

SXP–RAL Family Filarial Protein, rWbL2, Prevents Development of DSS-Induced Acute Ulcerative Colitis

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Abstract Helminthic infections lead to the release of various molecules which play an important role in modulation of the host immune system. Such filarial proteins with immunomodulatory potential can be used for therapeutic purpose in inflammatory and immune mediated diseases. In the present study, we have explored the prophylactic effect of filarial SXP–RAL family protein of *Wuchereria bancrofti* i.e. rWbL2 protein in DSS induced inflammatory ulcerative colitis in a mouse model. Prior treatment of rWbL2, followed by induction of colitis, showed significantly reduced disease severity as indicated by the decreased disease manifestations and improved macroscopic and microscopic inflammation. This preventive effect was found to be associated with increased release of anti-inflammatory cytokine IL-10 and decreased release of proinflammatory cytokines IFN- γ , TNF- α , IL-6 and IL-17 by the splenocytes of treated mice. From this study, it can be envisaged that pretreatment with filarial protein, rWbL2, can prevent the establishment of ulcerative colitis in BALB/c mice. The underlying immunological mechanism may involve the up-regulation of Th2 immune response with down-regulation of Th1 response.

Keywords Helminths · Ulcerative colitis · Recombinant WbL2 · Dextran sodium sulfate

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Introduction

The inflammatory conditions of the digestive tract are caused mainly due to the impaired intestinal barrier function. These conditions, designated as inflammatory bowel diseases (IBD), comprising Ulcerative colitis (UC) and Crohn's disease. IBD mostly affects young age individuals, hampering their normal working abilities and severely disturbing their social life [1]. Recent findings reveal the possible role of genetic predisposition, environmental factors, immune system and intestine microbial flora of an individual. These factors may trigger the aggressive immune response resulting in an increased proinflammatory response associated with increased expression of inflammatory and proinflammatory cytokines.

Though the IBD has been mostly prevalent in the Western countries, recent findings indicate that it is also increasing at an alarming rate and posing a rising threat to the population of developing countries [2, 3]. Its increasing prevalence has been found to be correlated with the eradication of helminths and improved personal hygiene [4]. This condition, supported by the 'hygiene hypothesis' or microbial deprivation hypothesis [5] implies that helminths for their own survival and growth, secrete various proteins, which are crucial for the suppression of the host's proinflammatory immune response and prevention of immune mediated diseases. Accordingly, investigators explored helminths as a possible bio-therapeutic option for autoimmune diseases. Earlier studies provided evidences that helminth infections can inhibit the development of autoimmune and inflammatory diseases [6–8]. Experimental colitis mice and rats were protected when exposed to *Hymenolepis diminuta*, *Heligmosomides polygyrus* and *Schistosoma mansoni*. However, the risk of pathogenesis cannot be excluded due to whole worm infection.

Therefore, several investigators used helminth derived molecules/proteins that could suppress the severity of colitis [9–11]. In previous studies from our laboratory, by immunoscreening of *Wuchereria bancrofti* larval (L3 stage) cDNA library, a recombinant protein (rWbL2) of the filarial parasite which belongs to SXP/RAL-2 family (NCBI accession numbers for: Nucleotides sequence: EU183493 and Protein sequence: ABW38023) was identified to be of a diagnostically useful protein. It detected high levels of IgG4 antibodies in the sera of actively infected filarial subjects (Siva Prasad et al. 2007, unpublished observation). The increased level of IgG4 antibodies against SXP/RAL-2 family of proteins were demonstrated in actively infected filarial cases [12]. The increased IgG4 secretion associated with high levels of IL-10 is believed to play important role in host immune modulation [13]. Hence, we selected for the first time SXP–RAL family protein to test for its anti-inflammatory and preventive effect in dextran sodium sulfate (DSS) induced colitis in BALB/c mice model.

Materials and Methods

rWbL2 Protein

Filarial protein rWbL2 was prepared as described earlier using *Escherichia coli* (*E. coli*) based expression system [14]. Briefly, pRSETA-*Wbl2* plasmid was constructed, transformed into BL21 (DE3) pLysS *Escherichia coli* expression host and expressed as His-tag proteins by inducing with 1 mM isopropyl β -D-1-thiogalactopyranoside (Merck Millipore, Bangalore, India). The recombinant protein was purified using a nickel affinity chromatography column (Thermo Fisher Scientific, Mumbai, India) and the protein content was estimated using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Mumbai). The endotoxin content was measured using Limulus amoebocyte lysate chromogenic quantitation kit (Thermo Fisher Scientific, Mumbai) which was found to be well below the recommended cut-off level [15].

Induction of Colitis in Mice

Colitis was established in the mice, as described previously [16]. The female BALB/c mice (6–8 weeks of age) were in-house bred in the CPCSEA (Committee for the Purpose and Control of Supervision on Experimental Animal) registered animal house of our institute and maintained in a pathogen free environment and in standard laboratory conditions with free access to normal diet and drinking water ad libitum. All experiments were approved by the

Institutional Animal Ethics Committee. To induce colitis, the mice were fed with 5% (w/v) ad libitum DSS (30–50 kD, MP Biomedicals, Mumbai) in autoclaved drinking water. Control healthy mice received normal autoclaved drinking water.

Pre-treatment of Mice with rWbL2

Mice were divided into different groups ($n = 6$ –8 mice/group) viz; the Control-Alum (mice treated with Alum only), Alum-DSS (mice treated with alum and later on induced for colitis) and rWbL2-DSS (mice treated with rWbL2 and later on induced for colitis). The treatment with rWbL2 consists of intra-peritoneal (i.p.) administration of mice, each with 4 doses of rWbL2 in alum adjuvant (25 μ g/dose/200 μ L) in intervals of 15 days. Initial dose–response experiments on colitis mice showed 25 μ g/dose of rWbL2 as the optimal quantity needed to achieve the significant therapeutic effect (data not shown).

The colitis induction was done after the last dose of Alum or rWbL2. Mice were monitored for the signs of colitis and sacrificed on the day when all Alum-DSS group of mice developed colitis [17]. From sacrificed mice, colons were isolated, scored for mucosal edema and changes in the colon length were recorded. Distal colon samples were used to determine myeloperoxidase activity and histopathological examination.

Assessment of Disease Activity Index (DAI) of Colitis

The DAI was measured using a score of 0 to 4, which is the sum of the scores given for weight loss (0, none; 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 fecal occult blood test; 4, gross bleeding) [18].

Macroscopic and Histopathological Scoring of Colon Damage

After sacrificing the mice, the colons were macroscopically observed for the degree of mucosal edema, which was scored from 0 (normal) to 3 (severe), and the changes in their length were also recorded [19]. For histopathological scoring, colonic segments/tissues were fixed in 10% formalin, embedded in paraffin, cut into 5- μ m-thick sections and were stained with hematoxylin and eosin (HE). Inflammation was graded based on the parameters: the extent (0–3) and severity (0–3) of inflammation, crypt damage (0–4), colon wall thickness (0–3), leucocyte (0–3) and lamina propria mononuclear cell infiltration (0–3) [20].

Assessment of Myeloperoxidase (MPO) Activity

Myeloperoxidase enzyme activity in the colon samples was assayed as previously described [21]. Briefly, the colonic segments were blotted dry, weighed and placed in a potassium phosphate buffer (0.05 mmol/L, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma-Aldrich, Mumbai) at a concentration of 5 g tissues per 100 ml buffer. The samples were placed on ice, minced and homogenized. The homogenates were subjected to two sonication cycles (30 s each) and freeze–thawing cycles. The suspensions were then centrifuged at $15,000\times g$ for 15 min at 4 °C. The supernatant of each sample (0.1 ml) was added to 2.9 ml of o-Dianisidine solution (Sigma-Aldrich, Mumbai) and the change in absorbance was recorded at 460 nm over 5 min using a spectrophotometer (Elico SL-159, Hyderabad). The MPO activity was presented as U/g of tissue, with a unit being equivalent to the amount of MPO necessary to degrade 1 μM of H_2O_2 to H_2O per min at 25 °C.

In Vitro Stimulation of Splenocytes

Spleens were aseptically collected from the mice and minced in a RPMI 1640 medium, pelleted and re-suspended in erythrocyte lysis buffer (Sigma Aldrich, Mumbai). The cells were washed thrice in RPMI 1640 medium supplemented with 2 mM/L L-glutamine, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 25 mM/L HEPES buffer and 10% heat-inactivated fetal calf serum. The cells were enumerated and then plated in duplicate in flat bottomed 24-well plates (Thermo Fisher Scientific, Mumbai) at a density of 2×10^6 cells/well. The cells were stimulated separately with rWbL2 (10 $\mu\text{g}/\text{well}$) or Concanavalin A (2 $\mu\text{g}/\text{well}$; positive control) (Sigma-Aldrich, Mumbai) or medium alone (negative control) and were incubated at 37 °C in 5% CO_2 atmosphere for 48 h. After incubation, the cells were pelleted and stored in Trizol reagent (Thermo Fisher Scientific, Mumbai) at -80 °C until further used for cytokine mRNA expression analysis.

Assessment of Relative mRNA Expression of Cytokines in Splenocytes

Total RNA was isolated from the splenocytes of the mice of different groups and reverse transcribed to cDNA by using a TRIzol reagent and High Capacity cDNA Reverse Transcription Kit (Invitrogen, Mumbai) according to the manufacturer's instructions. Real-time PCR was performed on a StepOne Plus machine (Applied Biosystems, Mumbai) using TaqMan gene expression assays (Applied Biosystems, Mumbai) for cytokines IFN- γ , TNF- α , IL-6, IL-10 and IL-17. Glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) was used as an endogenous control. Relative mRNA expression was analyzed using StepOne analysis software 2.2.2 (Applied Biosystems, Mumbai).

Estimation of Immunoglobulin G (IgG) Isotypes

Sera collected, on the day of sacrifice, from the caudal veins of all the mice in the experimental and control groups were used to measure the levels of IgG1 and IgG2a against rWbL2 by using a Rapid ELISA Mouse Antibody Kit (Thermo Fisher Scientific, Mumbai) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were carried out using software SPSS 21.0 (IBM, Armonk, NY, USA). Continuous parameters were expressed as mean \pm standard error of mean (SEM) and categorical variables were expressed as numbers and percentages. The variables were checked for normality assumptions. Abnormally distributed variables were analyzed using suitable non-parametric test. Normally distributed data were analyzed using suitable test for comparison of means. p values ≤ 0.05 were considered as statistically significant.

Results

Effect of Preventive Treatment with rWbL2 on DSS Induced Colitis

The animals pre-treated with alum alone (as medium) developed severe colitis by 9th day after the initiation of DSS feeding with 21% of loss in the mean body weight (Fig. 1a). Compared to this group, the mice pre-treated with rWbL2 and induced with colitis showed only 4% of loss in the mean body weight and this difference was significant ($p \leq 0.05$).

The mice pre-treated with alum alone and induced for colitis showed gradually increased DAI leading to mean DAI of 3.95 ± 0.04 (Mean \pm SEM) by the end of 9th day (Fig. 1b). In contrast, the mice pre-treated with rWbL2 and induced for colitis had DAI of only 1.94 ± 0.51 and this difference was significant ($p \leq 0.05$).

While the decrease in colon length in the control group of mice (Alum-DSS) was 33%, it was only 16% in the mice subjected to pre-treatment with rWbL2 and induced for colitis (Fig. 1c) and the difference was significant ($p \leq 0.05$).

The preventive effect of rWbL2 was also reflected in the reduced degree of mucosal edema which was significantly ($p \leq 0.0001$) lower in the colitis mice that had received

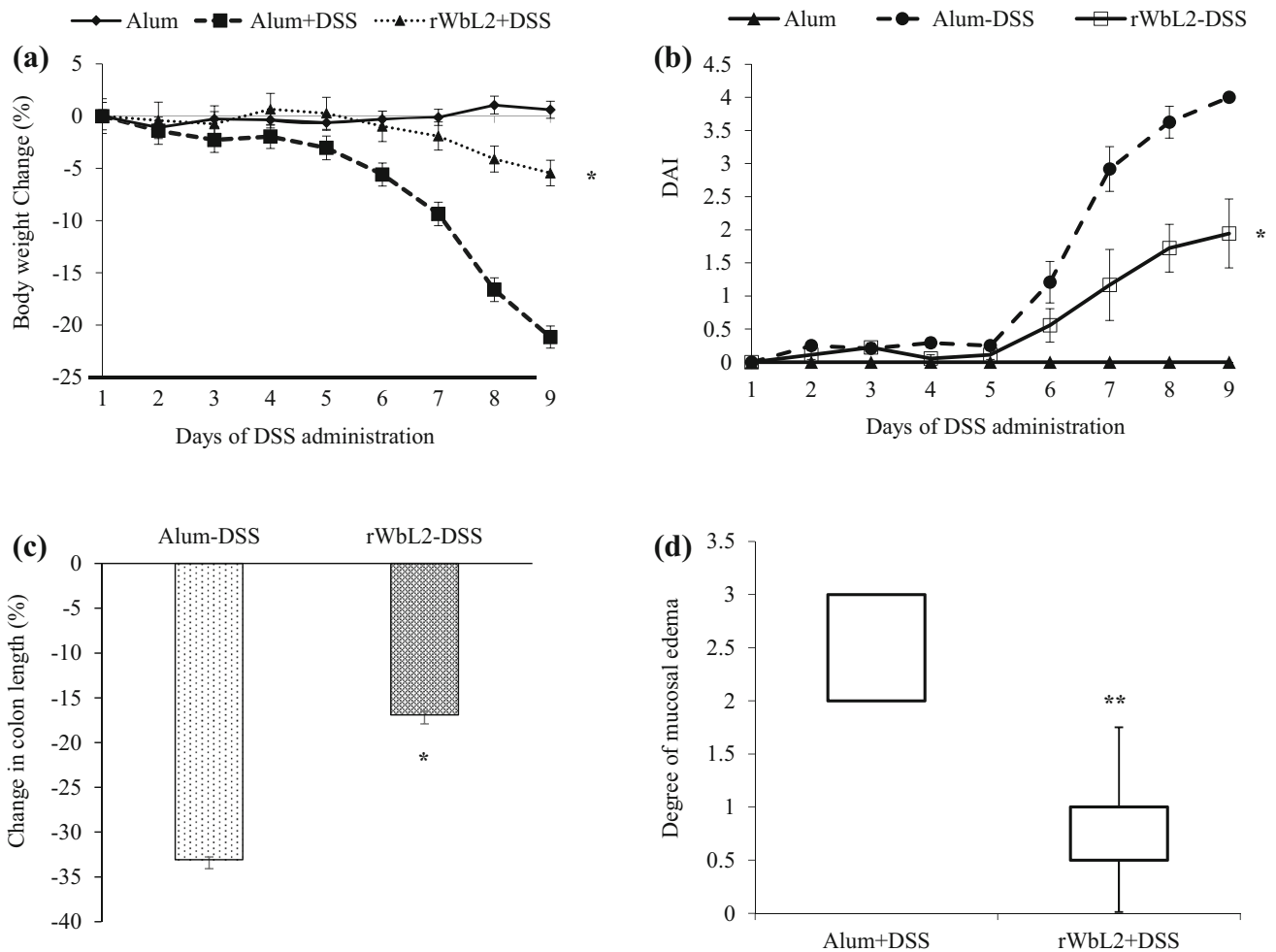


Fig. 1 Effect of the pre-treatment of colitis mice with rWbL2 on the **a** body weight. **b** Disease activity index (DAI). **c** Change in colon length. **d** Degree of mucosal edema. Each *data point* represents

Mean ± SEM. n = 6–8 mice per group; **p* ≤ 0.05; ***p* ≤ 0.0001 in comparison with the Alum-DSS group as analyzed by Mann–Whitney *U* test and change in colon length (%) by student *t* test

rWbL2 pre-treatment compared to the colitis mice that were pre-treated with alum alone (Fig. 1d).

Effect of the Pre-treatment with rWbL2 on Histopathological Changes

The extent and severity of inflammation, superficial crypt damage, colon wall thickness, leucocyte infiltration and the presence of lamina propria mononuclear cells were assessed in the H&E stained distal colon tissues of mice. The control mice (Alum-DSS group) had extensively severe and transmural inflammation with superficial crypt damage. There was moderate increase in lamina propria mononuclear cells and neutrophils infiltration into mucosal and submucosal areas in these mice. In contrast, the pre-treatment with rWbL2 resulted in decreased histopathological score along with intact structural integrity of the colon reflecting reduced damage (Fig. 2; Table 1).

Effect of rWbL2 Treatment on Myeloperoxidase (MPO) Activity

The control group (Alum-DSS) of mice after induction of colitis had significant increase in the MPO activity in the colon tissues. In contrast, the mice pre-treated with rWbL2 and induced with colitis showed significantly reduced MPO activity indicating lowered inflammation compared to that in the control mice (*p* ≤ 0.001) (Table 1).

Effect of the Treatment on the IgG Isotypes

Levels of IgG1 and IgG2a antibodies in the sera samples from the respective groups were measured and expressed in terms of their ratio. Significantly increased levels of IgG1 (*p* ≤ 0.0001) and decreased levels of IgG2a (*p* ≤ 0.05) antibodies were noted in the rWbL2 pre-treated colitis mice, which also reflected in a significant increase in the IgG1:IgG2a ratio (Fig 3a, b).

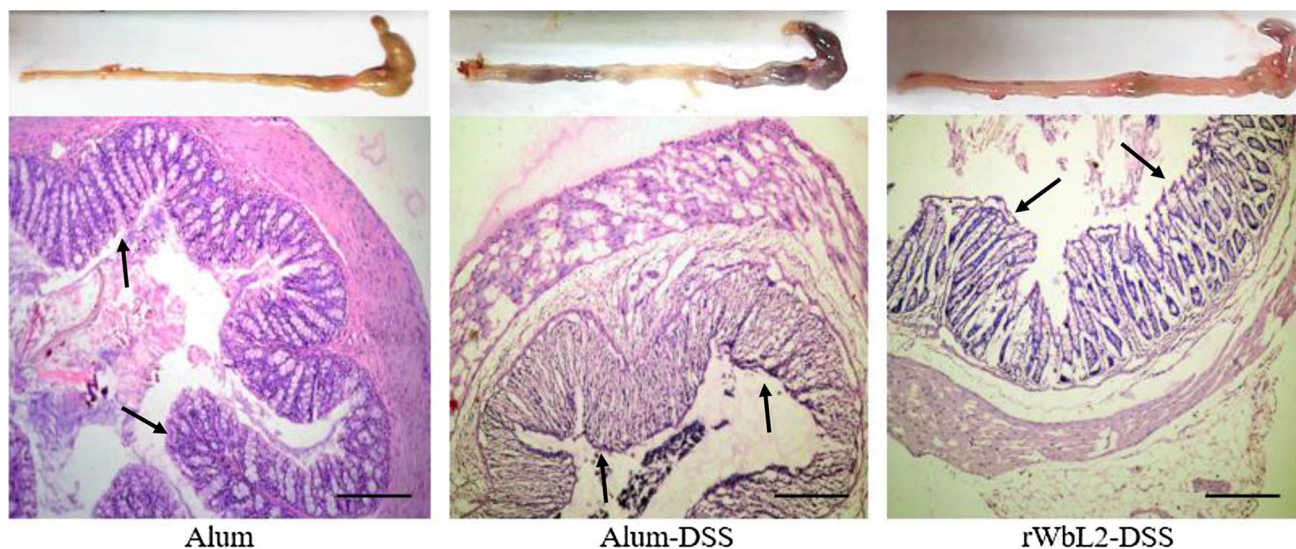


Fig. 2 Effect on the colon damage. Normal mice (Alum) showed normal colon histology with intact epithelial structure. In Alum-DSS mice, administration of DSS caused complete loss of epithelium with crypt distortion. However, pretreatment with rWbL2 ameliorated

histological damage with restoration of structural integrity of colon. Arrow indicates respective changes. Representative photographs of the colon sections of different groups of mice (Original magnification $\times 40$)

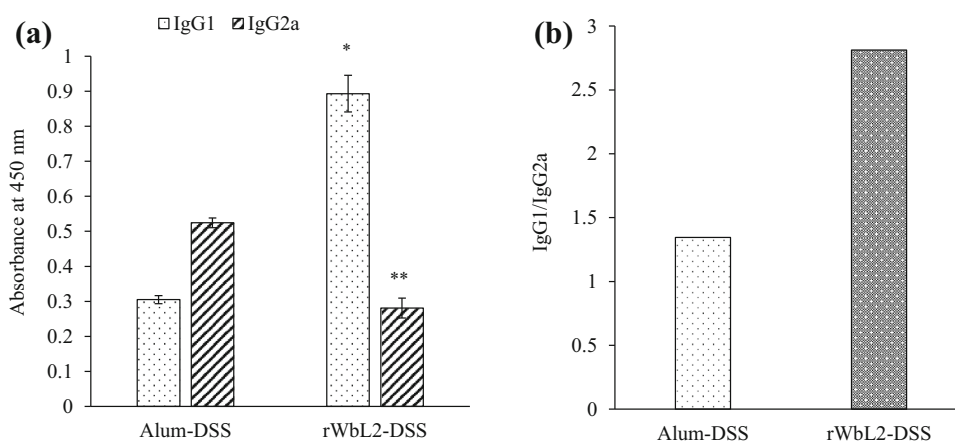
Table 1 Histopathological changes and MPO activity in colon tissues of colitis mice pretreated with rWbL2

Group	Histopathological Score (%)	MPO activity (U/g tissue sample; mean \pm SEM)
Alum-DSS	100	76.96 \pm 8.55
rWbL2-DSS	61.61*	28.75 \pm 5.69**

n = 6–8 in each group

* $p \leq 0.05$; ** $p \leq 0.001$ in comparison with Alum-DSS group as analyzed by Mann–Whitney *U* test

Fig. 3 Mean levels (\pm SEM) of **a** IgG1 and IgG2a antibodies in the sera. **b** Ratio of IgG1/IgG2a. * $p \leq 0.0001$; ** $p \leq 0.05$ in comparison with the Alum-DSS group



Effect of the Pretreatment with rWbL2 on Cytokine Expression

Upon re-stimulation with rWbL2, the expression of inflammatory cytokine IFN- γ in the splenocytes was found to be significantly ($p \leq 0.05$) reduced in colitis mice pretreated with rWbL2 compared to the level in colitis mice pretreated with alum only. Additionally, there was down-regulated

expression of TNF- α , IL-6 and IL-17 cytokines in the splenocytes compared to the levels in the splenocytes of colitis mice pretreated with alum only, though the difference was not significant. On the other hand, the expression of anti-inflammatory cytokine IL-10 was found to be significantly ($p \leq 0.0005$) up-regulated in the splenocytes of colitis mice pretreated with rWbL2 compared to the levels in the splenocytes of colitis mice pretreated with alum only (Fig. 4).

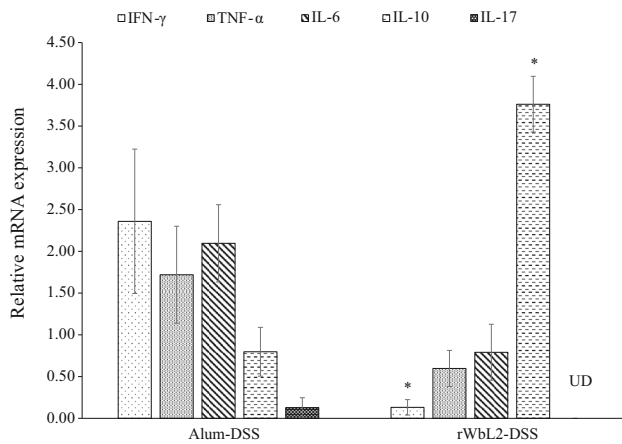


Fig. 4 Effect of the preventive treatment with the rWbL2 on cytokine expression. Each bar represents mean \pm SEM, $n = 5$ in each group; * $p \leq 0.05$; ** $p \leq 0.0005$ in comparison with Alum-DSS group as analyzed by Student t test. UD, mRNA expressions of IL-17 was undetected in the sample

Discussion

Many parasitic helminths including filarial nematode *Wuchereria bancrofti*, for their long time survival in the host, secrete specific filarial proteins and modulate host immune response towards favorable anti-inflammatory environment. The down-regulation of inflammatory host immune response is suggested to be induced by secreted products of the nematodes [22]. Helminths mediated immunomodulation is, thus, potentially effective in curing or preventing the immunological disorders like autoimmune and allergic diseases [23–25]. However, although they have immunosuppressive activity, they play a vital role in the establishment of active parasitism within the host. Therefore, this study has been undertaken to assess the preventive effect of a purified secretory filarial protein rWbL2 against inflammatory ulcerative colitis disease.

rWbL2 is one of the secretory SXP/RAL family of proteins [14]. Increased rWbL2 specific IgG4 antibody levels was reported in the sera of actively infected individuals (Shivprasad B unpublished data), which is consistently associated with the increased secretion of IL-10 [13]. IL-10 plays an important role in modulating host immune response towards activation and secretion of Th2 type anti-inflammatory cytokines [26]. Hence, SXP/RAL family proteins from various parasites and helminths are suitable candidate molecules to be explored as novel therapeutic and prophylactic agents in immunological disorders.

DSS induced colitis mimics the human ulcerative colitis in clinical parameters observed in the acute phase like progressive weight loss, diarrhea and stool consistency with increased DAI, macro and microscopic score and increased myeloperoxidase activity [27]. In the present

study, we found significantly decreased weight loss and DAI in the mice pre-treated with rWbL2, followed by induction of colitis. The disrupted colon morphology of colitis mice treated with alum alone indicate intestinal inflammation with increased infiltration of the neutrophils in the lamina propria, which is evidenced by increased myeloperoxidase activity. The colon sections of the mice pre-treated with rWbL2 (later on induced for colitis) showed reduced MPO activity. Also the reduction in the colon length shortening and decreased mucosal edema was found in the pre-treated mice. These improvement in the clinical parameters in the mice pre-treated with rWbL2 indicate prophylactic effect of this protein.

In alum pre-treated mice, after induction of colitis, the massive lymphocyte infiltration was observed, which is extended up to submucosa with complete loss of the epithelial lining of the colon. In contrast, rWbL2 pre-treated mice after induction of colitis showed significantly reduced lymphocyte infiltration and decreased epithelial damage. Myeloperoxidase is found in polymorphonuclear leucocytes, monocytes and some macrophages and its activity is a marker of accumulation of these inflammatory cells [28]. The reduced MPO activity in the rWbL2 pre-treated mice confirms its usefulness as an effective preventive agent against colitis.

For the validation of therapeutic responses and utilization of immunomodulators, understanding of mechanism of action, of rWbL2, in terms of cellular and humoral immune responses is necessary. For this purpose, splenocytes from the treated mice were stimulated with 10 $\mu\text{g}/\text{mL}$ of rWbL2 for 48 h (pre-optimized condition) [14, 17, 29]. Following this treatment, anti-inflammatory and pro-inflammatory gene expression analysis was performed. The increased levels of inflammatory and proinflammatory cytokines such as IFN- γ and IL-17a is an immunological characteristic of experimental colitis, which resembles the immune profile of UC in humans [6]. The involvement of T cell mediated immune response plays an important role in the colitis progression [30]. Characterized Th1/Th2-biased type of immune response with increased level of inflammatory cytokines such as TNF- α , IFN- γ , IL-1 β , IL-5 was found in the DSS-induced acute colitis [31]. On the other hand, helminths induce Th2 type immune response with an increase in anti-inflammatory cytokine IL-10 in the host to protect themselves from host pro-inflammatory immune response [32]. In our study after treatment with rWbL2, increased levels of the expression of anti-inflammatory cytokine IL-10 and decreased levels of expression of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6 and IL-17a was found. This protein has already shown to have immunomodulatory activity through alteration of cytokine levels [14]. This study demonstrated that such alteration in cytokine levels might be directly associated with change in

gene expression. Similar work by earlier workers also showed rSjcystatin induced decrease in pro-inflammatory cytokines and increases in anti-inflammatory cytokines at mRNA as well as at protein level [33]. This shift from pro-inflammatory cytokine profile to anti-inflammatory cytokine response was also supported by concomitant and significant ($p \leq 0.05$) increase in the levels of IgG1 antibodies in the sera of pre-treated mice, followed by induction of colitis.

Some of the other parasitic proteins have been shown to be able to induce protection from the development of colitis. The mice pre-treated with recombinant *Trichinella spiralis* (rTsP53) antigen attenuated the severity of trinitrobenzene sulfonic acid (TNBS)-induced colitis as evident from the significant reduction in the macroscopically and histopathologically evaluated colon damage and reduction in MPO activity in colon tissues [34]. Pre-treatment with As-MIF from *Anisakis simplex* has shown amelioration of DSS-induced colitis by the induction of IL-10 expression via the ERK pathway in intestinal epithelial cells [35]. From the results of this study, it can be envisaged that pre-treatment with the rWbL2 induces protection against ulcerative colitis and its preventive effect is associated with the generalized shift in immune response from Th1 to Th2.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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