Acta Biochim Biophys Sin 2012, 44: 259–268 | © The Author 2012. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr120.
Advance Access Publication 18 January 2012

Original Article

Comparative evaluation of T11 target structure and its deglycosylated derivative nullifies the importance of glycan moieties in immunotherapeutic efficacy

Sirshendu Chatterjee[1,2†], Sagar Acharya[1†], Pankaj Kumar[1], Ananya Chatterjee[2], Suhnrita Chaudhuri[1], Anirban Ghosh[1,3], and Swapna Chaudhuri[1*]

[1]Department of Laboratory Medicine, Calcutta School of Tropical Medicine, Kolkata 700073, West Bengal, India
[2]Department of Biochemistry, University College of Medicine, I.P.G.M.E&R, Kolkata 700020, West Bengal, India
[3]Department of Zoology, Panihati Mahavidyalaya, Sodepur, Kolkata 700110, West Bengal, India
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +91-33-22414900/+91-33-22414965 (Ext: 207); Fax: +91-33-22414915; E-mail: schaudhuri.cmiil@gmail.com or swapna.chaudhuri@gmail.com

Sheep red blood cell (SRBC), a non-specific biological response modifier that has long been used as a classical antigen, has been shown to exert an immunomodulatory and anti-tumor activities in experimental animals. The active component of SRBC, which is responsible for such effects, was found to be a cell surface acidic glycoprotein molecule, known as T11 target structure (T11TS). In the present study, T11TS was isolated and purified to homogeneity using a five-step protocol involving isolation of sheep erythrocyte membrane from packed cell volume, 20% ammonium sulfate cut of the crude membrane proteins mixture, immunoaffinity purification using mouse anti-sheep CD58 mAb (L180/1) tagged matrix, preparative gel electrophoresis, and gel electroelution process. Finally, the purity and identity of the proteins were confirmed by the matrix-assisted laser desorption/ionization (MALDI) mass spectrometric analysis. The *in silico* glycosylation site analysis showed that the extracellular domain contained three N-glycosylation sites (N-12, N-62, and N-111) and one O-glycosylation site (T-107). However, the experimental analysis negated the presence of O-linked glycan moieties on T11TS. To investigate the role of glycan moieties in the current immunotherapeutic regime, T11TS and its deglycosylated form (dT11TS) were administered intraperitoneally (i.p.) in *N*-ethyl-*N*-nitrosourea-induced immune-compromised mice at 0.4 mg/kg body weight. It was observed that both the forms of T11TS could activate the compromised immune status of mice by augmenting immune receptor expression (CD2, CD25, CD8, and CD11b), T-helper 1 shift of cytokine network, enhanced cytotoxicity, and phagocytosis activity. Therefore, the results nullify the active involvement of the N-linked glycan moieties in immunotherapeutic efficacy of T11TS.

Keywords ENU; MALDI; N-linked glycan; SRBC; T11TS

Received: August 19, 2011 Accepted: October 24, 2011

Introduction

Almost all of the key molecules involved in the innate and adaptive immune response are glycoproteins. In the cellular immune system, specific glycoforms are involved in the folding, quality control, and assembly of peptide-loaded major histocompatibility complex (MHC) antigens and the T cell receptor complex [1]. T11-target structure (T11TS) is a sheep red blood cell (SRBC) membrane acidic glycoprotein with an apparent molecular mass of 42 kDa [2]. It belongs to the immunoglobulin (Ig) superfamily and is a well-known classical ligand of the E (erythrocyte) receptor (CD2) [3], expressed on T cells, NK cells, and other immunocytes [4,5]. It plays a key role in transitory initial encounters between T cells and antigen presenting cells and, thereby, stimulates T cell activation process via the alternative Ag-independent pathway, which is considered as an important mechanism for intrathymic T cell proliferation [6–8]. The therapeutic relevance of this bioactive molecule had been established in our laboratory. It was found that intraperitoneal administration of this protein in immune-suppressed glioma-bearing rat model boosted up the dampened immune system [9–13]. T11TS can also act as an apoptotic [14] as well as cell cycle modulatory [15] agent by inducing cytotoxic T-lymphocyte-mediated immune activation. The *in silico* CD2–T11TS interaction analysis has highlighted that the main interacting residues (Met27,

Phe29, Lys32, Asp33, Lys34, Glu37, Asp39, Gln40, and Phe47) are highly conserved and located in a heterophilic, charged AGFCC′C″ face on the N-terminal IgV domain (D1 domain) of T11TS [16], as previously documented in the case of human lymphocyte function-associated antigen-3 (LFA-3) [17,18]. Although the CD2-binding domain of T11TS contains two potential N-type glycosylation sites, none is found to be present within 4 Å of the T11TS–CD2 interacting surface [16]. The aim of the present study was to experimentally analyze the types of glycosylation present in T11TS and to understand the differences in immunotherapeutic potential, if present, between T11TS and its deglycosylated derivative, which is an important prerequisite to anticipate the therapeutic efficacy of synthetic or recombinant T11TS. To explore this, an immune-compromised murine model was used to compare the effect of glycosylation with normal cases. Activation of immune system was assessed by receptor modulation (CD2, CD25, CD8, and CD11b), T-helper (Th)1/Th2 cytokine expression, and some related biochemical parameters.

Materials and Methods

Chemicals

Calbiochem® Glycoprotein Deglycosylation kit from Merck Biosciences Ltd (Darmstadt, Germany), streptavidin–alkaline phosphatase conjugate from Bio-Rad Laboratories Ltd (Hercules, USA), biotin-hydrazide, Bradford Protein Assay kit, AminoLink Plus Coupling Gel, MicroLink™ Protein Coupling kit, C18 spin column, TCEP (Tris (2-carboxyethyl) phosphine), iodoacetamide, cytokines tumor necrosis factor-alpha (TNF)-α, interleukin (IL)-10, and IL-12 ELISA kits from Pierce Biotechnology (Rockford, USA) were used. GeneCAPSULE™ was purchased from G-Biosciences (St Louis, USA). Amicon Centricon (10 kDa cutter) was purchased from Millipore (Billerica, USA). Anti-sheep CD58 mAb (L180/1) from AbD Serotec (Oxford, UK), anti-mouse PE conjugated CD2, anti-mouse PE conjugated CD25, anti-mouse PE conjugated CD11b, and anti-mouse PE conjugated CD8 antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, USA) were used. Alsever's solution, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and *N*-ethyl-*N*-nitrosourea (ENU) were purchased from Sigma-Aldrich chemical Co. (St Louis, USA). All the other chemicals (analytical grade) were procured from reputed manufactures.

Preparation of erythrocyte membranes

Sheep blood was obtained from an authorized local supplier in Alsever's solution and stored at 4°C. After centrifugation at 400 *g* for 25 min, the plasma and buffy coat were removed. The erythrocytes were suspended in an equal volume of cold 100 mM phosphate buffer containing 150 mM NaCl, pH 7.4, and again centrifuged at 400 *g* for 20 min. The supernatant and the buffy coat were again removed. This procedure was repeated thrice. The resulting packed volumes of erythrocytes were lysed with nine volumes of 10 mM phosphate buffer (pH 7.4) containing 0.01 mM ethylenediaminetetraacetic acid (EDTA) and 0.01 mM phenylmethylsulfonyl fluoride (PMSF). The membranes were pelleted at 16,000 *g* for 60 min at 4°C. The supernatant was aspirated, and the loosely packed membrane layer was decanted from the red pellet. The procedure was repeated until the membranes were creamy white, and the material was freeze dried.

Purification of T11TS

The freeze-dried material was solubilized using NP40 buffer (pH 7.4) containing 100 mM sodium phosphate, 150 mM NaCl, 1% NP40, 0.04% deoxycholate, 70 μg aprotinin, 0.01 mM PMSF, and 0.01 mM EDTA. The solubilization mixture was kept at 4°C for 24 h, with occasional vortexing. The insoluble portion was pelleted out at 16,000 *g* for 60 min at 4°C. The supernatant was collected and stored at 4°C. The protein concentration of the chilled supernatant was measured using a Bradford Protein Assay kit at 595 nm, and an appropriate amount of solid ammonium sulfate was added to fractionate the proteins; 0%–20%, 20%–40%, and 40%–60% ammonium sulfate cuts were carried out. For each cut, the precipitate was collected by centrifugation at 16,000 *g* at 4°C, solubilized with NP40 buffer (pH 7.4), and dialyzed extensively against cold dilute NP40 buffer. Each dialyzed fraction was then concentrated using an Amicon Centricon 10 kDa concentration device. The protein concentration was measured, and the presence of T11TS was assayed via immunoblot with anti-sheep CD58 mAb (L180/1). AminoLink Plus Coupling Gel was coupled with the anti-sheep CD58 mAb (L180/1) at 4°C based on the manufacturer's instructions. T11TS-containing fractions were then incubated with the mAb-tagged immunoaffinity matrix, washed with 100 mM sodium phosphate buffer (pH 7.4), and eluted with the elution buffer supplied in the MicroLink™ Protein Coupling kit. The eluted fractions containing T11TS were pooled and neutralized with 1 M Tris (pH 9.0). The purity of the T11TS fraction was checked using sodium dodecyl sulfate polyacrylamide gel electrophores (SDS–PAGE) [15,19], followed by silver staining and immunoblotting [15,20], following the standard procedure. The purified T11TS was separated from the leached antibody and some other non-specific proteins using preparative gel electrophoresis and electroeluted using GeneCAPSULE™. Finally, the pooled pure protein was subjected to MALDI-time-of-flight mass spectrometry (TOF MS) and MS/MS (Applied Biosystems 4700 Proteomics Analyzer) analyses for identification and purity confirmation.

In-gel protein digestion

The protein bands of interest were excised from the silver-stained 1D gel, sliced into 1-mm^3 pieces, and destained in 200 μl of destaining solution (25 mM of ammonium bicarbonate solution in 50% acetonitrile) at 37°C for 30 min with continuous shaking. The destaining solution was removed, and the gel pieces were washed thrice first with 100 μl of destaining solution, followed by 100 μl of acetonitrile. Proteins were then reduced by 35 μl of reducing buffer (50 mM TCEP in 25 mM ammonium bicarbonate solution) at 60°C for 10 min. The samples were allowed to cool, and the reducing buffer was removed. Subsequently, the samples were alkylated using 35 μl of alkylating buffer (100 mM iodoacetamide in 25 mM ammonium bicarbonate solution) in the dark for 1 h. Another washing process (same as that mentioned earlier) was performed before the in-gel tryptic digestion. Air-dried shrunk gel pieces were swelled with 10 μl of activated trypsin (10 ng in 25 mM ammonium bicarbonate solution), and the enzymatic digestion reaction was carried out overnight at 37°C. The peptides were extracted in 100 μl of 25 mM ammonium bicarbonate solution by gentle vortexing for 20 min, then in 2×100 μl of 1% trifluoro acetic acid/50% acetonitrile mixture, and finally in 100 μl of acetonitrile. The supernatants were collected and pooled at each step, and the entire extraction process was performed in duplicate. The supernatants were then dried with a vacuum concentrator without heat. The peptides were suspended in 2 μl of 15% acetonitrile/1% trifluoro acetic acid and 13 μl of distilled water prior to loading into the C18 Spin Column for desalting purpose, following the manufacturer's protocol. The desalted peptides were dried using the vacuum concentrator without heating and preserved dried at −80°C [21].

MALDI-TOF MS, MS/MS analysis, and protein identification

Molecular mass of the protein was determined using immunoblot analysis and thereafter confirmed by MALDI mass spectrometry. Prior to MALDI-TOF MS/MS analysis, the peptides were resuspended in 30% acetonitrile/0.1% trifluoroacetic acid, mixed with α-cyano-4-hydroxycinnamic acid matrix, and 0.45 μl was spotted on to a target plate. Des-Arg1-Bradykinin (904.468 Da), Angiotensin1 (1296.685 Da), Glu1 Fibrinopeptid (1570.677 Da), adrenocorticotropic hormone (ACTH) 1–17 (2093.087 Da), ACTH 18–39 (2465.199 Da), and ACTH 7–38 (3657.929 Da) were used as external standards for MS calibration. The Internal Calibration-Peak Matching criteria were: (i) minimum $S/N = 100$; (ii) mass tolerance = ± 1 m/z; (iii) minimum peaks to match= 4; and (iv) maximum outlier error = 3 p.p.m.

The product ions obtained from the MS and MS/MS analyses were submitted to MASCOT ion search (www.MatrixScience.com), and the NCBI nr database was searched for protein identification using the parameters of maximum number of missed cleavage of 1. MS and MS/MS tolerance limits were 100 p.p.m. and 0.2 Da, respectively, with possible modifications either of oxidation or carbamidomethylation. The other associated parameters involved were peptide charge 1+ for MALDI and trypsin as the digesting enzyme.

In silico N-glycosylation sites and O-glycosylation sites prediction

NetNGlyc1.0 [22] and NetOGlyc3.1 [23] web servers were used for the identification of N- and O-linked glycosylation sites.

Enzymatic deglycosylation

Deglycosylation of the purified protein was assessed using the Calbiochem® Glycoprotein Deglycosylation kit, according to the instruction of the manufacturer [24,25]. The enzymes used in this study included *N*-glycosidaseF, α2,3,6,8,9-neuraminidase, endo-α-*N*-acetyl-galactosaminidase, β1,4-galactosidase, and β-*N*-acetyl-glucosaminidase.

Identification of glycoprotein

The purified native protein and its different glycosidase-treated forms were electrophoresed by SDS–PAGE [19]. After SDS–PAGE, the protein was electrophoretically transferred to a nitrocellulose membrane. The membrane was equilibrated with 0.1 M acetic acid for 10 min, and the carbohydrates were oxidized with 10 mM sodium meta periodate in 0.1 M acetic acid by incubation for 20 min at room temperature in the dark. The membrane was washed twice with 0.1 M acetic acid and once with 0.05% Tween 20/0.1 M acetic acid. Biotin-hydrazide in 0.05% Tween 20/0.1 M acetic acid was then added and allowed to react for 60 min at room temperature to label the aldehydes that resulted from carbohydrate oxidation. After three washes with 0.05% Tween 20 in TBS (TBST), the membrane was blocked for 30 min and incubated with a 1 : 2000 solution of streptavidin–alkaline phosphatase conjugate. The membrane was washed again with TBST and stained with BCIP/nitro blue tetrazolium chloride (NBT) as the substrate [25].

Similarly, immunoblot analysis of the purified native protein and its different glycosidase-treated forms was carried out as stated previously [20] using mouse anti-sheep CD58 (L180/1) antibody.

Enrichment of T11TS and dT11TS

As stated earlier, 1000 ml of freshly drawn sheep blood was processed according to the purification protocol of T11TS. A portion of the pure T11TS was deglycosylated using *N*-glycosidaseF and gel-purified. Gel-purified pure T11TS and dT11TS were concentrated using vacuum

concentrator and stored at 4°C. Protein concentration was checked by Bradford Protein Assay kit at 595 nm.

Experimental animal grouping and treatment

Healthy newborn male Swiss albino mice, 2–3 days old, supplied by CNCI (Kolkata, India) and subsequently maintained in our laboratory, were used. The animals were separated into four groups before dosing schedule as follows: Group 1 (N), Group 2 (E), Group 3 (ET), and Group 4 (EdT). Each group consisted of 15 animals (three animals/cages). The animals were fed with autoclaved pellet diet (Hind Lever, Chandigarh, India and Tetragon Chemie Pvt. Ltd (Vetcare), Bangalore, India) and water *ad libitum*. They were kept in 12-h light/dark cycles and housed at 25° ± 1°C.

After 2–3 days acclimatization in our laboratory, mice of Groups 2–4 were injected once with freshly prepared ENU (10 mg/ml of ENU in sterile saline, pH was adjusted 4.5 with crystalline ascorbic acid) intraperitonially (i.p.) at an acute dose of 80 mg/kg body weight [26–28] for induction of immune suppression. Mice of control group (Group 1) were treated with sterile saline (pH 4.5) only. Three-month-old ENU-administered animals of Groups 3 and 4 were treated with T11TS (0.4 mg/kg body weight animal) and deglycosylated T11TS (0.4 mg/kg body weight animal), respectively. They were examined weekly throughout the experimental period. Maintenance and animal experimental procedures were strictly in accordance with the 'Principles of Laboratory Animal Care' (NIH) as well as the local 'Ethical Committee' regulation.

Isolation of peripheral blood lymphocytes, macrophages, and assaying some functional parameters

Lymphocytes and macrophages were separated from the blood of each group of animals according to standard procedure [28]. E-rosetting [28], cytolytic efficacy of lymphocytes (by HO-33342 release assay) [10,28], and assessment of reactive oxygen species (ROS) (by NBT reduction assay) [12,28] were also done using standard protocols. Flowcytometry (FACS) was used to assess the differential expression of CD2, CD25, and CD8 on lymphocytes and the CD11b expression on macrophages of different experimental groups using anti-mouse PE conjugated CD2 mAb, anti-mouse PE conjugated CD25 mAb, anti-mouse PE conjugated CD8 mAb, and anti-mouse PE conjugated CD11b mAb [11,12,28], respectively. Concentrations of Th1 cytokines (IL-12 and TNF-α) and Th2 cytokine (IL-10) in the serum were measured using commercially available ELISA kits.

Statistical analysis

All the experiments were repeated five times. The data are expressed as mean ± standard deviation, unless mentioned otherwise. Comparisons were made between different treatments (ANOVA) using the software GraphPad InStat (GraphPad Software Inc., San Diego, USA), where an error protecting multiple comparison procedure, namely Turkey–Kramer multiple comparison tests, was applied for the analysis of significance of all the data.

Results

Isolation, purification, and identification of T11TS

In the present study, we isolated and purified bioactive protein (T11TS) to homogeneity from SRBCs according to the method developed by Hunig [2], with some modifications. The purity of T11TS was checked at each step of the purification process by SDS–PAGE and immunoblot analysis (**Fig. 1**). Immunoblotting of the ammonium sulfate fractions revealed that the 0%–20% fraction contained majority of the T11TS [**Fig. 1(D)**]. Therefore, the 0%–20% fraction was further affinity purified. Affinity-purified T11TS contained a significant amount of affinity matrix leached antibody and two or three non-specific proteins [**Fig. 1(E)**]. The T11TS band was diffused in nature and appeared near the 43 kDa molecular weight marker. As a final purification step, preparative PAGE was performed, and T11TS was electroeluted. SDS–PAGE and immunoblot of the electroeluted protein showed a single band near 43 kDa, as indicated in **Fig. 1(F,G)**. In-gel digestion followed by MALDI MS and MS/MS analysis showed that five tryptic peptides with molecular masses of 874.6136, 1075.6200, 1759.9606, 1857.1755, and 1971.1174 Da, released during trypsin digestion, have sequence identity with LFA-3 (Ovis) or T11TS (NCBI nr database; accession no. BAA05920, version: gi 540538) (MS and MS/MS spectra are not shown here).

Experimental and *in silico* analyses of the presence and types of glycan moieties on T11TS

In silico analysis showed that T11TS contained three potential N-glycosylation sites, namely Asn12, Asn62, and Asn111, and one O-glycosylation site, namely Thr107.

Experimental analysis showed that the T11TS was highly glycosylated as detected using glycoprotein staining reagents. After the *N*-glycosidaseF treatment, the molecule was not detected in the blot using the glycoprotein detection procedure, implying probable loss of all glycan moieties. Immunoblot analysis of the *N*-glycosidaseF-treated sample using L180/1 showed a faint band near the 32-kDa region. Presence of terminal sialic acid was also confirmed by enhanced mobility of the α2-3,6,8,9-neuraminidase-treated protein from 43 to 37 kDa (**Fig. 2**). To confirm the absence of O-glycosylation, the protein was treated together with endo-α-*N*-acetylgalactosaminidase, β1,4-galactosidase, and β-*N*-acetylglucosaminidase and analyzed

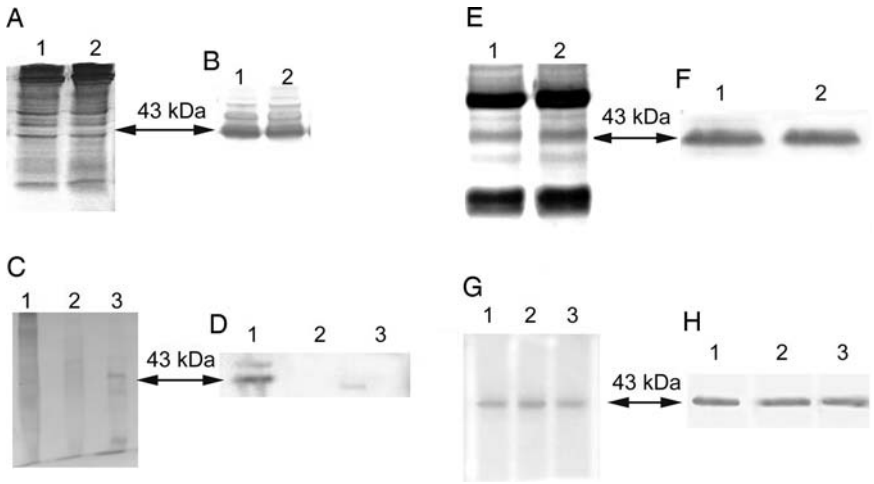

Figure 1 Isolation, purification, and identification of T11TS SDS–PAGE of (A) crude membrane protein mixture (lanes 1 and 2), (C) ammonium sulfate cut (lane 1, 0%–20%; lane 2, 20%–40%; lane 3, 40%–60%), (E) immunoaffinity purified (lanes 1 and 2), and (G) pure T11TS (lanes 1–3). Immunoblot analysis of (B) crude membrane protein mixture (lanes 1 and 2), (D) ammonium sulfate cut (lane 1, 0%–20%; lane 2, 20%–40%; lane 3, 40%–60%), (F) immunoaffinity purified (lanes 1 and 2), and (H) pure T11TS (lanes 1–3).

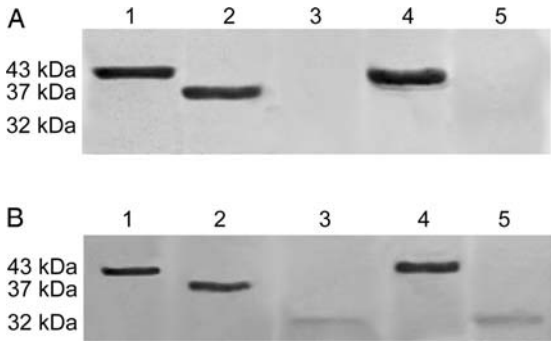

Figure 2 Experimental analysis of glycosylation present in T11TS T11TS was enzymatically deglycosylated using different glycosidases and analyzed using immunoblot techniques. Blots were stained with glycoprotein detection reagents (A) as well as L180/1 (mAb of T11TS) (B). In both (A) and (B), lane 1, T11TS; lane 2, neuraminidase-treated T11TS; lane 3, *N*-glycosidaseF-treated T11TS; lane 4, endo-α-*N*-acetylgalactosaminidase, β1,4-galactosidase, and β-*N*-acetylglucosaminidase-treated T11TS; lane 5, *N*-glycosidaseF, endo-α-*N*-acetylgalactosaminidase, β1,4-galactosidase, and β-*N*-acetylglucosaminidase-treated T11TS.

by the immunoblot technique using glycoprotein staining reagent as well as anti-sheep CD58 monoclonal antibody. No band shift was observed; instead, the bands were detected at the same position as the native protein. In another experiment, T11TS was concomitantly treated with *N*-glycosidaseF and O-glycosidases. The band shift was found near the 32-kDa region where the single *N*-glycosidaseF-treated band was observed. This implies that T11TS does not contain any O-glycan moieties and clearly indicates the presence of only N-linked glycosylation.

E-rosette formation

The number of rosette-forming lymphocytes was significantly reduced in ENU-treated animals (74.2%) when compared with the normal group. Administration of T11TS or its deglycosylated form showed significant improvement in the number of rosetting lymphocytes from the immunosuppressed value [**Fig. 3(A)**].

ROS-mediated phagocytosis of macrophage

Liberation of oxygen species during phagocytosis by macrophage decreased 2.8 folds in ENU-treated group when compared with the normal group. However, T11TS and deglycosylated T11TS administration drastically upregulated ROS liberation by 2.6 folds and 2.5 folds, respectively, when compared with the ENU-treated group [**Fig. 3(B)**].

Cytotoxic efficacy of the lymphocytes

Lymphocyte cytotoxicity was determined by percent lysis. When compared with the normal group, 62.39% decrease in cytotoxic efficacy was observed in the ENU-treated group. Administration of T11TS or its deglycosylated form in the ENU-treated group was found to significantly potentiate cytotoxic activity of the lymphocytes [**Fig. 3(C)**].

Surface receptors expression on immunocytes

Flow cytometry data demonstrated that the normal expression of the CD2, CD25, and CD8 on lymphocytes and CD11b on phagocytic macrophages were downregulated in the ENU-treated group by 6.2, 7.0, 3.8, and 2.9 folds, respectively. Considerable recovery of CD2, CD25, CD8, and CD11b expressions with respect to ENU-treated group were observed in T11TS-(4.7, 5.6, 3.0, and 2.5 folds, respectively) and deglycosylated-T11TS-(4.5, 4.2, 2.9, and 2.1 folds, respectively) treated groups, respectively (**Fig. 4**).

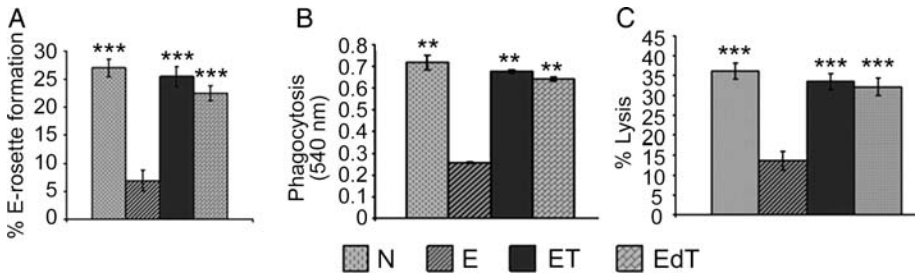

Figure 3 Effect of T11TS and deglycosylated T11TS on E-rosette formation, ROS-mediated phagocytosis of macrophage, and cytotoxicity of the lymphocytes in ENU-treated mice (A) Percent of E-rosette formation, (B) ROS-mediated phagocytosis of macrophage, and (C) cytotoxic efficacy of the lymphocytes were analyzed using microscopy and spectrophotometry, as described in 'Materials and Methods'. The values are mean ± standard deviation. $n = 5$. $^{***}P < 0.001$, $^{**}P < 0.01$ compared with the ENU (E)-treated group.

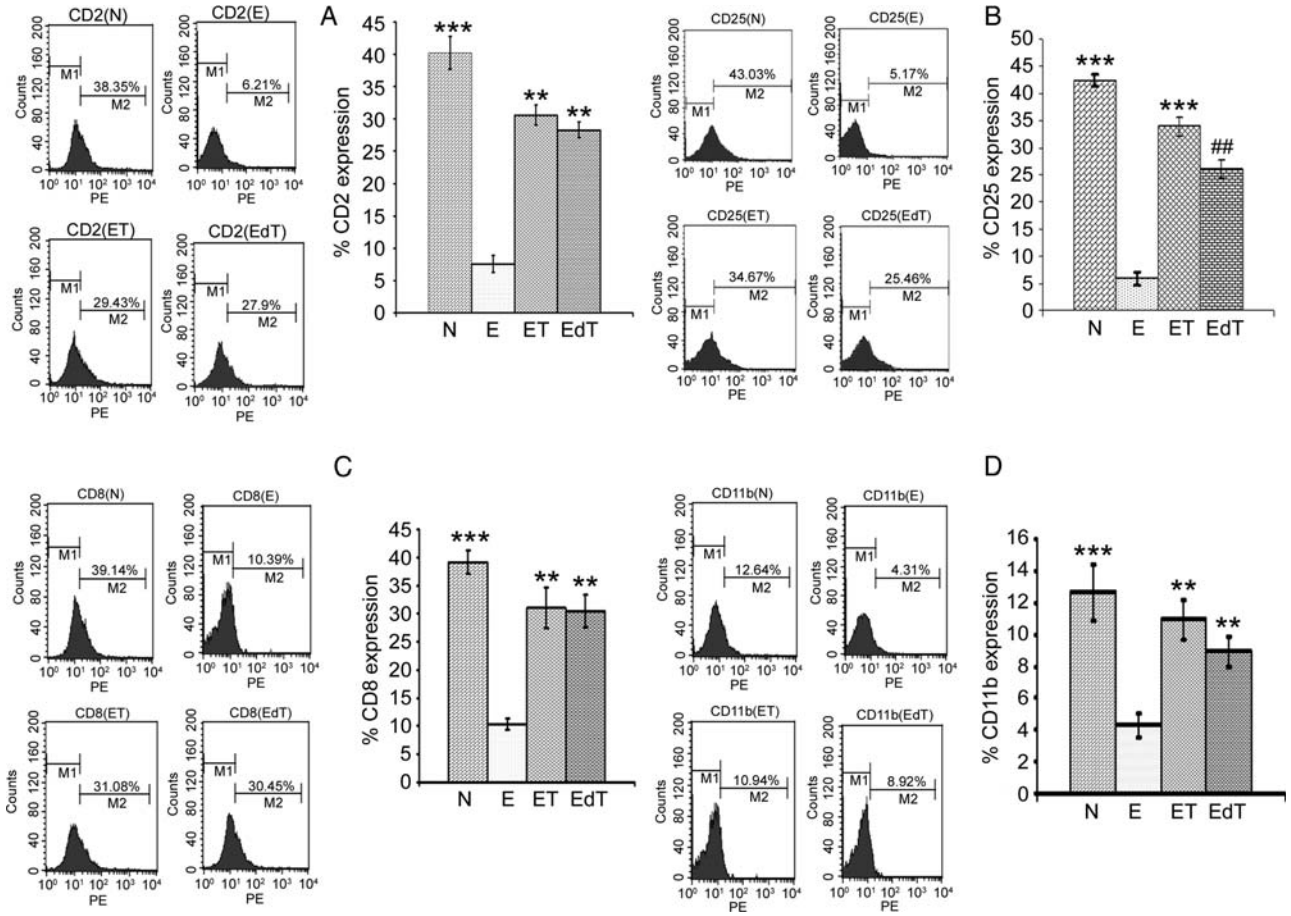

Figure 4 Effect of T11TS and deglycosylated T11TS on surface receptors expression in ENU-treated mice Expressions of CD2 (A), CD25 (B), and CD8 (C) on lymphocytes and CD11b (D) on phagocytic macrophages were analyzed by FACS, as described in 'Materials and Methods'. The values are mean ± standard deviation. $n = 5$. $^{***}P < 0.001$, $^{**}P < 0.01$ compared with the ENU (E)-treated group; $^{\#\#}P < 0.01$ compared with the T11TS (ET)-treated group.

Serum cytokines modulation

ELISA study depicted that ENU treatment reduced TNF-α, IL-12 levels (78.3% and 69.7%, respectively) and increased IL-10 level 1.4-fold when compared with the normal group. However, treatment with T11TS significantly increased the TNF-α level (4.3-fold) and the IL-12 level (2.9-fold) when compared with the ENU-treated group. Treatment with deglycosylated T11TS induced the level from the dampened state but not similar to that observed for native protein. But both the T11TS and its deglycosylated form significantly reduced the level of IL-10 by 0.8-fold, when compared with the ENU-treated mice. Thus, the treatment with T11TS and deglycosylated T11TS varied significantly in the case of TNF-α and IL-12 expressions [**Fig. 5(A,B)**] but did not show any significant difference in the case of IL-10 expression [**Fig. 5(C)**].

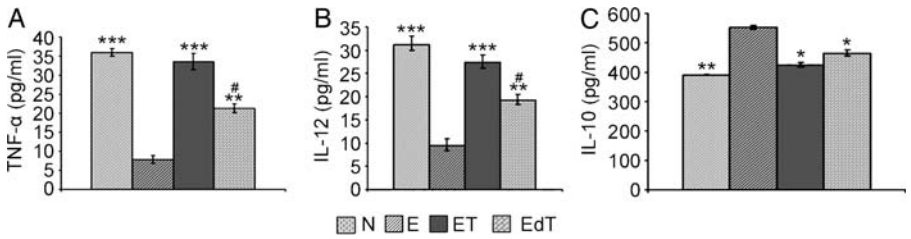

Figure 5 Effect of T11TS and deglycosylated T11TS on serum cytokines levels in ENU-treated mice Serum cytokine TNF-α (A), IL-12 (B), and IL-10 (C) levels were analyzed colorimetrically by ELISA as described in 'Materials and Methods'. The values are mean ± standard deviation. $n = 5$. $^{***}P < 0.001$, $^{**}P < 0.01$,$^{*}P < 0.05$ compared with ENU (E)-treated group; $^{\#\#}P < 0.01$ compared with T11TS (ET)-treated group.

Discussion

SRBC has long been used as non-specific biological response modifier to boost body's immune response. Previously, it was found that sheep erythrocyte could exert potent immunomodulatory and/or anti-tumor property in experimental brain tumor (glioma) model. T11TS, a minor acidic glycoprotein SRBC membrane, has the ability to bind with CD2/T11/E-rosette receptor on lymphocyte and form E-rosette-like constellation through a receptor–ligand complex formation [2,3]. T11TS-mediated actions have direct consequences in rejuvenating the immune defense with their receptor modulation, antigen presentation, and cytokine regulation [9–13]. Simultaneously, its active or passive regulation of glioma cell cycle to aid apoptosis and regulate immune cells to homeostasis indicates a complex and wide operative mode of the molecule at the cellular level [14,15]. Hence, the accurate purification and molecular detailing of T11TS have become the necessity at this backdrop to interpret its functional mechanism.

Therefore, for biochemical characterization, purification of the bioactive protein was carried out. The presence of T11TS was checked at each step of the purification process using immunoblot analysis. SDS–PAGE with immunoblot analysis of pure protein demonstrated a single symmetrical protein band near the region of 43 kDa molecular weight marker (**Fig. 1**). Finally, purity and identity of protein were checked and confirmed by using MALDI mass spectrometry, along with a conventional western blot technique. In the present study, we only used trypsin for peptide mass fingerprinting of T11TS. *In silico* trypsin digestion analysis of T11TS showed that 21 tryptic peptides (**Table 1**) might be generated, with a calculated mass range of 147.1128–3341.5493 Da. However, five tryptic peptides with molecular masses of 874.6136, 1075.6200, 1759.9606, 1857.1755, and 1971.1174 Da were released during digestion, and share sequence identity with LFA-3 (Ovis) (NCBI nr database; accession no. BAA05920, version: gi 540538). The observed molecular masses of the five peptides were higher than the theoretical value (**Table 1**) due to pre-analysis fixed (carbamidomethylation) and variable (oxidation) modifications. Furthermore, 28% amino acid sequence coverage of the mature protein was obtained. However, sequence analysis showed that 3 of the remaining 16 tryptic peptides (peptides nos. 1, 7, and 11) contain glycosylation sites, and one peptide (peptide no. 17) contains a glycosylphosphatidylinositol link site [16]. Their absence in the MS/MS spectrum probably indicates that they might be post-translationally modified. A further consideration for MALDI analysis is that low-mass peptides ($\lesssim$500 Da) are obscured by the presence of matrix peaks. Hence, small peptides were not identified. However, some peptides from trypsin were also present in the spectra.

Detailed computational analysis showed that T11TS contains three potential N-glycosylation sites (Asn12, potentiality: 0.7778; Asn62, potentiality: 0.6940; and Asn111, potentiality: 0.7748) and one O-glycosylation site (Thr104). Although there are more than the three Asn residues present in T11TS, all of them could not be glycosylated; N-glycosylation is specific to the consensus sequence Asn-X-Ser/Thr [29,30]. The presence of Ser/Thr in this position is essential for N-glycosylation [30], but not sufficient, as protein folding also plays an important role in the regulation of N-glycosylation. Furthermore, it is evident from the statistical analysis based on previous documentation that the presence of a Pro residue between Asn and Ser/Thr always inhibits N-glycosylation [29]. Therefore, Asn10, Asn52, Asn164, Asn171, and Asn222 were excluded from the most probable N-linked glycosylation sites. Experimental analysis of the types of glycosylation observed in T11TS was carried out using five different enzymes, namely *N*-glycosidaseF, α2,3,6,8,9-neuraminidase, endo-α-*N*-acetyl-galactosaminidase, β1,4-galactosidase and β-*N*-acetyl-glucosaminidase. The result showed that T11TS is heavily glycosylated with N-linked glycans only and negated the presence of O-linked glycans. There are mainly three types of N-glycans present in the mature protein, namely oligomannose, complex, and hybrid. *N*-glycosidaseF is an amidase that cleaves between the innermost GlcNAc and asperagine residues of all the three N-linked glycans if they are not modified with core α1–3 fucose. Enhanced mobility of the band of T11TS after

Table 1 *In silico* tryptic digestion analysis of T11TS

Peptide no.	Position of cleavage site	Name of cleaving enzyme(s)	Resulting peptide sequence	Peptide length (aa)	Theoretical peptide mass (Da)
1	29	Trypsin	VSQDIYGAMNGNVTFYVSESQPFTEIMWK	29	3342.742
2	30	Trypsin	K	1	146.189
3	32	Trypsin	GK	2	203.241
4	34	Trypsin	DK	2	261.278
5	51	Trypsin	**VVEWDQTSGLEAFQSFK**	**17**	**1971.154**
6	53	Trypsin	NR	2	288.307
7	70	Trypsin	VHLDIVSGNLTITGLTK	17	1781.082
8	84	Trypsin	LDEDVYEIESPSVK	14	1622.746
9	85	Trypsin	K	1	146.189
10	92	Trypsin	**SSQFHLR**	**7**	**873.967**
11	124	Trypsin	VIEPPPTPSASCFLTEGGNITLTCSIPEGDPK	32	3271.702
12	133	Trypsin	**ELDDSDLIR**	**9**	**1075.140**
13	146	Trypsin	**YLWECPPTIQCHR**	**13**	**1645.914**
14	175	Trypsin	GSISSEAFVSAESDLSQNVQCIVSNPLFR	29	3085.392
15	192	Trypsin	**TSASVSLSTCLPEDYAR**	**17**	**1799.970**
16	194	Trypsin	HR	2	311.344
17	212	Trypsin	YVLFAILPAVICGLLFLK	18	1993.565
18	217	Trypsin	CFLGR	5	594.729
19	218	Trypsin	R	1	174.203
20	221	Trypsin	SQR	3	389.412
21	225	End of sequence	NSGP	4	373.366

T11TS was digested with trypsin theoretically. The number of probable peptides to be generated on complete digestion, along with their calculated mass is given in the table. The five peptides that were identified by MALDI MS and MSMS analysis are given in bold. Probable glycosylation sites are highlighted by gray box.

N-glycosidaseF treatment signifies that N-glycans are not modified with core α1–3 fucose. α2,3,6,8,9-neuraminidase, which catalyzes the hydrolysis of all non-reducing terminal sialic acid residues from glycoprotein, was also observed to enhance the mobility of T11TS, and band shift was observed from 43 to 37 kDa. Therefore, it can be concluded that T11TS contains complex or hybrid N-linked glycans that are rich in terminal sialic acids moieties. The desialated and completely deglycosylated derivatives of T11TS have apparent molecular masses of 37 and 32 kDa, respectively, which demonstrate that the molecular mass of T11TS consists ∼13.9% of sialic acid and 25.5% of N-linked glycan. However, the molecular mass of the deglycosylated form (32 kDa) is still greater than its theoretically derived mass (25 kDa), indicating the potential presence of different types of post-translational modification. This discrepancy remains unsolved and should be investigated further.

After the purification and glycosylation analysis, it is important, at this point, to decipher the involvement of the glycan moieties in its therapeutic efficacy. In the present study, generation of immune suppression in young Swiss-albino mice was achieved within a period of 3 months by i.p. administration of ENU. T11TS and dT11TS were applied separately on the ENU-treated mouse model in two booster doses of 0.4 mg/kg body weight at the age of 3 months [13–15] at an interval of 6 days. Immune activation profiles, such as E-rosette formation, lymphocyte cytotoxicity, ROS generation, receptor (CD2, CD25, CD8, and CD11b) activation, and Th1 cytokines (IL-12 and TNF-α) and Th2 cytokine (IL-10) expressions, were screened and compared.

It was evident that patients with primary malignant central nervous system neoplasm manifest a variety of anomalies in cell-mediated and humoral immunity. Previously, it has been shown by several investigators that malignant-brain-tumor-derived immunosuppressive factor(s) can exert strong immunoregulatory effects on systemic cellular immunity [31,32], as well as at the site of the primary tumor [9,10,12,13]. The current data provide direct evidence of dysregulation of the Th1–Th2 shift in ENU-treated animal, as indicated from an increased secretion of anti-inflammatory cytokines (IL-10) and reduced secretion of inflammatory cytokines (IL-12 and TNF-α) in peripheral blood. Th2 cytokine IL-10 can directly inhibit CD2 signaling in T cells by utilizing the src-homology-2 domain containing tyrosine phosphatase (SHP-1). Secretion of

immunosuppressive Th2 cytokine IL-10 was elevated in the ENU-treated group ($P < 0.001$), when compared with the normal group, and thus, downregulated the T cell costimulator, CD2 expression, and E-rosette formation. The tumor burden also downregulated the T cell growth factor receptor CD25, which is also known as T cell activation marker. The extent of the T cell activation and proliferation was found to be directly dependent on the concentration of IL-2 and the level of IL-2R (CD25) expression on the cell. Thus, downregulation of CD25 on T cell depicts strong immune suppression that may be attributed to brain-tumor-derived TGF-β and IL-10. Downregulation of CD8 surface receptor of the peripheral blood lymphocytes resulted in decreased cytotoxicity of the ENU-induced brain tumor state ($P < 0.001$) and highlighted the diminished tumoricidal activity of the dampened immune system. The phagocytic activity of the macrophages was also found to be severely damaged, and this might be due to the decrease in CD11b expression and IL-12 secretion. Another Th1 cytokine, TNF-α, that produces wide spectrum of proinflammatory functions, including adhesion molecule expression, leukocyte movement to the site, NO production, effector maturation, etc., was found to be reduced in peripheral blood in ENU-induced immune-compromised mouse model. This observation is in agreement with earlier studies and depicts the perturbation of immune status in glioma environment [13,31,32].

After initially observing impaired immune functions in developed glioma, activation of the immune cells was observed both with the application of T11TS and dT11TS. The two molecules act with dampened immune system in a similar way. Intraperitoneal route is known to mainly consist of macrophages and small amount of lymphocytes. As the CD2-binding region is evolutionarily conserved [16], the part of i.p.-injected T11TS and dT11TS may directly interact with mice CD2 present on lymphocytes and macrophages, and the other portion of the injected protein might have been taken up by the macrophage for antigenic presentation through MHC. Thus, activation of the immune system by costimulatory molecules and non-specific immune modulation through antigen-dependent pathway may be responsible for the reversal of the dampened immune function in ENU-induced immune-compromised mice.

In antigen-independent pathway, both the molecules interact with the CD2 receptor on intraperitoneal lymphocytes, NK-cells, and macrophages. T11TS–CD2 interaction helps in the upregulation of the CD2 receptors from the clavicular pockets of the membrane. CD2 upregulation causes alteration of the CD2 adaptor proteins CD2BP1/BP2 and, thereby, accentuates the cytoskeletal architecture involving the immune synapse. This causes alteration of nuclear factor-κB/nuclear factor of activated T cells signaling cascades, resulting in increased Th1 cytokine secretion [7,33]. Similarly, in antigen-dependent pathway, the non-self-region of the molecule is presented through macrophage, which interacts with the T cell receptor and, thus, strengthens the tri-molecular complex. This causes augmentation of CD2, CD25, CD8, and CD11b of the dampened immune system and results in improved rosette-forming capacity ($P < 0.001$), cytotoxicity ($P < 0.001$), and phagocytosis ($P < 0.001$). Similarly, the IL-12 and TNF-α upregulation and IL-10 downregulation from glioma condition ($P < 0.001$) were also observed. However, IL-12 and TNF-α secretion did not increase in a similar manner in both the T11TS and dT11TS conditions. The slight difference may be attributed to the glycan moieties. On the contrary, in the case of IL-10, this observed difference was less prominent. Still, in general, glycosylated and deglycosylated T11TS can revert the Th2 cytokine environment of glioma to the Th1 or proinflammatory rank. In all instances, it was observed that T11TS and its deglycosylated form act similarly by augmenting the immune status of the ENU-treated animal by inducing immune effector functions and expression of immune receptors and reverting the Th1/Th2 balance conducive to glioma killing. Otherwise, the differences in receptor expression and immune activities observed were insignificant ($P > 0.001$).

Thus, T11TS, isolated and characterized more precisely with advanced methodology, was tested for their functional efficacy in relation to their glycan moieties. In view of this comparative result, it can be concluded that the synthetic or recombinant form of T11TS may maintain the immunotherapeutic potential of the native bioactive protein where glycosylation shows marginal significance. This study, therefore, widens the possibility of synthetic or recombinant T11TS as a therapeutic probe and requires further attention with regard to its application.

Acknowledgements

S.C. and S.A. are grateful to the Council of Scientific and Industrial Research (CSIR), Govt. of India, for providing research fellowship. The authors thank Dr Sen A.K. (Jr) (IICB, Kol), Dr Chatterjee N.S. (NICED, Kol), and Dr Bandopadhyay S.K. (IPGME&R, Kol) for their constant support throughout the work and valuable discussion. Authors are also thankful to Mr Sandip Chakroborty (MALDI Mass Div., IICB, Kol) for his technical support and assistance.

Funding

This work was supported by grants from the CSIR (Ministry of HRDG, India) and UGC (New Delhi, India).

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