weight loss, lesser histopathological damage along with protection from reduction in colon length. rTsP53, a 53-kDa recombinant protein of Trichinella spirallis effectively prevented 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced inflammation in colon with reduction in all the inflammatory parameters including DAI, macroscopic and microscopic score, degree of mucosal damage.

Akin to the chronic inflammatory pathology in the inflammatory bowel disease (IBD) patients caused by activated dendritic cells (DCs) and macrophages which stimulate T cell differentiation, enhanced Th1 and Th17 response by splenocytes was observed in DSS- induced colitis model. In the present study, we observed decreased IFN-γ and IL-6 mRNA expression in rBmALT2 treated colitis mice. As IL-6 is involved in the induction of Th17 cells; decreased IL-6 expression is expected to suppress IL-17 expression concomitantly and the same was noted in this study. Increased expression of IL-10, IL-4 or TGF-β in mice treated with rBmALT2 indicate the upregulation of anti-inflammatory Treg directed Th2 immune response. Helminth molecules have been
shown to promote a regulatory phenotype in naïve T cells and amelioration of severity of DSS colitis has been found to be correlated with upregulation of regulatory cytokines IL-10, TGF-β and increased Treg cells in the spleen, myentric lymph node and colon\textsuperscript{28}. Cho \textit{et al.} demonstrated that administration of rAs-MIF suppressed the intestinal inflammation induced by DSS and the same was found to be associated with reduced production of IL-6, TNF-α, IL-1β and IFN-γ mediated through the recruitment of Treg cells and via binding with TLR\textsubscript{2}\textsuperscript{25}. Ferreira and co-workers showed that the curative effect of ES products of \textit{Ancylostoma caninum} with involvement of Th2 type cytokine response, characterized by a distinct population of IL-4/IL-10 double-positive CD4\textsuperscript{+} T cells, is involved in the suppression of colitis disease pathology\textsuperscript{28}. The beneficial effect of \textit{S. mansoni} soluble worm protein (SmSWP) protein in amelioration of the severity of DSS-induced colitis was shown to be associated with downregulation of IFN-γ and IL-17A cytokines and upregulation of IL-4 cytokine in the colon\textsuperscript{12}.

ALT2 from \textit{B. malayi} filarial parasite plays critical role in immune evasion and has already been shown to induce IL-10 secretions and Th1 suppression\textsuperscript{19}. The observed effect of ALT2 protein in this study is in accordance with these studies and further confirms the immune suppressive potential of this filarial protein.

As the improvement in DSS colitis observed is more pronounced when BmALT2 was used in prophylactic mode, we hypothesize the role of immunoregulatory impact on antibody production and B cell signaling behind this disease attenuation effect of BmALT2. This antibody mediated modulation seems to be maintained for longer period of time and, thus suppressing the disease establishment right from the beginning of the disease condition. The molecular governing process behind BmALT2 mediated regulation need to be further investigated in detail. This study, however, has put forward supportive evidence in favour of BmALT2 as a future biotherapeutic protein in interventional strategies for IBD. It also can be envisaged from the results of these study that the helminth molecules are potentially useful as preventive tools for wide spectrum of immune dysregulatory disorders.

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\textbf{References}


