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Evaluation of preventive effect of *Brugia malayi* recombinant cystatin on mBSA-induced experimental arthritis

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Epidemiological and experimental studies have demonstrated the therapeutic efficacy of the helminths derived immunomodulatory molecules. In this study, we investigated the preventive effect of *Brugia malayi* recombinant cystatin (r*Bm*Cys) in methylated bovine serum albumin (mBSA)-induced arthritis. *Mastomys coucha* rats were treated with 4 doses of r*Bm*Cys (intraperitoneal) in alum adjuvant (25 µg/dose/200 µL) in intervals of 15 days. Control rats received alum only. mBSA-arthritis induction was done 10 days after the last dose of r*Bm*Cys/alum. Rats were sacrificed when all the rats in mBSA group developed arthritis. Administration of r*Bm*Cys significantly (P=0.0005) protected rats from arthritis by reducing paw swelling and arthritic index. In r*Bm*Cys treated rats, histopathology of hind paw joints showed decreased synovitis, bone erosion, fibrosis and influx of inflammatory cells. This protective effect was found to be associated with significantly (P <0.005) decreased levels of IFN-γ & TNF-α and increased levels of IL-4 and IL-10 cytokines. These results have shown that prior administration of r*Bm*Cys can benefit in rheumatoid arthritis prevention.

Keywords: *Brugia malayi* recombinant cystatin, mBSA-induced arthritis, Rheumatoid arthritis, Roundworm

Rheumatoid arthritis (RA) is a chronic autoimmune disease with unknown aetiology, affecting about 1% of the world population[1]. Available therapies *viz.* anti-rheumatic drugs, nonsteroidal anti-inflammatory drugs and cytokine inhibitors[2] are not only non-effective and its continuous usage may also result in serious side effects[1,3]. RA, hence requires new therapeutic strategies.

Helminths are highly adaptive parasites and quite prevalent in humans[4]. During their survival in the host, they modulate the hosts' immune system to create a favourable environment essential for their survival. "Hygiene hypothesis" suggests that these parasitic infections can provide protection against autoimmune or allergic disorders as evident from the fact that several autoimmune disorders are less prevalent in countries which are endemic for helminth infections[5,6]. Further, the protective efficacy imparted by helminth infections or their products has been confirmed by experiments conducted in animal models of several immuno-functional disorders[4]. We have also demonstrated the therapeutic efficacy of filarial protein, *Brugia malayi* recombinant abundant larval transcript 2 (r*Bm*ALT2) against ulcerative colitis[5].

Acanthocheilonema viteae excretory-secretory (ES)-62 has been widely tested for RA and has been found to protect mice from developing arthritis by suppressing Th1/Th17 immune responses and increased IL-10 production by splenocytes[2]. A recent study has demonstrated the protective effects of *Schistosoma mansoni* and *Trichinella spiralis* derived antigens against adjuvant arthritis[7]. Till now, proteins from filarial parasite *B. malayi* have not been tested for their protective effects in RA. We have earlier shown the therapeutic potential of *B. malayi* cystatin in mBSA-induced RA[8]. Hence, this study was undertaken to investigate the protective effect of *B. malayi* recombinant cystatin in antigen-induced RA in rats.

Cystatins are the secreted immunomodulators known to regulate the cytokine production in macrophages[9,10]. They are crucial for the parasitic establishment within the host and suppress the MHC class II antigen presentation, thus significantly impacting the T-cell responses[10,11]. Recombinant cystatin of *S. japonicum* or *B. malayi* or *A. viteae* has been shown to ameliorate the inflammatory condition of the gut in experimental mice models[12-14]. In light of the evidence from these studies, *B. malayi* cystatin was considered as a promising candidate to be used for the preventive treatment of RA.

Materials and Methods

Brugia malayi recombinant cystatin

r*Bm*Cys was expressed as His-tag protein in *Escherichia coli* by transforming pRSET-A-*bmcys*

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plasmid into BL21 (DE3) *E. coli* cells and was purified using nickel affinity chromatography column (Thermo Fisher Scientific, Mumbai). Purified protein was confirmed by western-blot analysis using monoclonal anti-His G-HRP antibody (Invitrogen, Mumbai) and protein content was quantified. Endotoxin level was measured by LAL chromogenic endotoxin quantitation kit (Thermo Fisher Scientific, Mumbai). Endotoxin levels in the final purified preparations were <10 EU/mL.

Experimental animals

Female *Mastomys coucha* (6-8 wk) were maintained at CPCSEA registered Central Animals House facility on standard animal chow and drinking water *ad libitum*. Study was undertaken after obtaining prior approval from the Institute's Animal Ethics Committee (approval no: MGIMS/IAEC/3/2009).

Treatment of rats with r*Bm*Cys

Rats (n = 8) were grouped as: Control-Alum group (normal rats administered with alum adjuvant only); mBSA group (un-treated rats induced for arthritis only); Alum-mBSA group (rats administered with alum adjuvant followed by induction of arthritis); and r*Bm*Cys-mBSA group (rats administered with 25 µg/dose of r*Bm*Cys in alum adjuvant followed by the induction of arthritis). Treatment with r*Bm*Cys consisted of administration of rats, each with 4 doses (intraperitoneal) of r*Bm*Cys in alum adjuvant (25 µg/dose/200 µL) in intervals of 15 days.

Induction and assessment of mBSA-arthritis

The rats pre-treated with r*Bm*Cys (r*Bm*Cys-mBSA group) or with alum alone (Alum-mBSA group) along with another control group of rats (mBSA group) were sensitized with mBSA; 500 µg of mBSA (Merck, Bangalore) in 0.2 mL of an emulsion containing 0.1 mL of PBS (0.05 M; pH 7.2) and 0.1 mL Freund's complete adjuvant (Sigma-Aldrich, Mumbai). Booster injections of mBSA dissolved in Freund's incomplete adjuvant (Sigma-Aldrich, Mumbai) were given on 7th and 14th day after the first dose of mBSA. On 21st day, arthritis was induced by intra articular injection of mBSA (30 µg in 10 µL PBS)[15].

Assessment of arthritis was done by measuring swelling of paws thrice a week after induction of arthritis. Arthritic index was evaluated based on the score ranging from 0-4. Scoring parameters were: 0, no swelling; 1, erythema and mild swelling; 2, erythema and swelling extending from ankle to the tarsals; 3, erythema and moderate swelling extending to ankle, foot and digits; and 4, erythema and severe swelling encompassing entire paw including ankle[1]. The arthritic index was presented as mean of the summative scores.

Assessment of histopathological changes

All the experimental rats were sacrificed when all the normal rats treated with mBSA (mBSA group) developed arthritis. Hind paws of rats were removed, fixed in 10% formal-saline, decalcified and embedded in paraffin. Five micrometre cross sections of tissues were cut and stained with haematoxylin and eosin. Sections were scored in the range of 0-3: 0, normal; 1, mild; 2, acute; and 3, chronic based on the parameters: synovitis, cartilage & bone erosion and fibrosis[16].

***In vitro* culture of splenocytes for estimation of cytokines**

Spleens were aseptically harvested from the sacrificed rats and minced in RPMI 1640 medium. Single-cell suspension were pelleted and suspended in erythrocyte lysis buffer (Invitrogen, Mumbai). Cells were washed thrice in RPMI 1640 medium and plated in duplicates (2×10^6 cells/well/1 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 foetal calf serum. Cells were stimulated in the presence of r*Bm*Cys (10 µg/1 mL), Concanavalin A (2µg/1 mL; ConA; Sigma-Aldrich, Mumbai; positive control) or medium alone (negative control). After incubation (37°C in 5% CO_2 atmosphere for 72 h) culture supernatants were collected separately by centrifugation and stored at −80°C until used for estimation of cytokines. The levels of IL-10, IL-4, TNF-α and IFN-γ in the culture supernatants of splenocytes were measured by using ELISA kits (Invitrogen, Mumbai) as per the manufacturer's instructions.

Assessment of anti-mBSA specific IgG antibodies

Sera were collected from rats through caudal vein before they were sacrificed. The wells of microtitre plates (Thermo Fisher Scientific, Mumbai) were coated with mBSA (10 µg/well/100 µL) in coating buffer (0.06 M carbonate buffer, pH 9.6) and kept overnight at 4°C. The wells were washed once with PBS/T (0.05% tween 20 in 0.01 M PBS; pH 7.2), blocked by adding BSA (2% w/v; 200 µL/well) and incubated at 37°C for 1 h. After washing thrice, serum samples (1:50 diluted in PBS; 100 µL/well) were

added in duplicates and further incubated (37°C for 1 h). Followed by another washing, wells were incubated with HRP conjugated anti IgG1/IgG2a/ IgG2b/IgG3 antibodies (1:5000 diluted in PBS; 100 µL/well) for 1 h at 37°C. Finally, wells were washed 5 times and the colour was developed by addition of TMB/H_2O_2 substrate (100 µL/well). After 10 min. of incubation, reaction was stopped by addition of H_2SO_4 (2 N; 50 µL/well) and the optical density was measured at 450 nm[17].

Statistical analysis

Results were presented as Mean ± SEM. Data was checked for normality assumptions and normality distributed data was analysed using one way ANOVA followed by Tukey post-hoc test for multi group comparison, data which was not normally distributed was analysed using Kruskal-Wallis test. P values <0.05 were significant and statistical analysis was performed using SPSS 21.0 version software (IBM, India).

Results

Preventive treatment with rBmCys suppresses severity of mBSA-induced RA

In control group of rats, the signs of development of arthritis were visible from the 4th wk after arthritis induction with gradual increase in severity till the end of experimental period (Fig. 1 A & B). Compared to the control groups of rats (mBSA and Alum-mBSA groups), prior treatment of rats with r*Bm*Cys

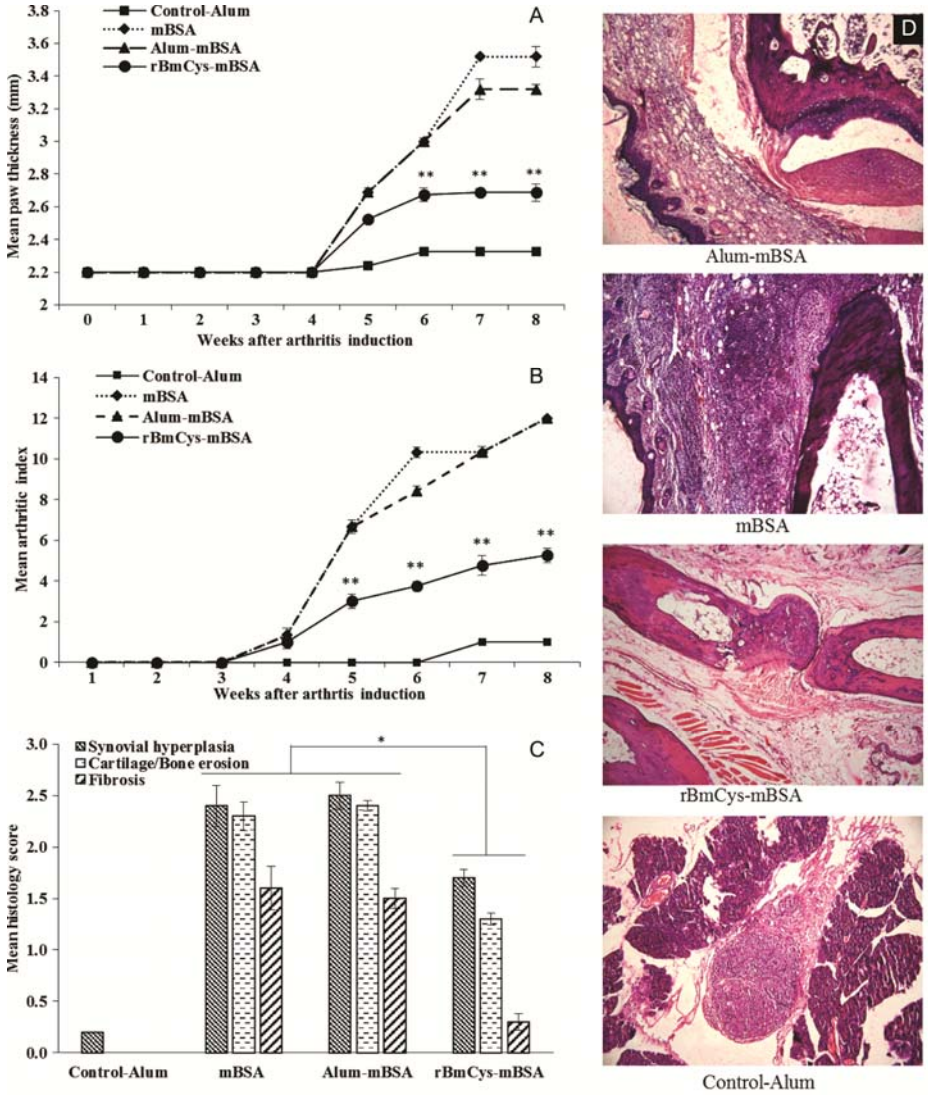

Fig. 1 — Preventive treatment with r*Bm*Cys suppresses severity of mBSA-induced RA. (A) Mean paw thickness; (B) mean arthritic index; (C) histopathological score; and (D) representative images of H&E stained tissue sections (magnification 40X). [Each data point represents Mean±SEM; n=8; **P* ≤0.0005 in comparison with mBSA and Alum-mBSA groups as analysed by Kruskal-Wallis test]

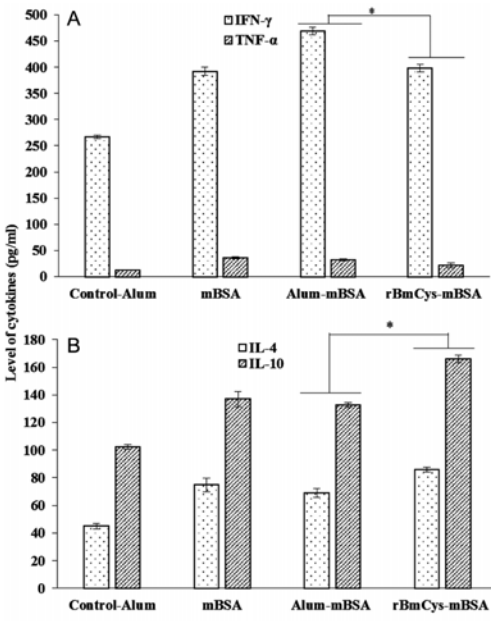

Fig. 2 — Effect on cytokines response. (A) Levels of IFN-γ and TNF-α cytokines; and (B) levels of IL-10 and IL-4 cytokines. [Each bar represents Mean±SEM; n=8; *P <0.05 in comparison with Alum-mBSA group as analysed by One way ANOVA followed by Tukey HSD test (IFN-γ, IL-10 & IL-4) or Kruskal-Wallis test (TNF-α)]

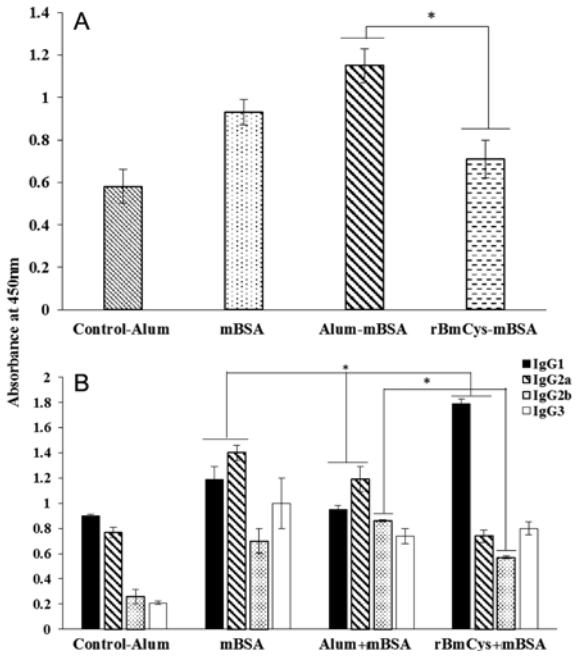

Fig. 3 — Effect on anti-mBSA IgG antibodies. Levels of (A) IgG; and (B) IgG antibody isotypes. [Each bar represents Mean±SEM; n=8; *P <0.05 in comparison with mBSA or Alum-mBSA group as analysed by One way ANOVA followed by Tukey HSD test (IgG) or Kruskal-Wallis test (IgG1, IgG2a and IgG2b)]

significantly prevented the development of arthritis as evident from the significantly (P =0.0005) reduced paw swelling and arthritic index among r*Bm*Cys treated rats (Fig. 1 A & B).

Marked synovitis and infiltration of mononuclear cells inflammation with alleviated destruction of bone and cartilage was observed in rats that were not pre-treated (mBSA group) or that were pre-treated with alum alone (Alum-mBSA group; Fig. 1 C & D). No such apparent changes were observed in the rats pre-treated with r*Bm*Cys followed by induction of arthritis (Fig. 1 C & D).

Effect on cytokines response

The prevention of development of arthritis in animals pre-treated with r*Bm*Cys was correlated with the changes in cytokine profiles in culture supernatants of splenocytes of these animals. The levels of pro and inflammatory cytokines IFN-γ & TNF-α were significantly (P =0.0005) reduced as compared to the group of rats treated with alum alone (Alum-mBSA; Fig. 2A). The shift of immune response towards anti-inflammatory milieu due to the administration of r*Bm*Cys was also evident from the significantly (P =0.0005) increased levels of IL-4 and IL-10 cytokines as compared to the Alum-mBSA and mBSA groups (Fig. 2B).

Effect on anti-mBSA IgG antibodies

To evaluate the effect of r*Bm*Cys treatment on anti-mBSA IgG response, serum samples from rats were analysed for anti-mBSA IgG and its isotype antibodies. In the rats pre-treated with r*Bm*Cys followed by induction of arthritis, levels of anti-mBSA IgG and IgG2a antibodies were significantly (P <0.004) lower compared to the arthritic rats treated with alum alone (Fig. 3 A & B). Shift of immune response towards Th2 type was again confirmed by the increased level of IgG1 antibody in r*Bm*Cys treated rats as compared to the control groups of arthritic rats (Fig. 3 A & B).

Discussion

Methylated BSA induced arthritis is a T-cell dependent experimental animal model which produces acute inflammation and the disease pathology closely related to as observed in humans[18]. The histopathological characteristics of antigen-induced RA are marked synovial hyperplasia, proliferation of sub-lining cells, infiltration of inflammatory cells, pannus formation and articular cartilage destruction[18]. In the present study, we have demonstrated that the preventive role of *B. malayi* recombinant cystatin which clearly averted the establishment of disease condition. Significant

suppression in the paw swelling and arthritic index in the r*Bm*Cys treated rats were noted. Although, initially there was inflammation in the paws of the r*Bm*Cys treated rats, however 6th wk onwards after induction of arthritis, no significant increase in the inflammation of knee joints and arthritic index was observed. RA patients showed edema in joints, lymphocytic infiltration and cartilage degradation. The histopathological examination of paws and knee joints confirmed the anti-arthritic effect of r*Bm*Cys treatment with reduction in inflammatory cells, fibrosis with damage of bone and cartilage.

The observations of this study are in congruence with the previous studies which have employed whole worms or the products derived from them for preventive treatment of RA[19]. Products derived from *Acanthocheilonema viteae* and *Ascaris suum* have been shown to exhibit IL-22 dependent reduction in the severity of inflammation in experimentally induced-arthritis[2,20]. Treatment utilizing rSj16 protein has been found to significantly supress paw swelling in complete Freund's adjuvant (CFA) induced arthritis in dose-dependent manner[21].

In the present study, we have used alum to deliver the filarial protein to the experimental groups of rats. Alum has been known to influence the immune system and upregulate the Th2-mediated immune responses. The normal and arthritic groups of rats pre-treated with alum alone have not shown any significant changes in the clinical and histopathological conditions of the arthritis. Moreover, no significant increase in the secreted levels of IL-4 cytokine by the splenocytes has been observed in these groups of rats. Thus, the possibility of immunomodulatory effects of alum alone influencing the disease condition might practically be ruled out.

Cytokines play an important role in the pathogenesis of RA. Levels of Th1 and Th17 cytokines are important regulatory parameters and play an important role in the disease progression[21]. The results of this study showed that, administration of r*Bm*Cys has significantly supressed the levels of IFN-γ and TNF-α cytokines and increased the levels of IL-10 and IL-4 cytokines. As helminths are known modulators of immune system resulting from skewing of Th1/Th2 balance[22]. Reduction in mBSA-specific IgG and IgG2a antibodies with increased secretion of IgG1 antibody in the sera of r*Bm*Cys immunized rats again indicates the possible shift of immune balance towards Th2 type. The r*Bm*Cys mediated Th2-polarization of the immune system would be able to suppress the pro-inflammatory and Th1 dominant immune responses in the arthritic condition. Recently, it has been shown that the role of Foxp3+ T regulatory cells (Tregs) in the *Schistosoma mansoni* antigen (ASMA) and *Trichinella spiralis* antigen (ATSA) mediated prevention of RA[7]. Filarial cystatin is known to modulate the antigen specific T-cell responses along with the suppression of the antigen presentation system[10,11]. In colitis, treatment with rSj cystatin has up-regulated the Tregs in the mesenteric lymph nodes and splenocytes[12].

In Conclusion, pending detailed study of the cellular mechanism behind the r*Bm*Cys mediated effect in RA, the results from this study support the idea of utilizing the immunosuppressive capabilities of r*Bm*Cys as an alternative approach for the prevention of RA.

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