Immuno-Modulatory Effect and Therapeutic Potential of *Brugia malayi* Cystatin in Experimentally Induced Arthritis

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Abstract Helminths are known to modulate host's immune system and understanding this modulation can help in identification of novel therapeutic agents for autoimmune diseases. In this study, we have assessed the immuno-modulatory activity and the therapeutic effect of *Brugia malayi* recombinant cystatin (rBmCys) in methylated BSA (mBSA) induced arthritis using rodent model. Administration of rBmCys has suppressed the severity of mBSA-arthritis in mastomys by reducing paw swelling and other clinical disease parameters as evident from significantly decreased arthritic index. The anti-arthritic effect of rBmCys was also confirmed by decreased histopathological score for synovitis, bone erosion and fibrosis in the tissue sections of paws. Further, this therapeutic effect of cystatin was found to be associated with significantly decreased production of IFN- γ and TNF- α and increased release of IL-4 and IL-10 cytokines. These results implied that rBmCys treatment has alleviated mBSA-induced arthritis and thus can be a promising alternative agent for the treatment of arthritis.

Keywords *Brugia malayi* cystatin · Methylated BSA · Rheumatoid arthritis · Therapeutic effect

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint swelling, synovial inflammation and cartilage destruction [1]. Though the aetiology of the disease is not fully understood, dysregulation of IL-17 production and activation of both T and B cells as well as macrophages are supposed to be the cause behind disease progression [1]. Current therapy for RA is based on generalized suppression of immune response through disease modifying anti-rheumatic drugs (DMARDs) and non-steroidal anti-inflammatory drugs [1]. However, these drugs have been shown to cause serious side effects in gastrointestinal tract, kidneys and central nervous system [2]. Therefore, there is need to develop novel and safe drugs for the treatment of RA.

Infection with helminths could confer a concomitant benefit to both the parasites and host. Several studies on experimentally induced autoimmune disorders have shown that helminths or the products derived from them can alter the severity of allergy and autoimmune conditions via modulation of the host immune system, suppression of IFN- γ cytokine production and up-regulation of IL-4/IL-10 [3, 4]. Infection with *Schistosoma mansoni* or *Schistosoma japonicum* or *Acanthocheilonema viteae* has been shown to effectively ameliorate experimentally induced RA [5–7]. *S. japonicum* recombinant protein (rSj16) resulted in the suppression of the severity of experimentally induced arthritis in rats [8]. Based on these studies, utilization of helminths or their products in the treatment of RA has been conceptualized.

Parasite cystatins are highly expressed protease inhibitors with defined immunomodulatory roles. Cystatins from several helminth parasites have been demonstrated to modulate hosts antigen presentation and processing

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mechanisms and reduce T cell priming [9–12]. Treatment with recombinant cystatin (rCsStefin-1) from *Clonorchis sinensis* has ameliorated the severity of DSS induced colitis in mice [13]. But, cystatin has never been tested for its immune-modulatory activity and for its ability to cure RA. Hence, the present study was carried out to assess the therapeutic effect of *Brugia malayi* recombinant cystatin (rBmCys) in mBSA induced arthritis in rat model.

Materials and Methods

Brugia malayi Recombinant Cystatin (rBmCys)

rBmCys was expressed as His-tag protein in *Escherichia coli* by transforming pRSET-A-BmCys plasmid into BL21 (DE3) pLysS *E. coli* cells and purified using nickel affinity chromatography column (Thermo Fisher Scientific, Mumbai). The protein content was quantified and endotoxin content was measured by LAL chromogenic endotoxin quantitation kit which was found to be <0.2 EU/ml (Thermo Fisher Scientific, Mumbai).

Experimental Animals

Multi mammary mastomys rats (*Mastomys coucha*, 6–8 weeks old) used in this study were housed in a CPCSEA registered Central Animal House facility and maintained under standard conditions with free access to pelleted animal diet and drinking water ad libitum. Approval from Institutional Animal Ethics Committee was obtained for this study.

Induction of Arthritis Using mBSA

Arthritis was induced in female mastomys using mBSA by the method described by Grespan et al. [14]. Briefly, mastomys 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. Twenty-one days after first dose of mBSA, arthritis was induced by intra articular injection of mBSA (30 µg in 10 µl PBS).

Assessment of mBSA-Induced Arthritis

Development of arthritis in mastomys was monitored by measuring paw swelling thrice a week after induction of arthritis. Arthritic index was evaluated based on the score ranging from 0 to 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; (4) erythema and severe swelling encompassing entire paw including ankle [6]. The arthritic index was presented as mean of the summative scores. All the groups of mBSA administered mastomys developed signs of arthritis around a week after induction of arthritis.

Treatment of Mastomys with rBmCys

Mastomys were grouped (n = 8–10) as *Control-Alum* group (normal mastomys treated with alum alone), *mBSA* group (arthritic mastomys without any treatment), *mBSA-Alum* group (arthritic mastomys treated with alum alone) and *mBSA-rBmCys* group (arthritic mastomys treated with rBmCys in alum adjuvant). The treatment of arthritic mastomys with rBmCys was initiated when all the animals of that group developed arthritis (evident from significantly higher arthritic index value compared to normal rats). The rBmCys was administered intraperitoneally in four doses in alum adjuvant (25 µg/dose/200 µl) at the intervals of 15 days. All the rats were sacrificed 10 days after the last dose of rBmCys treatment.

Assessment of Histopathological Changes

The paws were removed from sacrificed mastomys, fixed in 10 % formal-saline followed by decalcification and embedding in paraffin. 5 µm cross sections were cut and stained with haematoxylin and eosin. Sections were scored in the range of 0–3 for the parameters: synovial hyperplasia, cartilage and bone erosion and fibrosis [15].

In Vitro Culture of Splenocytes for Estimation of Cytokines

Spleens were aseptically harvested and minced in RPMI 1640 medium. Single-cell suspensions were pelleted and suspended in erythrocyte lysis buffer (Invitrogen, Mumbai). Cells were washed three times in RPMI 1640 medium and plated in duplicates (2×10^6 cells/well) 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 rBmCys (10 µg/well) or Concanavalin A (2 µg/well; positive control) (Sigma-Aldrich, Mumbai) or medium alone (negative control). The final volume in each well was adjusted to 1 ml and incubated at 37 °C in 5 % CO₂ atmosphere for 72 h. The culture supernatants from wells were collected separately by

centrifugation and stored at -80°C until further used for estimation of cytokines.

Measurement of Cytokines

The levels of IL-10, IL-4, TNF- α and IFN- γ in the culture supernatants of splenocytes were measured by ELISA using kits from 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 96 well microtitre plates (Thermo Fisher Scientific, Mumbai) were coated with mBSA (10 $\mu\text{g}/\text{well}/100\ \mu\text{l}$) 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) and blocked by adding BSA (2 % w/v; 200 $\mu\text{l}/\text{well}$) and incubating at 37°C for 1 h. After washing thrice, serum samples (1:50 diluted in PBS; 100 $\mu\text{l}/\text{well}$) were added in duplicates and incubated (at 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 $\mu\text{l}/\text{well}$) for 1 h at 37°C . Finally, wells were washed five times and the colour was developed by addition of TMB/ H_2O_2 substrate (100 $\mu\text{l}/\text{well}$). After 10 min. of incubation, reaction was stopped by addition of H_2SO_4 (2 N; 50 $\mu\text{l}/\text{well}$) and the optical density was measured at 450 nm [16].

Statistical Analysis

Statistical analysis was performed using SPSS 21.0 version software (IBM, India). Results were presented as Mean $\pm$ SEM. Data was checked for normality assumptions and accordingly suitable parametric test (One way ANOVA followed by Tukey's post hoc HSD test) or non-parametric test (Kruskal–Wallis test) was applied. p values ≤ 0.05 were considered to be significant.

Results

rBmCys Suppresses Severity of Rheumatoid Arthritis

One day after the arthritis was clinically evident in the mBSA administered group of mastomys, rBmCys treatment was initiated. In mBSA and mBSA-Alum groups of mastomys, paw swelling was found to be significantly increased by 3rd week after initiation of the treatment associated with increased arthritic index (Fig. 1a, b). In

contrast, there was a significant suppression in the severity of arthritis reflected from the reduced paw thickness and arthritic index in the rBmCys treated arthritic rats (Fig. 1a, b).

Histopathological examination of the ankle joint tissues of mBSA and mBSA-Alum groups of rats showed clear synovial hyperplasia, inflammatory cell infiltration, cartilage and bone erosion, and fibrosis (Fig. 1c, d). These derangements were significantly reduced in the rBmCys treated arthritic rats (Fig. 1c, d).

Effect of Treatment with rBmCys on Cytokines Profile

To explore the possible effect of rBmCys on T cells as part of the mechanism in the suppression of arthritis, changes in cytokine levels in culture supernatants of splenocytes followed by stimulation with rBmCys were analysed. The release of both IFN- γ and TNF- α cytokines was higher in the control groups of arthritic mastomys (Fig. 2a, b). Treatment with rBmCys has significantly (<0.001) reduced the production of these cytokines (Fig. 2a, b).

The significantly increased (<0.001) release of IL-10 and IL-4 cytokines in the rBmCys treated arthritic rats as compared to the control groups suggests the anti-inflammatory milieu initiated by the administration of this protein (Fig. 2a, b).

Effect of Treatment with rBmCys on mBSA-Specific IgG Antibodies

To evaluate the effect of rBmCys treatment on the humoral anti-mBSA response, levels of anti-mBSA IgG and its subclasses in the sera were analysed. The levels of anti-mBSA IgG antibodies were significantly lower ($p < 0.001$) in the rBmCys treated arthritic mastomys compared to the control (untreated) arthritic animals (Fig. 3a). rBmCys treatment resulted in predominant Th2-type of immune response with significantly increased levels of anti-mBSA IgG1 and decreased levels of anti-mBSA IgG2a, IgG2b and IgG3 antibodies as compared to control (untreated) arthritic rats (Fig. 3b).

Discussion

In the present study, we demonstrated the therapeutic effect of *B. malayi* recombinant cystatin on the antigen induced model of arthritis. The treatment of arthritic mastomys with rBmCys resulted in the significant suppression of the severity of arthritis evident from reduction in paw swelling and arthritic index. The anti-arthritic effect of the rBmCys treatment was also confirmed by histopathological changes

Fig. 1 Therapeutic effect of rBmCys on severity of antigen-induced arthritis in mastomys. Differences in **a** mean paw thickness, **b** mean arthritic index, **c** histopathological score in H&E stained tissue sections of paws of arthritic mastomys treated with rBmCys and control groups of mastomys and **d** representative figures of H&E stained tissue sections of paws (magnification ×40). Each data point

represents Mean ± SEM; n = 8 mice/group; * $p < 0.005$ in comparison with mBSA-Alum and mBSA groups as analyzed by Kruskal-Wallis test; ** $p < 0.05$ in comparison with Control-Alum group as analyzed by Kruskal-Wallis test; arrow indicates an area of cartilage/bone destruction, synovial hyperplasia with a prominent influx of inflammatory cells

of the arthritic paws, which indicated that rBmCys treatment can significantly suppress the progression of arthritis.

Onchocystatin of *Onchocerca volvulus* has been shown to modulate host immune responses and shift the immune balance towards Th2/Th3 responses [17]. Cystatins of parasites have been shown to have important role in the suppression of the immune responses and also for subsequent establishment of active parasitism within the host. Cystatin from *B. malayi* is shown to have suppressed the antigen presentation system in human host and also modulation of antigen-specific T cell proliferation by induction of nitric oxide [12, 18].

The disease attenuation effect of rBmCys in antigen-induced arthritis in mastomys in the current study is in agreement with the previous reports. ES-62 protein derived from *A. viteae* has been shown to exhibit reduction in the severity of inflammation in collagen induced arthritis (CIA) [1]. The administration of *Ascaris suum* or the treatment of whole worm extract effectively prevented zymogen induced arthritis in rats with amelioration of clinical symptoms of arthritis and synovitis in a dose dependent manner [19]. Prophylactic administration of *S. mansoni* worms reduced the severity of CIA reflected from decreased arthritis score and marked reduction in synovial

Fig. 2 Effect of treatment with rBmCys on cytokine profile in antigen-induced arthritic mastomys. **a** Levels of IFN- γ and TNF- α cytokine and **b** Levels of IL-10 and IL-4 cytokine. Each bar represents Mean $\pm$ SEM; $n = 8$ mice/group; * $p < 0.001$ in comparison with mBSA-Alum group as analyzed by One way ANOVA followed by Tukey's post hoc multiple comparisons test

hyperplasia, inflammatory cell influx and destruction of bone and cartilage as apparent from histopathological examination [5]. Treatment with rSj16 protein alleviated complete Freund's adjuvant (CFA) induced arthritis in rat model with significant reduction in paw swelling in dose-dependent manner [8].

Helminths have been shown to modulate the host immune response from Th1 to anti-inflammatory Th2 response [20]. Though the aetiology of RA is not completely understood, dysregulated immune responses with TNF- α , IL-1 and IL-10 cytokines play an important role in the disease progression [8]. Administration of rBmCys in the current study has significantly decreased the level of pro and inflammatory cytokines IFN- γ and TNF- α and increased the levels of anti-inflammatory cytokines IL-10 and IL-4. These results indicate that, the anti-arthritic effect

Fig. 3 Effect of treatment with rBmCys on mBSA-specific IgG antibodies in antigen-induced arthritic mastomys. **a** IgG antibody levels and **b** levels of IgG antibody isotypes. Each bar represents Mean $\pm$ SEM; $n = 8$ mice/group; * $p < 0.001$ in comparison with mBSA-Alum and mBSA groups as analyzed by Kruskal–Wallis test

of rBmCys may be through skewing of Th1 immune response towards Th2 type. This is again confirmed by observed changes in humoral immune response in the sera of immunized mastomys. It is well demonstrated that antibody class switching depends on T cell cytokines as IL-4 secretion promotes switching of activated B cells towards IgG1 isotype and IFN- γ production shifts towards IgG2a isotype. The decreased levels of total IgG and IgG2a in rBmCys treated mastomys suggest the skewing of immune response towards Th2 type.

Thus, from the results of this study it can be envisaged that rBmCys can modulate the regulatory immune response and can be a promising novel therapeutic candidate against RA.

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Compliance with Ethical Standards

Conflict of interest All the authors have declared that they have no conflict of interest.

Ethical Approval This study does not contain any studies with human participants performed by any of the authors. This study was approved by Institutional Animal Ethics Committee and national guidelines as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for the care and use of animals were followed.

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