Filarial Abundant Larval Transcript Protein ALT-2: An Immunomodulatory Therapeutic Agent for Type 1 Diabetes

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Abstract Type 1 diabetes (T1D) that accounts for about 5–10 % of all diabetes cases results from the autoimmune destruction of the insulin-producing beta cells in the pancreas. It is characterized by severe inflammatory reaction mediated by pronounced T helper type-1 response. Parasitic infections having the ability to skew the host immune responses towards type-2 type as a part of their defense mechanism are able to induce protection against autoimmune diseases like T1D. Hence, the present study is undertaken to explore a recombinant abundant larval transcript protein of the human lymphatic filarial parasite *Brugia malayi* (*rBmALT-2*), a known anti-inflammatory molecule for its therapeutic effect on streptozotocin (STZ)-induced T1D in mice. The diabetic mice on treatment with *rBmALT-2* showed a significant ($p < 0.0005$) decrease in their fasting blood glucose levels. By the end of the second week after the initiation of treatment with the *rBmALT-2*, 28 % of the diabetic mice became normal and none of them were diabetic by the end of 5th week. The anti-diabetic effect of *rBmALT-2* significantly correlated with the concomitant redressal of the pancreatic histopathological damage caused by STZ assault ($\rho = 0.87$; $p < 0.0005$).

The sera of *rBmALT-2* treated diabetic mice had increased levels of IgG1 antibodies associated with decreased IgG2a antibodies against the principal autoantigen insulin. The splenocyte proliferative response and the cytokine release in the treated mice showed marked bias against inflammation skewing the immune response to Th-2 type. From this study, it can be envisaged that that filarial proteins like *rBmALT-2* with effective immunomodulatory activity and anti-diabetic effect are promising alternative therapeutic agents for T1D.

Keywords Therapeutic · Abundant larval transcript-2 · Autoimmune diseases · Type 1 diabetes · Immunomodulators · Streptozotocin

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of β -cells of islets of Langerhans due to infiltration of lymphocytes and monocytes and concomitant chronic inflammatory response. This results in the disturbance of glucose homeostasis and associated metabolic derangement. Although the emergence of type 2 diabetes (T2D) in both children and adolescents has attracted much attention, T1D remains as the predominant form during childhood in many countries [1]. While the incidence of T1D is increasing globally, there are marked variations in different geographic locations [2]. The conventional therapy for T1D comprises of dietary care and oral administration of insulin as a primary line of treatment. Other treatment methods aimed to hinder the disease progression typically relied on the use of immunosuppressive agents. However, these drugs lack long lasting

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effects and may also lead to renal toxicity [3, 4]. As the attempts made to explore new treatment strategies for T1D have hardly met with significant success so far, helminth based immunotherapy has been proposed as one of the options to be explored.

Growing body of evidence supports an inverse relationship between worm infection and T helper type 1/17 (Th1/Th17)-based inflammatory disorders including T1D [5]. Experimental studies on animal models have shown that infection with helminth parasites like *Heligmosomoides polygyrus*, *Schistosoma mansoni*, *Trichinella spiralis*, *Litomosoides sigmodontis* or treatment with the molecules derived from these parasites can subvert the severity or prevent the onset of T1D [6–10]. The individuals with chronic filarial infections were shown to have lower rates of T1D compared to those without any evidence of this infection living in the same environment [11]. Filarial nematodes showcase a classic model of parasitism to establish long-term infection in human hosts via suppression and/or modulation of the host immune system. The infected host's immune response to these worms is characterized by reduced production of interferon (IFN)- γ and increased production of interleukin (IL)-4/IL-10 along with elevated levels of IgG4 antibodies against parasite antigens suggesting a predominant Th2/anti-inflammatory phenotype, that is conducive to parasite survival without any major effect on host [12, 13]. In a previous study, we had shown that the somatic and excretory-secretory proteins of *B. malayi* attenuate the severity of STZ-induced T1D in mice [14]. However, in lieu of logistical problems associated with the use of crude extracts of parasites and also, as such extracts may contain other immunogenic substances that could subside or inhibit the protective components within the extract, it is preferable to identify and use specific immuno-modulatory molecules as therapeutics.

In this context, filarial abundant larval transcript (ALT) proteins are of interest as they are expressed abundantly and implicated in protective immunity that may play a role in immune evasion [15]. *B. malayi* ALT proteins have also been shown to be involved in the up-regulation of host's Th2 immune responses [15]. Some of the T cell epitopes of *B. malayi* ALT-2 could induce high levels of IL-10 secretion in BALB/c mice suggesting their possible involvement in immunomodulation within the host [16].

Hence, we have undertaken the present study to assess whether the *Brugia malayi* ALT-2 could be used in the treatment of mice with STZ-induced T1D based and to understand the associated modulated responses in the host's immunity.

Materials and Methods

Brugia malayi Recombinant Abundant Larval Transcript-2 Protein

Recombinant BmALT-2 was expressed as his-tag protein as described earlier using pRSETA as vector and BL21 (DE3) pLysS *Escherichia coli* (Invitrogen, Bangalore, India) as expression host [17]. The endotoxin content was measured by Limulus amoebocyte lysate (LAL assay, Thermo fisher scientific) chromogenic quantitation kit and it was <0.2 EU/ml, which is below the recommended cut-off level [18]. The Protein concentration was estimated using micro bicinchoninic acid (BCA) Protein Assay kit (Thermo fisher scientific).

Experimental Animals

Clearance from the Institutional Animal Ethics Committee (IAEC) was taken to use female BALB/c mice of 6–8 weeks of age bred and maintained in the animal house of the institute for this study. The mice were housed under the standard laboratory conditions with 12 h of light/dark cycle and had free access to normal diet and water ad libitum.

Induction of T1D in BALB/c Mice

T1D was induced in the female BALB/c mice as described by Santos-junior et al., (2009) by injecting low dose Streptozotocin (STZ; Sigma-Aldrich, Mumbai, India) (40 mg/kg/day, made freshly in 0.1 M sodium citrate buffer pH 4.5, injected intraperitoneally (i.p.) within 15 min. of preparation) for five consecutive days [19]. The mice were monitored for fasting glucose levels in blood samples (obtained from the tail vein of mice after 6 h of fasting) once in a week starting from 7 days after the last dose of STZ. The glucose in the blood samples was evaluated using glucometer (Gluco Check, Major Biochemical Corporation, Taiwan). The mice with blood glucose levels of ≥ 200 mg/dl for two consecutive weeks were considered as diabetic and the incidence of diabetes was expressed as the percentage of mice in a group that presented with diabetes.

Treatment of Diabetic Mice with rBmALT-2

Mice (n = 6–8 in each group) were divided into different sub-groups' viz., **STZ + rBmALT-2** group (mice induced with diabetes followed by treatment with rBmALT-2 in alum adjuvant), **STZ + Alum** group (mice induced with

diabetes followed by treatment with alum adjuvant only), **Alum** group (normal mice treated with alum adjuvant only) and **Normal** group (normal mice without any treatment). The treatment with *rBmALT-2* consisted of four doses of *rBmALT-2* (25 µg/dose/200 µl; i.p.) in alum adjuvant in intervals of 1 week. Initial dose–response experiments on diabetic mice showed 25 µg/dose of *rBmALT-2* as the optimal quantity needed to achieve the significant therapeutic effect (data not shown). All the samples of mice were monitored for fasting blood glucose levels for 5 weeks after the initiation of treatment.

Histopathological Assessment of Pancreas

A week after the last dose of treatment the mice were sacrificed, the pancreas was removed immediately, fixed in 10 % formal-saline and then processed for histological analysis as described by Hübner et al. [10]. 5-µm longitudinal sections were stained with haematoxylin and eosin and evaluated microscopically for inflammatory changes by a qualified expert who was blinded for the treatment groups of mice. The grading of insulinitis was done as normal (non-infiltrated), minor (peri-insulinitis), moderate (intra-insulinitis with infiltration ≤50 %) and severe (intra-insulinitis with infiltration ≥50 %).

Assessment of Cellular Proliferation

Splenocytes were harvested from the mice in aseptic conditions and cultured (2×10^6 viable cells/100 µl/well) in triplicate wells at 37 °C with 5 % CO₂ in a 96-well flat-bottomed tissue culture plate (Thermo fisher scientific) in the presence of *rBmALT-2* (10 µg/100 µl/well, antigen stimulated) or Concanavalin A (Con A, 2 µg/100 µl/well, positive control) or medium alone (unstimulated control). 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 (Invitrogen, Bangalore) was used for culturing the splenocytes and the final volume in each well was adjusted to 200 µl. After 48 h the cell proliferation was measured using a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Promega, New Delhi, India). The absorbance in the wells was measured at 570 nm using microplate reader (Biotek, Mumbai, India). The stimulation index (SI) was calculated as the absorbance of mitogen (Con A) or antigen (*rBmALT-2*) stimulated cells divided by the absorbance of unstimulated control wells.

Measurement of Release of Cytokines in Culture Supernatants of Splenocytes

The splenocyte cells culture supernatants from parallel triplicate cultures maintained as described above were collected after 72 h of incubation in 5 % CO₂ at 37 °C and stored at −70 °C for the estimation of the levels of cytokines. The levels of cytokines (IL-10, IL-4, IFN-γ and TNF-α) were measured by two by two-site sandwich ELISA as per the protocol supplied by the manufacturer (Invitrogen Bioservices Pvt. Ltd, Mumbai).

Measurement of Anti-insulin Antibodies

Blood samples were collected through the caudal veins of the mice, sera were separated and used to analyze the anti-insulin antibodies (IgG1, IgG2a, and IgE) as described by Imai et al. [20] by ELISA. Wells of 96-well microtiter plates (Thermo Fisher Scientific, Mumbai) were coated with human recombinant insulin (10 µg/100 µl/well; Sigma-Aldrich) in carbonate-bicarbonate buffer (100 mM, pH 9.5) and incubated overnight at 4 °C. After a single wash with PBS/T (0.05 M PBS containing 0.05 % of tween 20, pH 7.2), the wells were blocked by incubating with BSA (2 % in PBS, 300 µl/well) for 1 h at 37 °C. After washing thrice, the mice sera samples (1:50 diluted in PBS) were added and incubated for 1 h at 37 °C. After washing five times, the bound antibodies were detected by addition of HRP conjugated goat anti-mouse IgG1, IgG2a or IgE antibodies (1:5000 diluted in PBS; IgG1 and IgG2a from Invitrogen and IgE from Southern biotech, New Delhi, India). The colour developed using the substrate tetramethylbenzidine (TMB) was measured as absorbance at 450 nm.

Statistical Analysis

The levels of various analytes studied are represented as mean ± SEM values for each control or test group. Suitable statistical tests were carried out for the detection of differences among the groups using SPSS 21.0 software (IBM, India). After confirmation of fulfillment of normality assumption, One-way analysis of variance followed by the Tuckey's post hoc test for multiple comparisons was used to establish significant differences among three or more groups. The data which has not followed a normal distribution (composite scores for histopathology of pancreatic damage) was analyzed by the Kruskal–Wallis test. Spearman's correlation analysis was also performed to find any

association between the alteration in the pancreatic composite damage and alteration in blood glucose levels and cytokine responses.

Results

Effect of the Treatment with *rBmALT-2* on the Status of STZ-Induced T1D in BALB/c Mice

The treatment of diabetic mice with *rBmALT-2* reduced the severity of disease as reflected by a significant decrease in the blood glucose levels ($p < 0.0005$, Fig. 1a). After the administration of initial 2 doses of *rBmALT-2*, 28 % of the mice showed recovery from diabetes and none of the mice were diabetic after giving four doses (Fig. 1a, b). In contrast, all the diabetic mice treated with alum alone (STZ + Alum) remained diabetic throughout the experimental period (Fig. 1a, b).

Fig. 1 Treatment with *B. malayi* protein reduces the severity of STZ-induced T1D. Effect of treatment of diabetic mice with *rBmALT-2* on **a** fasting blood glucose levels and **b** incidence of diabetes. Each data point represents mean $\pm$ SEM ($n = 6-8$ mice in each group). * $p < 0.0005$ in comparison with glucose level in STZ + Alum group as analysed by One Way ANOVA followed by Tukey's HSD post hoc test

Effect of Treatment with *rBmALT-2* on Pancreatic Islets

The results of evaluation of histopathological changes in diabetic mice treated with *rBmALT-2* compared to the untreated mice or mice treated with alum alone as shown in Fig. 2. Diabetic mice treated with *rBmALT-2* showed significantly ($p < 0.0005$) reduced cellular infiltration and islets damage. There was also significant positive correlation between the reduction of histopathological damage score and the decrease in blood glucose level ($\rho = 0.87$; $p < 0.0005$).

Effect on Cellular Proliferation and Cytokine Response

The splenocytes of diabetic mice treated with *rBmALT-2* after re-stimulation with the same protein had significantly higher ($p \leq 0.0005$) proliferative response with stimulation index (SI) value of 1.84 ± 0.07 (mean $\pm$ SEM) compared to the splenocytes of diabetic mice treated with alum alone (for which the SI was 1.21 ± 0.04).

The levels of cytokines released in the culture supernatants of splenocytes of different groups of mice followed by re-stimulation with *rBmALT-2* are summarized in Table 1. Compared to the splenocytes of diabetic mice treated with alum alone, the splenocytes of diabetic mice treated with *rBmALT-2* had increased production of cytokine IL-10 (mean $\pm$ SEM; 372.8 ± 36.9 vs. 147.5 ± 12.9 pg/ml) and IL-4 (97 ± 5.9 vs. 77 ± 2.6 pg/ml) and decreased production of TNF- α (87.1 ± 4.1 vs. 129 ± 9.7 pg/ml) and IFN- γ (142.8 ± 23.8 vs. 466.2 ± 81.3 pg/ml). The differences in the levels of all the cytokines in these two groups of mice were significant except for the IL4 levels, where the difference was not significant statistically.

The Spearman's correlation analysis between the histopathological damage score and the cytokine level was performed which suggested a significant negative correlation between the increase in the release of cytokines like IL-4 ($\rho = -0.75$; $p = 0.005$) and IL-10 ($\rho = -0.77$; $p = 0.003$) and the decrease in the histological damage scores. Similarly, significant positive correlation was found between the increase in the release of TNF- α ($\rho = 0.93$; $p < 0.0005$), IFN- γ ($\rho = 0.69$; $p = 0.01$) and the higher histopathological damage score.

Effect on the Insulin-Specific Antibodies in the Sera of *rBmALT-2* Treated Mice

Diabetic mice treated with *rBmALT-2* showed significantly higher ($p \leq 0.05$) levels of IgE and IgG1, and lower levels of IgG2a antibodies compared to the mice treated with

Fig. 2 Treatment with *rBmALT-2* decreases the severity of insulinitis in diabetic mice. **a** Representative photographs of histopathological changes in different groups of mice with different grades of pancreatic damage (original magnification $\times 40$): (i) Normal group (ii) STZ + Alum group (iii) STZ + *rBmALT-2* group and (iv) Alum group. **b** Percentage of pancreatic islet cells classified as normal (non-infiltrated), minor (peri-insulitis), moderate (intra-insulitis with infiltration $\leq 50\%$) and severe (intra-insulitis with infiltration $\geq 50\%$) insulinitis

alum alone (Table 2). The ratio of IgG1/IgG2a was accordingly quite higher in *rBmALT-2* treated mice.

Discussion

In the present study the treatment with ALT-2 protein of filarial parasite *B. malayi* (*rBmALT-2*) decreased fasting blood glucose levels in mice with STZ induced T1D. The

glucose lowering effect of *rBmALT-2* was so pronounced that all the treated mice became non-diabetic by the 5th week after the initiation of the treatment. Histopathological examination of the pancreas of these mice revealed that the progressively increased islet damage caused by STZ was minimised with significantly reduced cellular infiltration of their islets followed by treatment with *rBmALT-2*. There was significant correlation between its blood glucose lowering effect and its effect in reverting the pancreatic

Table 1 Effect of treatment of diabetic mice with *rBmALT-2* on cytokine release from the splenocytes

Group of mice	Cytokines level in pg/ml (mean $\pm$ SEM)			
	IL-10	IL-4	TNF- α	IFN- γ
STZ- <i>rBmALT-2</i>	372.8 $\pm$ 36.9*	97 $\pm$ 5.9	87.1 $\pm$ 4.1**	142.8 $\pm$ 23.8 [#]
STZ-Alum	147.5 $\pm$ 12.9	77 $\pm$ 2.6	129 $\pm$ 9.7	466.2 $\pm$ 81.3
Alum	79.1 $\pm$ 32.5	35.1 $\pm$ 0.9	33.5 $\pm$ 10.6	118 $\pm$ 44.2
Normal	58.7 $\pm$ 6.5	60.1 $\pm$ 1.6	45.4 $\pm$ 4.7	136.2 $\pm$ 16

Levels of IL-10, IL-4, TNF- α and IFN- γ cytokines measured by sandwich ELISA in the culture supernatants of splenocytes from the diabetic mice (treated with *rBmALT-2*) followed by re-stimulation with the same antigen. Each data point represents mean $\pm$ SEM (n = 6–8 mice in each group). * p = 0.002 in comparison with all the control groups; ** p < 0.05 and [#] p < 0.005 in comparison with STZ + Alum group as analysed by One way ANOVA followed by Tukey's HSD post hoc test

Table 2 Effect of treatment of diabetic mice with *rBmALT-2* on insulin-specific antibodies

Group of mice	Absorbance at 450 nm (OD) (mean $\pm$ SEM)			Ratio
	IgE	IgG1	IgG2a	IgG1/IgG2a
STZ- <i>rBmALT-2</i>	1.3 $\pm$ 0.2 [#]	1.6 $\pm$ 0.26*	0.75 $\pm$ 0.05**	2.13
STZ-Alum	0.74 $\pm$ 0.05	0.72 $\pm$ 0.03	1.16 $\pm$ 0.18	0.62
Alum	0.66 $\pm$ 0.04	0.59 $\pm$ 0.05	0.64 $\pm$ 0.06	0.93
Normal	0.69 $\pm$ 0.13	0.56 $\pm$ 0.03	0.6 $\pm$ 0.12	0.93

The levels of IgE, IgG1, IgG2a antibodies and ratio of IgG1/IgG2a in the sera of diabetic mice followed by treatment with *rBmALT-2*. Each data point represents mean $\pm$ SEM. (n = 6–8 mice in each group). * p < 0.002 and [#] p < 0.05 in comparison with all the control groups; ** p = 0.001 in comparison with STZ-Alum group as analysed by One-way ANOVA followed by Tukey's post hoc test

islet damage. In the earlier study from our laboratory, we had demonstrated that the whole excretory and secretory proteins of *B. malayi* could suppress or prevent the development of STZ induced T1D in mice [13]. These findings are consistent with the earlier reports that the administration of whole helminth worms [6, 21, 22] or the products derived from them [23] prevented the development of T1D by lowering the blood glucose levels and by minimising the histopathological damage to pancreas.

We have used alum as a vehicle to deliver the *rBmALT-2* to the mice to ensure that optimal immune response is generated against this protein in the treated diabetic mice resulting in its effective immunomodulation in the host with consequent therapeutic effect against diabetes, if there is any [24]. The possibility of alum alone influencing the immunomodulatory effect in the mice could be ruled out as there were no significant changes in the clinical status or histopathological scores in the diabetic mice treated with alum alone (STZ-Alum group).

To explore the plausible rationale for the reversal of pancreatic damage by this protein, we investigated the proliferative and cytokine responses of the splenocytes of the treated by mice after re-stimulating them with the same protein. There was significant splenocyte proliferation associated with the increased release of anti-inflammatory (IL-4, IL-10) cytokines and decreased levels pro-

inflammatory (TNF- α , IFN- γ) cytokines. This apparent bias of cytokine response correlated with the reduction in pancreatic histopathological damage score in the diabetic mice treated with *rBmALT-2*. The analysis of IgG subclasses in these mice showed that *rBmALT-2* treatment resulted in significantly elevated levels of insulin-specific IgG1 and decreased IgG2a antibodies. El-Wakil et al. [21] reported that T cells and their associated cytokines are the key players in switching of antibody class, as the IL-4 and IL-10 cytokines promote switching of activated B cells towards production of IgG1 antibody isotype (TH2 type) while inflammatory cytokine IFN- γ promotes the synthesis of IgG2a antibodies shifting immunity towards TH1 type [23]. The *rBmALT-2* treated diabetic mice also showed significantly elevated IgE antibodies, which is in line with the earlier report by Imai et al. [20] that the IgE antibodies induced by the parasites prevent the development of T1D in NOD mice. These results suggest that *rBmALT-2* is able to create an environment conducive of combating the pancreatic islet damage by lowering the inflammatory potential and by inducing generalized TH2 shift with characteristic cytokine and antibody responses. This larval transcript protein of filarial parasites as such is known for its potential to shift the immune response towards an apparent TH-2 bias [16]. Hence, it is quite comprehensible that the prevention of the profound TH-1 response

mediated pancreatic damage expected from STZ assault in mice is basically due to a favorable TH-2 skew in immune response induced by ALT-2 protein.

Though the exact function of ALT-2 protein is yet to be understood, it seems to have critical role in the host-parasite relationship. The infective larvae that get transferred from the mosquito vector to the host represent the crucial stage in the lifecycle of filarial parasite [24]. Quite strikingly this transcript gets abundantly expressed and secreted almost like ‘magic bullets’ at this stage of the parasite development; presumably, part of natural defense mechanism to curb the upheaval of host’s inflammatory response and to survive and safeguard the patency of the infection [25]. As already mentioned, the available evidence suggests on the development of some natural protection from type 1 diabetes among infected populace in a filarial endemic area [26] which might be part of this immune evasion mechanism by the parasite.

Growing body of evidence in the recent past supports the potential of several helminth-derived proteins as promising therapeutic agents against various auto-immune disorders [12, 14, 20, 27].

The remarkable reversal of diabetic state and minimization of the inflammatory damage of the pancreas in mice with T1D achieved by treatment with filarial protein rBmALT-2 makes it as one of the effective mediators to ameliorate diabetic pathology. The exact cellular mechanisms involved in its therapeutic effect need to be thoroughly investigated along with the determination of the longevity of its treatment effect and accounting of the possible side effects from its long term usage. The findings of the current study thus provide a proof of principle that selective immune modulation using parasitic proteins offers a valid therapeutic rationale and help in development of novel candidates for enriching anti-diabetic drug repertoire.

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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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