EXPLORATION OF IMMUNE MODULATION BY COMBINATION OF FILARIAL PROTEINS AGAINST DSS INDUCED ACUTE COLITIS IN MOUSE MODEL

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ABSTRACT

Background: Chronic helminthic infections lead to release of various immunomodulatory molecules which play an important role in modulation of the host immune system. In the present study, we assessed the therapeutic efficacy of the combination of immunomodulatory proteins from *Wuchereria bancrofti* and *Brugia malayi* filarial parasites (rWbL2, rBmALT2 and rBmCystatin), against dextran sulphate sodium (DSS) induced ulcerative colitis in BALB/c mice.

Methods: Colitis was induced by feeding female BALB/c mice on 5% DSS in autoclaved tap water. The colitis mice were treated intraperitoneally (i.p.) with different combinations of filarial proteins. The disease severity was monitored by measuring disease activity index (DAI), macroscopic score, microscopic score and myeloperoxidase activity. The changes in the immunological responses were checked by measuring Th1, Th2 and regulatory cytokine profiles in splenocytes.

Results: Colitis mice treated with a combination of rBmALT2 with rBmCystatin showed *significantly reduced disease severity as indicated by the decreased disease manifestations,* improved macroscopic and microscopic inflammation correlated with the up-regulation of anti-inflammatory cytokines IL-10 and down-regulation of pro-inflammatory cytokines such as IFN-γ, TNF-α in splenocytes and serum of treated mice. The rBmALT2+rBmCys combination was significantly more effective compared to the combination of rWbL2+rBmALT2 and rWbL2+rBmCys in reducing the severity of colitis.

Conclusions: The above findings provide evidence that cocktail of rBmALT2 and rBmCystatin has a therapeutic potential for alleviating colitis in mice. The underlying immunological mechanism seems to involve the up-regulation of Th2 immune response with concomitant down-regulation of Th1 response.

Keywords: helminth**s**, ulcerative colitis, recombinant Cystatin, recombinant BmALT2, recombinant WbL2.

REZUMAT

Introducere: Infecțiile cronice cu helminți conduc la eliberarea diferitelor molecule imunomodulatoare, care joacă un rol important în modularea sistemului imunitar al gazdei. În studiul prezent, am evaluat eficacitatea terapeutică a combinării proteinelor imunomodulatoare de la *Wuchereria bancrofti* și a paraziților filariali *Brugia malayi* (rWbL2, rBmALT2 și rBmCystatin), pentru tratarea colitei ulcerante induse de dextran sulfat de sodiu (DSS) la șoareci BALB/c.

Metode: Colita a fost indusă prin hrănirea șoarecilor femele BALB/c cu DSS 5% în apă de la robinet autoclavată. Șoarecii care prezentau colită au fost tratați intraperitoneal (i.p.) cu diferite combinații de proteine filariale. Severitatea bolii a fost monitorizată prin măsurarea indicelui de activitate a bolii (DAI), a scorului macroscopic, a scorului microscopic și a activității mieloperoxidazei. Schimbările răspunsurilor imunologice au fost verificate prin măsurarea profilurilor Th1, Th2 și a citokinelor reglatoare în splenocite.

Rezultate: Șoarecii bolnavi de colită, tratați cu o combinație de rBmALT2 cu rBmCystatin au prezentat o severitate semnificativ scăzută a bolii, după cum au indicat manifestările diminuate ale bolii, o ameliorare a inflamației evidențiată macroscopic și microscopic, corelată cu creșterea citokinelor pro-inflamatorii IL-10 și scăderea pro-citokinelor inflamatorii, cum ar fi IFN-γ, TNF-α în splenocite și serul șoarecilor tratați. Combinația rBmALT2 + rBmCys a fost semnificativ mai eficientă în reducerea severității colitei, decât combinația de rWbL2 + rBmALT2 și rWbL2 + rBmCys.

Concluzii: Constatările de mai sus oferă dovezi care indică potențialul terapeutic al cocteilului de rBmALT2 și rBmCystatin în ameliorarea colitei la șoareci. Mecanismul imunologic care stă la baza acestei ameliorări pare să implice creșterea răspunsului imun Th2 și scăderea concomitentă a răspunsului Th1.

Cuvinte-cheie: helminți, colită ulcerantă, Cystatin recombinant, BmALT2 recombinant, WbL2 recombinant.

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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), comprise 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 pro-inflammatory response associated with increased expression of inflammatory and pro-inflammatory cytokines.

Though the IBD has been mostly prevalent in the Western countries, the recent finding indicates that it is also 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 depletion hypothesis, implies that helminths for their own survival and growth secrete various proteins, which are crucial for the suppression of the host's pro-inflammatory immune response. 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 [5-7]. Also, several studies showed that use of helminth derived molecules/proteins could suppress the severity of colitis [8-10].

Previous studies from our laboratory showed that the use of recombinant proteins of human lymphatic helminths *Brugia malayi* and *Wuchereria bancrofti viz.,* rBmCystatin, rBmALT2 and rWbL2 are useful to treat acute colitis in mice [11, 12].

The present study aims to further enhance the therapeutic efficacy of these filarial proteins in colitis using different combinations of these proteins to treat DSS induced acute colitis in BALB/c mice.

MATERIALS AND METHODS

Filarial protein preparation: Filarial proteins rWbL2, rBmALT2 and rBmCystatin protein were prepared as described by earlier using *Escherichia coli* (*E. coli)* based *expression* system [12, 13]. Briefly, pRSET-A-rWbL2 or pRSET-A-rBmALT2 or pRSET-A-rBmCystatin plasmid was transformed into BL21(DE3) pLysS *Escherichia coli* expression host and expressed as His-tag protein by inducing with 1mM Isopropyl β-D-1-thiogalactopyranoside (Merck Millipore, Bangalore) at 37°C. The recombinant protein was purified using a nickel affinity chromatography column (Thermo Fisher Scientific, Bengaluru). The protein content was estimated using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Bengaluru). Endotoxin content was removed by triton 114 method as described earlier [14].

Induction of colitis: Colitis was established in the mice, as described previously [15]. 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.

Treatment with filarial proteins: Prior to treatment, mice in different groups were recruited (n = 6–8 mice/group) *viz*; the PBS group (control mice treated with PBS alone), DSS+PBS group (DSS induced colitis mice treated with PBS) and DSS+rWbL2+rBmALT2, DSS+rWbL2+rBmCys, DSS+rBmALT2+rBmCys (DSS-induced colitis mice treated with filarial proteins). The mice were treated with 25 μg of combination of recombinant filarial proteins intraperitoneally on days 4, 5 and 6 of DSS administration and monitored for the signs of colitis and sacrificed on the day when all DSS-Colitis group of mice developed colitis [16].

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 mean 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) [17].

Macroscopic and histopathological scoring of colon damage: After sacrifying 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 [18]. For histopathological scoring, colonic segments were fixed in 10% formalin, embedded in paraffin, cut into 5-µm-thick sections and stained with hematoxylin and eosin (HE). Inflammation was graded based on the parameters adapted from with some modifications [19]. The parameters scored included 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).

Assessment of Myeloperoxidase (MPO) activity: Myeloperoxidase enzyme activity in the colon samples was assayed as previously described [20]. 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 15000 × g for 15 min at 4°C. The supernatant of each sample (0.1 mL) was added to 2.9 mL 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 µmol of H_2O_2 to H_2O per min at 25°C.

Cytokine analysis in *in vitro* culture 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 µg/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, Bengaluru) at a density of 2×10^6 cells/well. The cells were stimulated separately with Concanavalin A (2*µg/well; positive control) (Sigma-Aldrich, Mumbai*) or medium alone (negative control) and were incubated at 37°C in 5% CO_2 atmosphere for 48h. After incubation, the cells were pelleted by centrifugation supernatant, were collected and stored at -80°C for the estimation of the cytokines using Enzyme Linked Immunosorbent Assay (ELISA) kits (Invitrogen, Mumbai) as per the manufacturer's instructions and cell pellets were stored in Trizol reagent at -80°C until further used for cytokine mRNA expression analysis.

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 by using a Rapid ELISA Mouse Antibody Kit (Thermo Fisher Scientific, Bengaluru) according to the manufacturer's instructions. Briefly, 50 µl of pooled sera samples from each group were applied to the pre-coated (with anti-mouse capture antibodies) 96 well plate supplied with kit. Followed by washing, wells were incubated with peroxidase conjugated goat anti-mouse monoclonal IgG1 or IgG2a antibody (1:5000 diluted in PBS; 100 µl/well). After incubation for 45 min at 37°C, wells were washed five times and color was developed by the addition of TMB substrate. Optical density was measured at 450 nm.

Assessment of relative mRNA expression of cytokines in splenocytes: Total RNA was isolated 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, Maharashtra, India) using TaqMan gene expression assays (Applied Biosystems) for cytokines IFN-γ, TNF-α, 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).

Statistical analysis: Statistical analyses were carried out using software SPSS 21.0 (IBM, Armonk, NY, USA). Continuous parameters were expressed as mean ± standard error of mean (SEM) and categorical variables were expressed as numbers and percentages. The variables were checked for normality assumptions. Normally distributed parameters were analyzed using a one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. P values ≤ 0.05 were considered as statistically significant.

RESULTS

Effect of combination of filarial proteins on DSS induced colitis

The disease progression in the colitis mice treated with PBS only was significantly higher. The combinations of rBmALT2+rBmCys and rBmCys+rWbL2 were significantly ($p \leq 0.05$) more effective in protecting the mice from the weight loss compared to combination of filarial proteins (rBmALT2+rWbL2) (Fig. 1A).

The colitis mice treated with combination of all three showed significant ($p \leq 0.05$) reduction in DAI compared to the control group of colitis mice (DSS+PBS) (Fig. 1B). The colitis mice treated with rBmALT2+rBmCys combination resulted in reduced colon length shortening and degree of mucosal edema when compared to DSS-induced colitis mice treated with PBS only, though the difference was not significant (Figs. 1C and D).

Effect of the treatment on histopathological damage and myeloperoxidase activity-

Upon histopathological examination, the colon sections from the untreated colitis mice showed severe inflammation with superficial crypt damage, colon wall thickness and increased neutrophils infiltration which was evident from the increased myeloperoxidase activity in colon sections.

Whereas mice treated with combination of these proteins showed significantly ($P \leq 0.05$) decreased myeloperoxidase activity in the protein treated groups compared to the untreated group of mice; however, there was no significant difference found among these three treated groups (Table 1) (Fig. 2).

Effect of the treatment on the IgG isotypes

Immunoglobulin G (IgG) isotyping analysis was done by using ELISA and the results were expressed in terms of their ratio.

Table 1. Histopathological changes and MPO activity in colon tissues of colitis mice treated with combinations of filarial proteins

Groups	Histopathological score (%)	MPO activity (U/g tissue sample; mean ± SEM)
DSS+PBS	100	23.77 ± 2.75
DSS+rWbL2+rBmALT2	12.76595745*	7.20 ± 0.60*
DSS+rWbL2+rBmCys	25.53191489*	12.01 ± 0.89*
DSS+rBmALT2+rBmCys	26.80851064*	11.80 ± 0.83*

Distal colon samples were labelled, fixed, paraffin embedded, HE stained and scored for histopathological changes and presented as % change in score compared to DSS+PBS group. The histopathological score consists of scoring of inflammation severity, inflammation extent, crypt and epithelial tissue damage, and recruitment of immune cells. MPO activity was measured in the colon tissue samples and expressed as Mean ± SEM values of units per gram (U/g) of tissue sample; n=7 in each group, *$p \leq 0.05$compared to control group as analyzed by One way ANOVA followed by Tukey's post hoc multiple comparisons test.

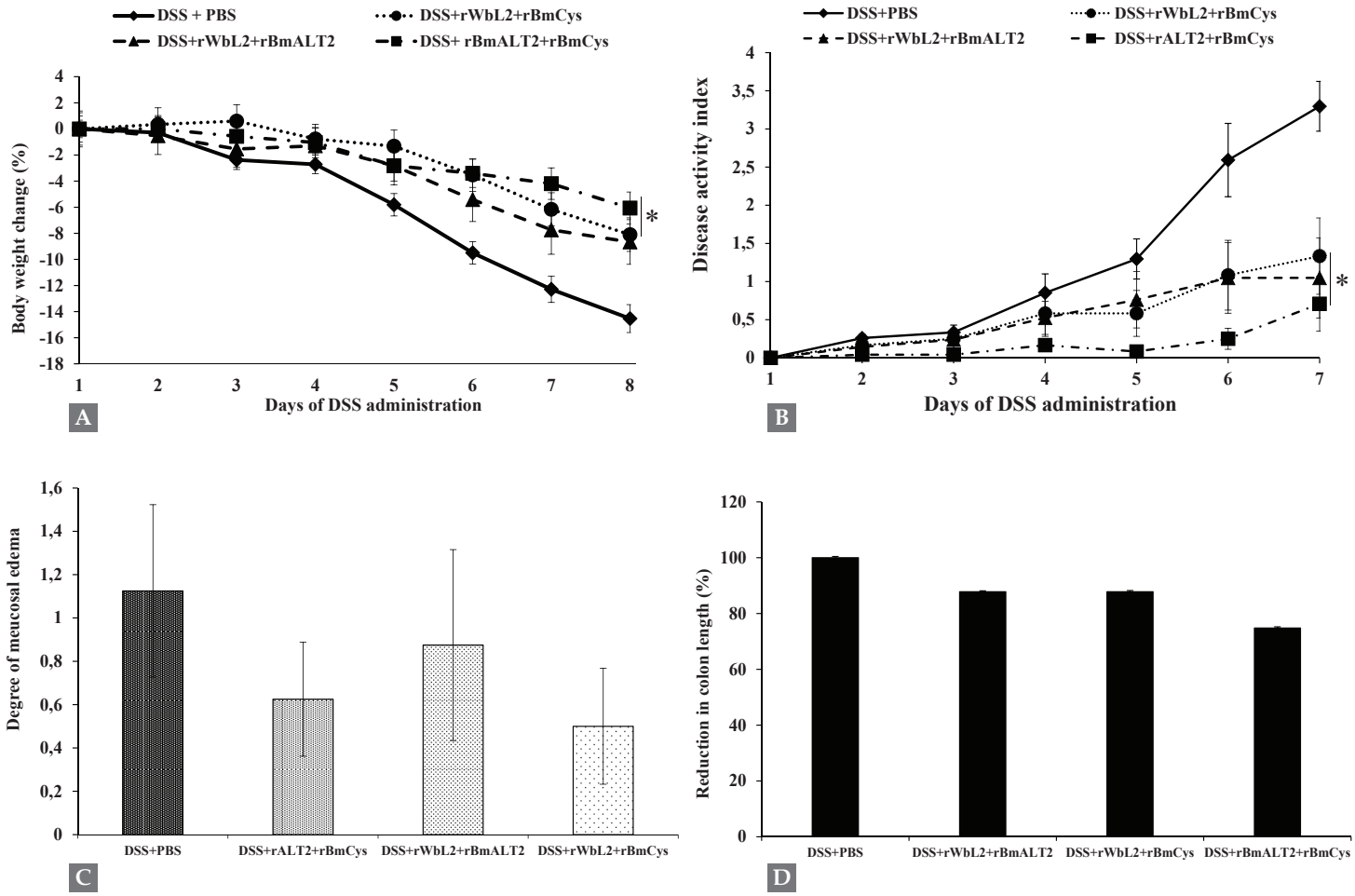

Fig. 1. Effect of treatment with combination of filarial proteins on colitis mice.
A) Body weight change (%); B) DAI; C) degree of mucosal edema; D) change in colon length.
Values are Mean ± SEM, n=6-8 in each group; $^{*}P \leq 0.05$ compared to untreated colitis mice group as analyzed by One way ANOVA followed by Tukey's post hoc multiple comparisons test.

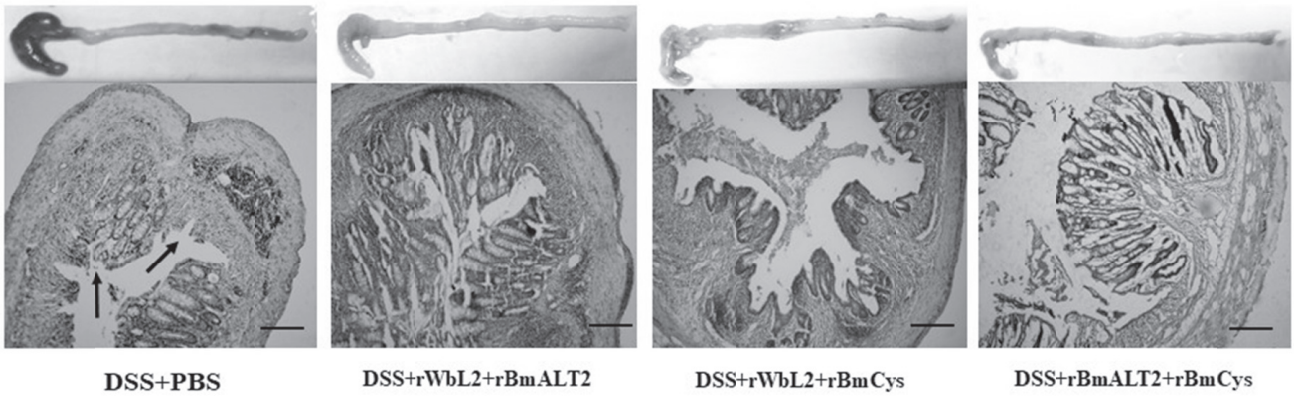

Fig. 2. Effect of treatment on colon damage.
Paraffin embedded colon tissue sections were stained with H & E
for microscopic assessment of colon damage. Representative photographs of H&E stained tissue sections of distal colon (original magnification x 40).
[Arrow indicates neutrophil infiltration and loss of crypt and surface epithelial lining of colon.]

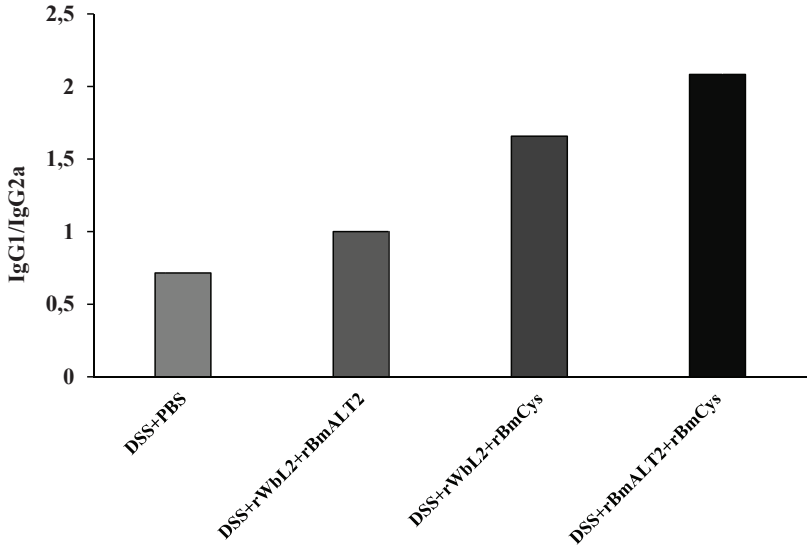

Fig. 3. Effect of treatment with rBmALT2+rBmCys on IgG antibodies in DSS induced colitis. Each bar represents the ratio of IgG1 and IgG2 isotypes

The increased IgG1 to IgG2 was observed in the colitis mice treated with the combinations of filarial proteins when compared with untreated colitis mice (Fig. 3).

Effect of the treatment with combination of rBmALT2+rBmCys on cytokine expression

Upon stimulation with ConA, the expression of inflammatory cytokine IFN-γ was found to be significantly ($p \leq 0.05$) reduced in rBmALT2+rBmCys treated colitis mice compared to the colitis mice treated with PBS. Additionally, there was down-regulated expression of TNF-α and IL-17a cytokines compared to untreated colitis mice, though the difference was not significant.

On the other hand, the expression of anti-inflammatory cytokine IL-10 was found to be significantly ($p \leq 0.05$) up-regulated in the splenocytes of rBmALT2+rBmCys treated colitis mice compared to the PBS treated colitis mice (Fig. 4).

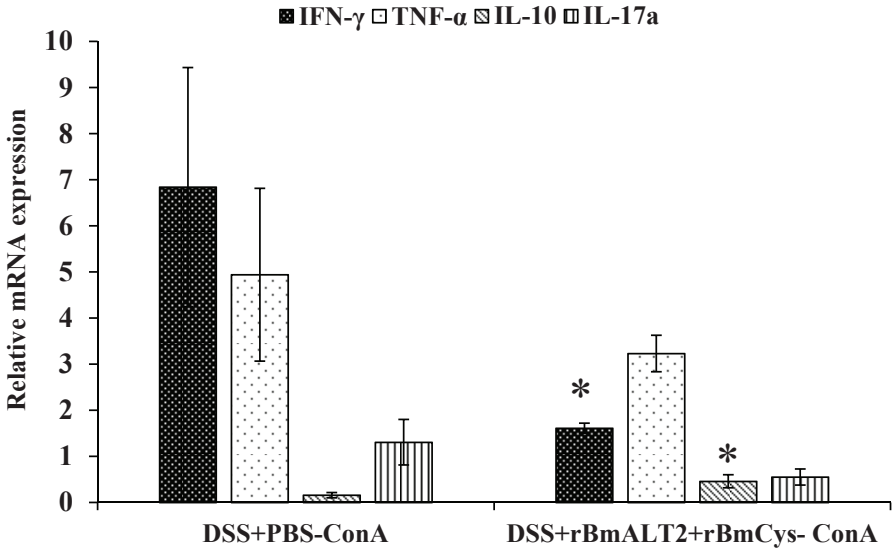

Fig. 4. Effect of the therapeutic treatment with the combination of both rBmCys and rBmALT2 on cytokine expression. Each bar represents mean ± SEM, n= 5 each group; *$p \leq 0.05$ statistically significant in comparison with DSS+PBS group as analyzed by analyzed by Student t-test.

DISCUSSION

Helminth parasites have been shown to induce protection against autoimmune and allergic disorders [21-23]. Earlier studies demonstrated the role of helminth and helminth derived products including soluble proteins, antigenic molecules as well as recombinant protein preparations with the ability to suppress the development of ulcerative colitis by down-regulating Th1 and Th17 immune responses [6, 24, 25].

Previous studies from our laboratory showed the recombinant proteins of filarial parasite *Brugia malayi* such as rBmCystatin and rBmALT2 having *in vitro* anti-inflammatory as well as *in vivo* amelioration effect on the DSS induced colitis in BALB/c mice model (11). Similarly, recombinant proteins from *Wuchereria bancrofti* i.e. rWbL2 protein were demonstrated to have anti-inflammatory activity in *in vitro* and therapeutic efficacy in *in vivo* by the suppression of pro-inflammatory condition in DSS induced UC [12]. To further enhance the efficacy of these proteins, the present study was aimed to check the therapeutic effect of different combinations of rBmALT2, rBmCystatin and rWbL2 to treat acute colitis in mice.

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 [26]. In the present study, we found significantly decreased weight loss and DAI in the mice treated with the three different combinations of these filarial proteins namely, rWbL2+rBmALT2, rWbL2+rBmCys or rBmALT2+rBmCys. The disrupted colon morphology of untreated colitis mice indicated intestinal inflammation with increased infiltration of the neutrophils in the lamina propria, which was evidenced by increased myeloperoxidase activity. On the other hand, colon sections of the mice treated with the different combinations of these proteins showed reduced MPO activity. Also the decline in the colon length shortening and decreased mucosal edema was found in the treated colitis mice. These improvements in the clinical parameters in the mice treated with all three combinations of filarial proteins suggest the curative effect of these combinations.

The combination of rBmALT2 and rBmCys proteins showed significantly ($p<0.05$) higher attenuation of disease severity, than that of other combinations, as evident from decrease in the weight loss, reduced DAI, conservation of colon length and decreased mucosal edema. Colon tissues from these mice also showed minimized damage in histopathological features and decreased MPO activity compared to other combinations (rWbL2+rBmALT2 and rWbL2+rBmCys), though it was not significantly different. For the validation of therapeutic impact in terms of cellular and humoral immune responses and further effective utilization of these filarial proteins in various combinations as immunomodulators, understanding of the mechanism of action is necessary. As rBmALT2+rBmCys combination showed more protective effect against the development of colitis compared to other combinations, the humoral and cellular responses were tested in DSS-induced colitis mice those were treated with this particular combination.

The increased level of inflammatory and pro-inflammatory cytokines such as IL-17 and IFN-γ are considered as immunological characteristics of experimental colitis, which resembles the immune profile of clinical UC [6]. The involvement of T cell mediated immune response plays an important role in colitis progression [27]. Characteristic Th1 biased type of immune response with increased level of inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-5 was found in the DSS-induced acute colitis [28]. On the other hand, helminth tends to induce Th2 type immune response with an increase in anti-inflammatory cytokine IL-10 in the host to protect themselves from pro-inflammatory immune response [25]. In our study after treatment with the combination of filarial proteins, increased levels of the IL-10 and TGF-β were found to display potential anti-inflammatory activity with concomitant decrease in the levels of inflammatory cytokines such as IFN-γ, TNF-α. Although not statistically significant but definitive down regulation in the expression level of IL-17 in the treated group of mice was noted as compared to untreated disease group. 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 colitis mice. Therefore, it is tempting to speculate that treatment with the combinations of filarial proteins could balance impaired host immune response and stimulate regulatory processes which results in the protection against the dreadful disorder.

CONCLUSION

Taken together, the decrease in the severity of the colitis in mice treated with filarial protein combinations was accompanied by the increase in the levels of anti-inflammatory cytokines and IgG1 antibodies indicating that the amelioration effect of these proteins might be associated with the generalized shift in immune response from Th1 to Th2.

Acknowledgements

We would like to extend our tribute to Late Dr. MVR Reddy, for his active contribution to this work. All authors gratefully acknowledge the financial assistance given by the Department of Science and Technology (DST) (Grant No. SR/SO/HS-0065/2012) and the Department of Biotechnology (DBT) (Grant no. BT/PR/4988/INF/22/55/2012) Ministry of Science & Technology, Government of India. Also, the infrastructure facility developed by DST-FIST grant (Grant No. SR/FST/LSI-470/2010) is thankfully acknowledged. Namdev Togre would like to thank Dr. Babasaheb Ambedkar, Research and Training Institute (BARTI), Pune, Government of Maharashtra for fellowship.

Conflict of interests: The authors declare no conflict of interest.

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