AN IN VITRO STUDY OF IMMUNOMODULATORY ROLE OF T11TS IN RESPECT TO CYTOTOXIC LYMPHOCYTES IN FOUR GRADES OF HUMAN GLIOMA

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ABSTRACT

T11 target structure (T11TS), a membrane glycoprotein has been documented with antineoplastic activity in animal model in our lab. Previously, in animal study we have documented T11TS induced cytotoxic abrogation of tumor cells. Encouraged by these established findings by our group and as prerequisite for clinical trial, this study has been designed to assess the cytotoxic potential of the patient’s lymphocytes in in vitro study of autologous human glioma as modulated by T11TS. Meningioma samples were chosen as disease control group. The data produced indicates T11TS induced up regulation of cytotoxicity of T lymphocytes in grade I and II glioma. Significant enhancement of cytotoxic protein, perforin and granzyme suggest cytotoxic death of T11TS induced target tumor. Also, T11TS downregulates the TGF-β secretion in grade I and II tumor cells. These preliminary findings may help in pushing this molecule into pharmaceutical domain.
KEY WORDS
Glioma, T11 Target Structure, Glycoprotein, Cytotoxicity, Meningioma, Immunotherapy

INTRODUCTION

Malignant glioma is most prevalent among neuro-oncology cases majorly affecting cerebral hemisphere of individuals. Eventual progression from lower grades to higher grades constitutes the pathological entities of these malignant tumors. World Health Organization grade these (gliomas of astrocytic origin) into pilocytic astrocytoma (grade I), astrocytoma (grade II), Anaplastic Astrocytoma (grade III), and Glioblastoma multiformes (grade IV). Immunosuppression as previously characterized in in vitro study of glioma patients included low peripheral blood lymphocyte counts and reduced delayed type hypersensitivity reactions to recall antigens. The diminished immunoglobulin synthesis by B-cells and impaired transmembrane signaling through the T-cell receptor/CD3 complex is evident in glioma patients. Recent impairments that are being identified include anergy, failure of co stimulation, lack of sufficient numbers of functional effector T-cells and the presence of T-suppressor cells within the tumor microenvironment.

Chemotherapy in combinations with surgery and radiation remains the primary treatments for glioblastoma. But, the major limitations of this approach is that malignant cells are either intrinsically resistant to chemotherapy or are able to acquire resistance during treatment. The development of immune based therapies which executes the eradication or suppression of the residual infiltrative component of malignant glioma has been heralded with much hope and optimism. Various approaches to manipulate the immune system have been reported under passive or active immunotherapies. During progression of the disease, tumor cell evolve strategies to avoid an immune response through loss of component responsible for the presentation of tumor specific antigen and loss or gain of cellular functions that renders the tumor cell resistant to death. These associated obstacles underline the importance of search for novel immunotherapy. The sequential data providing comparative status of relevant functional phenotypic markers correlating cytotoxicity in all four grade of glioma is still missing. This study is first of its kind where any possible immunomodulatory action of a novel agent over cytotoxicity of T lymphocytes and its relevant phenotypic markers in all four grades of glioma in in vitro system is being probed.

T11TS/SLFA-3, a membrane glycoprotein isolated from sheep erythrocytes reverses the immune-suppressed state of brain tumor-induced Druckrey rats by boosting the functional status of the immune cells. The T11 sheep erythrocytes binding glycoprotein, also known as CD2/E-rosette receptor/LFA-2 is expressed throughout human T-lymphocyte ontogeny. The complete reversal from the hyperplastic state to normal cellular homeostasis found in a highly invasive and ‘difficult to treat’ glioma model signifies the immunotherapeutic importance of exogenous administration of T11TS acting as a ligand of CD2 receptor on immunocytes/SLFA-3. Our group for the first time successfully established the role of T11TS as catalyzing cell cycle arrest and also specific apoptotic inducer of the brain tumor cells in animals. Earlier in vitro study of rat model documented that T11TS orchestrated the CTL killing of target tumor cell. Encouraged by the previous findings and as a prerequisite for clinical trial the proposed study has been designed to elucidate and assess any possible immunomodulatory role of T11TS in different established grades of human glioma in vitro experiments. The total effector cytotoxicity has been analyzed relating the phenotypic
markers with granular exocytosis and the resultant cytotoxic death of the glioma cells. The sequential analysis of surface markers, cytotoxic proteins in different grades of glioma and T11TS mediated immunomodulation remains the hallmark, as it substantiates its novel therapeutic potential.

MATERIALS AND METHODS
Procurement of glioma sample and patient blood
Procurement of excised and biopsied human glioma along with 5ml of their peripheral blood in acid-citrate-dextrose anticoagulant was conducted under the supervision of a neurosurgeon from Bangur Institute of Neurology &Psychiatry, IPGMER, Kolkata (Table-1). These tumor cells which were dissected stereotaxically by neurosurgeons during the actual operation and have been characterized by expert Neuropathologist at the above Institute and graded accordingly. They characterized the cell for tumor markers excluding normal cells. The procedure was adopted strictly in adherence to approved Institutional Ethical Committees of School of Tropical Medicine &IPGMER, Kolkata and followed schedule Y of Indian Drugs and Cosmetics Act. Moreover, all norms for research with human subjects were done as per the specification and methodologies described in Good Clinical Practices. Eight samples of grade I&II, and six samples each of grade III&IV were obtained during 2 year span of this study. Also, for control purpose six samples of benign tumor (meningioma) were collected. Clinical observation of the relevant patient was duly maintained. The frequency of accessing grade III and IV remain lower in comparison to grade I, II. Three samples of each grade were used in the beginning for dose estimation study of TII1TS.

<table>
<thead>
<tr>
<th>Grades and types of glioma specimen</th>
<th>No. of samples/specimen</th>
<th>Sex(M=Male F=Female)</th>
<th>Age(in years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meningioma</td>
<td>6</td>
<td>M=4,F=2</td>
<td>Between 6-56 yrs.</td>
</tr>
<tr>
<td><strong>Grade I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polycystic</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>2</td>
<td>F=2,M=6</td>
<td>Between 11-36 yrs.</td>
</tr>
<tr>
<td>Papilloma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Grade II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrillary astrocytoma</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protoplasmic astrocytoma</td>
<td>3</td>
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</tr>
<tr>
<td>Gemistocytic astrocytoma</td>
<td>1</td>
<td>F=3,M=5</td>
<td>Between 17-56 yrs.</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ependymoma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Grade III</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplastic oligodendroglioma</td>
<td>1</td>
<td>F=1,M=5</td>
<td>Between 36-64 yrs.</td>
</tr>
<tr>
<td>Anaplastic ependymoma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Grade IV</strong></td>
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<td></td>
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<td>Glioblastoma multiformes</td>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>Glioma sarcomatosum</td>
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<tr>
<td>Medulloblastoma</td>
<td>2</td>
<td></td>
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</tbody>
</table>

Table-1

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Tumor grouping for in vitro studies
Three groups were maintained namely group I benign (meningioma) tumor (as disease control), group II glioma control and group III glioma treated with T11TS. Groups II and III were subdivided into 4 groups each based on the four grades of glioma samples that were obtained.

Isolation of lymphocytes.
Lymphocytes were isolated from fresh peripheral blood by standard histopaque density gradient method. 185 ml of venous blood was collected aseptically from the antecubital vein of each patient (meningioma, Grade I, Grade II, Grade III and Grade IV glioma patients). Then the patient’s blood was separated with density gradient elutrition method with Histopaque (Sigma). The isolated lymphocytes were further panned out mixture of anti-CD4 and anti-CD56 and adherence of the antibody-coated cells to anti-human IgG-coated Petri dishes. The efficacy of T cell depletion was controlled by flow cytometry of the non-adherent population, which contained less than 2% CD4+ or CD56+ cell was collected.

Maintenance of tumor cell culture
Patient biopsies were immediately dissociated by trypsinization and subsequently grown as monolayer cell cultures. Cells were cultured in DMEM (Sigma-Aldrich), 10% fetal bovine serum, with NEAA, 100 units/mL Pen/Strep, and 400 mol/L L-glutamine (Cambrex). Until the 10th passage, when just small rounded cells were seen in the culture, a slow growth rate was observed (data not shown). At 11th passage, the cells entered into an exponential growth phase. The population doubling time was about 24 h at 37°C and the saturation cell density was reached at 10 × 10^5 cells/cm^2 (Figure). The high growth rate was observed for the successive passages. Cells were analyzed for GFP and CD133 positivity with CellQuest on a FACSCalibur (BD Biosciences).

Isolation of T11TS
T11TS was isolated from sheep erythrocyte (sheep red blood cell) membranes. Briefly, sheep red blood cells were trypsinized, treated with TCA and neutralized. The glycopeptides were separated by ion exchange chromatography on a DEAE-cellulose column and eluted with a five-gradient system. Elute fraction III was selected as the fraction of choice.

Dose estimation study of T11TS
The protein concentration of the 3rd elute fraction of T11TS was determined by Bradford assay (Bio rad, USA) and diluted to 1:10, 1:100, 1:1000, 1:10000. To establish most effective dose these dilutions were separately incubated with three samples (three samples of each grade, i.e. total 12 samples were included).
Effectivity was measured by estimating cytotoxic efficacy of peripheral blood activated lymphocytes (with four serial dilutions separately) against target autologous tumor cell by HO-33342 release assay. Lymphocytes (1×10^5 cells/ml) were incubated with each serial dilutions of T11TS separately for 24 hrs. Harvested lymphocytes were incubated with target (tumor cells, 1×10^5 cells/ml). Tumor cell labeled with HO-33342 fluorochrome dye (6 μ/10^6 / ml) for 15 minutes at 37° C and excess was washed off (total incorporation). Activated lymphocytes incubated with target (tumor) by maintaining 10:1 ratio in a CO₂ incubator (37°C, 4 percent CO₂ and humidified environment) for a period of 18 hrs. Fluorochrome released as per target lysis (experimental lysis) was measured in a spectrofluorimeter (Hitachi, Tokyo) and provided an index of cytotoxic efficacy of effectors. Result was calculated as follows:

<table>
<thead>
<tr>
<th>Experimental lysis - spontaneous lysis</th>
<th>× 100</th>
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<tbody>
<tr>
<td>Total incorporation</td>
<td></td>
</tr>
</tbody>
</table>

Serial dilution of T11TS showed highest and consistent cytotoxic efficacy chosen for further assessment.

Estimation of cytotoxic efficacy of peripheral blood lymphocytes against target autologous tumor cell by HO-33342 release assay.

A newer approach to this method has been adopted using a fluorochrome dye Hoechst-33342 (HO-33342, Sigma, USA). Three groups, group I benign (meningioma as disease control), grade II glioma control and grade III glioma samples treated with T11TS (experimental) was maintained for this and later studies. In this method, HO-33342 binds to DNA of cells irreversibly without leakage until lysed. Further, similar protocol (described above) followed for cytotoxicity estimation.

Analysis of phenotypic surface markers and cytotoxic proteins by flowcytometry

Lymphocytes of glioma patients were divided into 2 groups - T11TS treated and control using standard culture media. Separately, benign (meningioma) tumor group, as disease control was also maintained. Incubation of (treated) lymphocytes with T11TS was for 24 hrs, prior to FACS study. Cell populations of disease control and treated and untreated group of lymphocytes of approximately 1×10^6 cells/ml were incubated with 5 μl of respective FITC conjugated anti human monoclonal antibodies CD2, CD8, CD25, CD56 (BD Bioscience). Harvested cells were treated with Para formaldehyde (0.3% in PBS) at 4°C for 1 hr. After this cells were washed and resuspended in 300 μl PBS and subjected to flow cytometric analysis in FACS Caliber instrument (BD Biosciences). A total of 10,000 events were acquired and analyzed.

To estimate the cytotoxic protein perforin and granzyme, the above groupings were maintained. Incubation of (treated) lymphocytes with T11TS was for 24 hrs. After 24 hrs, T11TS activated were harvested and set up with autologous tumor cell for next 24 hrs. Cell populations of treated and untreated group of lymphocytes of approximately 1×10^6 cells/ml were collected to permeabilize the sample with Triton X-100 (0.5%) for 1 hr at 4°C. After this cells were incubated with 5μl of respective FITC conjugated anti human monoclonal antibodies perforin and granzyme. After 30 min washed cells were subjected to flow cytometric analysis in FACS Caliber instrument. A total of 10,000 events were acquired and analyzed.

*TGF-β analysis by ELISA*

ELISA test was performed to analyze secretory TGF-β cytokine in different grades of glioma. Method adopted, strictly follows the ELISA kit’s standard protocol (PK-EL-69006, Promokine, Germany).

Statistical analysis

The results of spectrofluorimeter, flow cytometry and ELISA were analyzed using the t-test for paired observations. The computed t score was then compared with the critical t scores with the same d.f. The difference between the paired observations was considered to be significant if the computed t equaled or exceeded the critical t.
for the chosen level of significance (P<a). On the contrary, the difference was considered not significant if the computed t was lower than the critical t for the chosen significance level (P>a). All results were evaluated statistically by applying the SPSSPC package (version 9.0, SPSS, Chicago, Illinois, USA.

**RESULTS**

*Figure-IA*

**Estimation of CTL activity using serial dilution 1:10, 1:100, 1:1000 of T11TS in each four grades of human glioma**

![Graph showing estimation of CTL activity using serial dilution 1:10, 1:100, 1:1000 of T11TS in each four grades of human glioma.]

#UT=Untreated, T=Treated, G1=Grade I glioma, G2=Grade II glioma, G3=Grade III glioma, G4=Grade IV glioma.

-Most effective dose among three serial dilutions 1:10, 1:100, 1:1000 of T11TS were estimated. The cytotoxicity of peripheral lymphocytes against autologous tumor cell by HO-33342 release assay was measured with three serial dilutions in all four grades of human glioma. Three samples of each grade of glioma were used for the purpose. Figure represents mean±SD value of untreated and treated cell of three samples each of all four grade glioma.

**Estimation of cytotoxic efficacy of peripheral blood lymphocytes against target autologous tumor cell by HO-33342 release assay.**

Compared to CTL activity in benign (control) tumor (2.96±2.73) (Fig-I) the value of grade I glioma control cytotoxic lysis raised to 51.95±1.46 and T11TS activation further significantly (p<0.0001) increased the value to 68.49±2.87. In grade II glioma control the cytotoxic result diminished to 43.47±6.30 when compared to grade I control and T11TS activation hiked the value significantly (p<0.0001) 53.069±9.168. There was no significant changes in grade III (p>0.05) where control value was 39.83±2.53 and with T11TS activation 40.05±2.15 and in grade IV (p>0.05) cytotoxic lysis without T11TS was 37.06±1.73 and with T11TS value was 37.64±1.541.
Figure-IB
Estimation of CTL

Cytotoxicity of peripheral lymphocytes estimated against autologous target tumor cells in all four grades of human glioma by HO3334 assay method. Comparative study were made between untreated and T11TS treated peripheral lymphocytes in all four grades of glioma. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples. Meningioma were used as disease control. Significant enhancement of cytotoxic effect were observed in T11TS treated cells of grade I and II glioma. No such significant change reported between T11TS treated and non treated groups of grade III and IV glioma.

Statistical analysis-
(I) Meningioma (Disease control)-M=29.048, SD=2.736, p=0.0001.
(II) Grade I (Untreated)-M=51.85, SD = 3.44, (Treated) M = 68.49, SD = 5.87, p=0.0001.
(III) Grade II (Untreated)-M=43.47, SD = 6.10, (Treated) M=53.06, SD = 9.06, p<0.0001.
(IV) Grade III (Untreated)-M = 39.83, SD = 2.52, (Treated) M = 40.05, SD = 2.157, p>0.05.
(V) Grade IV (Untreated)-M = 37.06, SD = 1.73, (Treated) M = 37.64, SD = 1.54, p>0.05.

M = Mean, SD = Standard Deviation.

Analysis of the result of phenotypic marker CD2
Expression of CD2 was 89.352 ±5.130 in benign (control) tumor. But this value drastically comes down in grade I control glioma to 47.116±8.197 but significant (p<0.0001) up regulation (83.158±6.681) was found with T11TS. Significant up regulation of CD2 was reported in grade II (p<0.0001) (49.45±5.62) with T11TS activation, compared to the value of 38.214±7.32 in glioma control. Insignificant (p>0.05) changes were observed in grade III (31.243±3.233) and grade IV (p>0.05) (9.778±2.579) activated with T11TS in comparison to glioma control values of grade III (30.220±3.394) and grade IV (10.158±1.927) respectively. (Graphical representation in Fig –II)

Figure-II
Estimation of CD2

Estimation of the membrane receptor protein CD2 of lymphocytes in all four grades of human glioma patients in vitro with and without T11TS by Flow Cytometry. Meningioma samples were used as disease control. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples.

Statistical analysis—
(I) Meningioma (Disease control)-M=89.352, SD = 5.130, p<0.0001.
(II) Grade I (Untreated)-M = 47.116, SD = 8.197, (Treated) M = 83.158, SD = 6.681, p<0.0001.
(III) Grade II (Untreated)-M = 38.214, SD = 7.32, (Treated) M = 48.45, SD = 5.62, p<0.0001.
(IV) Grade III (Untreated)-M = 30.220, SD = 3.394, (Treated) M = 31.243, SD = 3.233, p>0.05.
(V) Grade IV (Untreated)-M = 10.158, SD = 1.927, (Treated) M = 9.778, SD = 2.579, p>0.0001.

* M = Mean, SD = Standard Deviation.

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Analysis of CD25 phenotypic marker

Expression of CD 25 in benign (control) tumor was 80.552±4.815. In grade I glioma lymphocytes the immunosuppression down regulates this activation receptor to 31.147±3.593 and T11TS activation significantly (p<0.0001) increased the results to 44.692±4.854. In grade II glioma the expression of CD25 was lesser in control non activated lymphocyte (30.817±5.322), which was raised significantly (p<0.0001) (40.607±4.013) in T11TS activated lymphocytes. But immunosuppression down regulates the expression in grade III and IV control lymphocytes (non T11TS incubated) to 18.870±2.884 and 14.353±3.227 respectively. Insignificant alteration was observed in T11TS activated lymphocyte of grade III (p>0.05, 19.430±2.601) and grade IV (p>0.05, 14.362±3.699) (Graphical representation in Fig.III.)

Figure-III
Estimation of CD25

Estimation of the membrane receptor protein CD25 of lymphocytes in all four grades of human glioma patients in vitro with and without T11TS by Flow Cytometry. Meningioma samples were used as disease control. Mean±SD is based upon six meningioma,eight each of grade I & II and six each for grade III & IV glioma samples.

Statistical analysis-
(I) Meningioma (Disease control) -M = 80.552, SD = 4.815, p<0.0001.
(II) Grade I (Untreated)-M = 31.147, SD = 3.593 (Treated) M = 44.692, SD = 4.854, p<0.0001.
(III) Grade II (Untreated)-M = 30.817, SD = 5.322, (Treated) M = 40.607, SD = 4.013, p<0.0001
(IV) Grade III (Untreated)-M = 18.870, SD = 2.884, (Treated) M = 19.430, SD = 2.601, p>0.05.
(V) Grade IV (Untreated)-M = 14.353, SD = 3.227, (Treated) M = 14.362, SD = 3.699, p>0.05
* M = Mean, SD = Standard Deviation.

Result analysis of phenotypic marker CD 8

The high CD8 count of the meningioma cells can be accounted for by the purity of the CD8 cell obtained by the cell separation method. But in the Grade I untreated group this percentage comes down to 52.407±3.128 due to the immune suppression by the glioma generated suppressive materials, but this range again goes up to 83.583±2.374 due to the activation of the CD8 lymphocytic population with T11TS. Due to greater immune-suppression in CD8 lymphocytes of Grade II glioma this value comes down to 38.268±6.369 which is raised again to (p<0.0001, 51.341±7.391)with T11Ts activation. But T11TS fails to activate the GradeII (31.295±3.864) and Grade IV (30.642±3.633) CD8 lymphocytes, whose values are downregulated to (p>0.05, 32.175±3.630) (Fig-IV.A.iv.b) and IV (p>0.05, 30.413±3.803).

(Graphical representation in Fig-IV)
**Figure-IV**

**Estimation of CD8**

Estimation of the membrane receptor protein CD8 of lymphocytes in all four grades of human glioma patients in vitro with and without T11TS by Flow Cytometry. Meningioma samples were used as disease control. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples.

**Statistical analysis:**
(I) Meningioma (Disease control) - M = 83.386, SD = 4.815, p<0.0001.
(II) Grade I (Untreated) - M = 52.407, SD = 3.128, (Treated) M = 83.583, SD = 2.374, p<0.0001.
(III) Grade II (Untreated) - M = 38.268, SD = 6.369, (Treated) M = 51.341, SD = 7.391, p<0.0001.
(IV) Grade III (Untreated) - M = 31.295, SD = 3.864, (Treated) M = 32.175, SD = 3.630, p>0.05.
(V) Grade IV (Untreated) - M = 26.642, SD = 3.633, (Treated) M = 30.473, SD = 3.803, p>0.05

* M = Mean, SD = Standard Deviation.

**Result analysis by flow cytometry of phenotypic marker CD56**

Estimated expression of CD 56 in benign (control) tumor was 81.482 ± 6.287 which down regulates in grade I & II to 47.498±2.677 and 25.960±5.063 respectively. T11TS incubated lymphocytes of grade I showed significant up regulation (p<0.0001, 76.847±5.468). Also, T11TS incubated lymphocytes of grade II show significant rise in the values (p<0.0001, 37.739±7.118) respectively. There was further down regulation in expression of CD 56 in lymphocytes of grade III (14.690±1.763) & IV (11.788±2.890) which showed insignificant change after T11TS incubation. The insignificant change in grade III and IV was (p>0.05, 15.302±1.389) and (p>0.05, 11.675 ±3.520) respectively. (Graphical representation in Fig-V.)

**Figure-V.**

**Estimation of CD56**

Estimation of the membrane receptor protein CD56 of lymphocytes in all four grades of human glioma patients in vitro with and without T11TS by Flow Cytometry. Meningioma samples were used as disease control. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples.

**Statistical analysis:**
(I) Meningioma (Disease control) - M = 81.482, SD = 6.287, p<0.0001.
(II) Grade I (Untreated) - M = 47.498, SD = 2.677, (Treated) M = 76.847, SD = 5.468, p<0.0001.
(III) Grade II (Untreated) - M = 25.960, SD = 5.063, (Treated) M = 37.739, SD = 7.118, p<0.0001.
(IV) Grade III (Untreated) - M = 14.690, SD = 1.763, (Treated) M = 15.302, SD = 1.389, p>0.05.
(V) Grade IV (Untreated) - M = 11.788, SD = 2.890, (Treated) M = 11.675, SD = 3.520, p>0.05

* M = Mean, SD = Standard Deviation.
**Result analysis of cytotoxic protein perforin by flowcytometry**

Expression of perforin in benign (control) tumor was 74.962±5.317 which decrease down to 16.900±2.757 and 14.736±2.015 in grade I & II respectively. T11TS activated lymphocytes of grade I showed significant hike of perforin (p<0.0001, 25.942±4.905). Also, T11TS activated tumor cell of grade II reported significant increase of perforin level (p<0.0001, 23.957±4.168). Expression of perforin in tumor cell of grades III and IV decline further to 8.58±3.065 and 7.801±2.168 respectively. No significant change in the expression of perforin was evident in T11TS activated tumor cell of grade III (p>0.05, 9.86±3.625) and IV (p>0.05, 6.265±2.081). (Graphical representation in Fig-VI)

![Estimation of Perforin](image)

**Estimation of cyttoplasmic cytotoxic protein perforin in *in vitro* lymphocytes against autologous tumor cell of all four grades of human glioma patients by Flowcytometry. Meningioma samples were used as disease control. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples.**

**Statistical analysis:**
- (I) Meningioma (Disease control): M=74.962, SD = 5.317, p<0.0001.
- (II) Grade I (Untreated): M=16.900, SD = 2.757, (Treated) M=25.942, SD = 4.905, p<0.0001.
- (III) Grade II (Untreated): M=14.736, SD = 2.015, (Treated) M=23.957, SD = 4.168, p<0.0001.
- (IV) Grade III (Untreated): M=8.58, SD = 3.065, (Treated) M=9.86, SD = 3.625, p>0.05.
- (V) Grade IV (Untreated): M=1.265, SD = 2.168, (Treated) M=6.265, SD = 2.081, p>0.05

* M = Mean, SD = Standard Deviation.

**Result analysis of cytotoxic protein granzyme by flowcytometry**

The estimated expression of granzyme in benign (control) tumor is 75.152±7.306. But immunosuppression down regulates this expression to 18.924±2.005 and 10.463±1.505 in grade I & II tumor cell respectively. But T11TS significantly upregulates the expression in grade I to 24.100±5.338 (p<0.0001). Also grade II reported significant upregulation to 19.157±2.981 (p<0.0001) in grade II respectively. Expression of granzyme further declined in grade III (6.905±2.029) and IV (6.277±0.8144) which showed insignificant alteration after T11TS incubation (p>0.05, 6.953±1.957) in grade III and (p>0.05, 5.870±0.8132) in grade IV respectively. (Graphical representation in Fig-VII.)

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Figure-VII.
Estimation of Granzyme

![Diagram showing estimation of granzyme](image)

Estimation of cyttoplasmic cytotoxic protein granzyme in *in vitro* lymphocytes against autologous tumor cell of all four grades of human glioma patients by Flowcytometry. Meningioma samples were used as disease control. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples.

Statistical analysis-
1. (I) Meningioma (Disease control): M=75.152, SD=7.306, p<0.0001.
2. (II) Grade I (Untreated): M=18.924, SD=2.005, (Treated) M=24.100, SD=6.338, p<0.0001.
3. (III) Grade II (Untreated): M=10.463, SD=1.505, (Treated) M=19.157, SD=2.981, p<0.0001
4. (IV) Grade III (Untreated): M=6.905, SD=2.029, (Treated) M=6.953, SD=1.957, p>0.05
5. (V) Grade IV (Untreated): M=7.801, SD=0.8144, (Treated) M=5.870, SD=0.8132, p>0.05

*p* = Mean, *SD* = Standard Deviation.

Result analysis of TGF-β estimation by ELISA (Fig-VIII.)

Estimated expression of TGF-β in benign (control) tumor was 1.873±0.2609. There was significant (p<0.0001) down regulation of TGF-β in T11TS treated glioma of grade I (p<0.0001) to 6.087±2.142(Fig VIII.A ) compared to grade I control (9.183±0.9191). The T11TS treated value in grade II tumor cell diminished significantly (p<0.0001) to 11.898±1.615 in comparison to control value of grade II (15.027 ± 1.409). Higher expression of TGF-β observed in grade III (17.783±1.276) and grade IV (23.550±0.9774) which showed insignificant (p>0.05) change to 1.392±0.04719 in grade III and (p>0.05) 1.392±0.04719 in grade IV in T11TS activated tumor cells.

Figure-VIII.
Estimation of TGF

![Diagram showing estimation of TGF](image)

Estimation of TGF-β concentration (ng/ml) by ELISA in tumor cell of all four grades of human glioma patient samples in vitro. Mean±SD is based upon six meningioma, eight each of grade I & II and six each for grade III & IV glioma samples.

Statistical analysis-
1. (I) Meningioma (Disease control): M=1.917, SD=0.2609, p<0.0001.
2. (II) Grade I (Untreated): M=9.183, SD=0.9191(Treated) M=6.087, SD=2.142, p<0.0001.
3. (III) Grade II (Untreated): M=15.027, SD=1.406, (Treated) M=11.898, SD=1.615, p<0.0001
4. (IV) Grade III (Untreated): M=17.783, SD=1.276, (Treated) M=17.707, SD=1.186, p>0.05
5. (V) Grade IV (Untreated): M=23.550, SD=0.9774, (Treated) M=23.522, SD=0.9411, p>0.05

*p* = Mean, *SD* = Standard Deviation

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DISCUSSION

Glioma induces immune suppression by impairing T cell function. In the present study the immunotherapeutic efficacy of TIITS was tested on all four grades of human glioma patients in relation to cytotoxicity of peripheral blood lymphocytes. Dilution 1:100 of T11TS induced most effective and consistent CTL activity and was chosen for later functional estimation, phenotypic and other studies. When total cytotoxicity was studied the meningioma patients showed lower results than the glioma patients. The glioma cells present the newer antigens like GAGE,EGFR,MAGE that are unknown to the T lymphocyte in healthy condition. Due to breakage of blood brain barrier, such antigen enters the system circulation and activates the T lymphocyte. So, these activated T lymphocytes of the glioma patients show higher cytotoxic activities as compared to benign meningioma patients as studied in vitro assay. Our previous study regarding cytotoxicity of glioma patients revealed that there was no significant change in benign tumor patient and normal patients. But in all other parameters studied when the groups of glioma controls were analyzed against non malignant (benign) meningioma controls, there was distinct immunosuppression evident during analysis indicating again the immune suppression induced in the glioma patients. Electrostatic potential maps reveal the binding surface of T11TS/SLFA-3 with CD-2 on immunocytes to be nearly identical with human CD 58 and supports a 'hand shake 'model of CD2-CD 58 interaction. Exogenous administration of TIITS induces higher CD2 up regulation in grade I tumors suggesting higher receptor ligand receptivity . This may hint at induced quantitative increase in lymphocyte number. This receptivity plays a critical role in alternative antigen independent polyclonal T cell activation, proliferation and lymphokine secretion through antigen independent cytolytic machinery. Higher expression of CD 25 in grade I indicates higher activation of lymphocytes which is the resultant effect of CD2-T11TS ligation. Phenotypic CD2 expression in T11TS activated and non activated control lymphocytes of different grades of glioma indicates linear reduction in CD2 expression from lower to higher grade supporting our previous findings. The T11TS activated and non activated lymphocytes were subjected to cytolytic assay.

CTL activity estimated by HO3342 method produced high significant death in grade I & II glioma induced by T11TS incubated lymphocytes against autologous tumor cell. While Grade III produced insignificant cytotoxic potential of glioma lymphocyte and Grade IV shows no enhancement in CTL activity after incubation with T11TS.No significant enhancement of TIITS induced CTL activity and phenotypic markers CD2, CD25 up regulation in grade III and IV is a possible indication of immunosuppressive microenvironment which derails the TIITS induced lymphocytes activation. In glioma induced suppression, CD3 ξ chain, of TCR-CD3 complex cannot be detected indicating that with the disappearance of the ITAMs of the CD3 ξ chain the proper signaling cascade would not be operative. In presence of defective signaling cascade, the cytotoxic activity of the T lymphocytes hence would be severely impaired. In accordance with our previous findings, this study also reported the inverse relation between grades of tumor and CTL activity and CD2 upregulation.

To decipher the involved mechanistic approach in TIITS induced CTL death, we evaluated the comparative estimation of CD8 and CD56 expression in TIITS activated and non activated lymphocytes. Cellular immune response orchestrates the death of target tumor cell by operating CD8/NK cell regulated CTL activity. Disease control lymphocytes showed high expression of CD8 and CD56 marker which is further down regulated in glioma control. Significant enhancements of CD8 and CD56 phenotypic markers in T11TS treated lymphocyte suggest CD8/NK cell mediated CTL activation.
activity in grade I & II. Also, higher up regulation of CD8 (TIITS treated lymphocytes) in comparison to CD56 in grade I hints the execution of CTL mechanism by specific killing. The higher expression of CD8 in comparison to CD 56 indicates the greater specific killing by the cytotoxic T cells. Data clearly indicates the trend similar to CD2 expression in which there was significant change reported in grade I&II. In grade III and IV, like CD2 expression, no change was observed. To elucidate the role of perforin granzyme pathway in CTL mediated death of target tumor, expression of perforin and granzyme in TIITS activated and non activated lymphocytes of all four grades of tumors were estimated. TIITS activated lymphocytes of grade I and II showed significant increase in the expression in comparison to non TIITS incubated lymphocytes. Grade III and IV showed no alteration in its expression, with and without TIITS incubation. Activated T lymphocytes can kill tumor cell through two different mechanisms: the Fas-FasL pathway and the perforin-granzyme pathway. In perforin granzyme pathway, the pore forming molecule is stored in cytotoxic granules together with granzymes. On recognition of a target cell, the granules are released and perforin monomers insert themselves into the target cell membrane and interact to create pores. These perforin pores can cause osmotic lysis of the target cell and induce apoptosis through various downstream effector pathway.20 The granule exocytosis pathway used by NK cells and CTL predicts that perforin (pfp) released from cytotoxic granules facilitates the entry of serine proteases (granzymes) and other cytotoxic molecules into the target cell, probably through a process of endosomal disruption.21,22,23

TIITS have already been documented in our lab as pro apoptotic for glioma cells in vivo animal model.10 Increase in granzyme expression in TIITS activated lymphocyte possibly indicates the activation of caspase 9 and their downstream pathway leads to apoptotic death.10 The failure of effective potential of TIITS in grades III and IV instigated us to investigate the role of immunosuppressive cytokine TGF β secreted from glioma cells. Many tumors, including gliomas, produce a variety of immunosuppressive molecules including transforming growth factor-b1 (TGF-b1) and TGF-b2 (collectively referred to as TGF-b1,24,25,26,27) Inverse relation between expression and glioma grades hints towards immunosuppressive microenvironment results in neutralizing the effect of T11TS in higher grade glioma. Significant decline induced by T11TS in grade I & II, strengthen the immunopotentiating action of TIITS in in vitro glioma while there is no change in expression of TGF-β reported between, with and without TIITS incubated tumor cell of grade, III and IV.

The comparative and sequential status of cytotoxic T lymphocyte and its associated markers with and without TIITS of all four grade glioma, revealed that the cytotoxic potential in grades I &II glioma increased by T11TS activation but in grade III and IV no changes were observed. This cytotoxic potentiation was related with the phenotypic markers of the lymphocytes and their cytotoxic proteins, and in each case similar result have been obtained. The causative immune suppressant cytokine TGF-β secreted from glioma cells could be effectively down regulated by T11TS activation, which can be well correlated with increased cytotoxic effect of the grade I &II glioma.

In in vivo animal studies of glioma model three doses of T11TS was administered at an interval of six days, which showed complete regression of glioma with immune potentiation in the first two doses the highest being in the second dose. The third dose brought back this level to the homeostatic level. But in the present in in vitro study of human glioma patients a single dose which was used could not probably booster up the immunopotentiation in grades III and IV. Clinical trials will be proposed for three doses of intravenous T11TS administration in the glioma patients. The outcome of this preliminary study is a prerequisite for clinical trial, which would be pivotal in pushing this molecule TIITS into pharmaceutical domain.

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HIGHLIGHT OF STUDY

- Data indicates upregulation of cytotoxicity of T lymphocytes in T11TS treated in vitro grade I&II glioma.
- Significant enhancement of phenotypic markers CD2,CD8,CD25,CD56 reported in T11TS treated in vitro lymphocytes of grade I&II glioma.
- Upregulation of cytotoxic protein perforin and granzyme in T11TS treated lymphocytes against autologous target tumor cell of grade I&II glioma.
- No significant immunomodulation observed between T11TS treated and non treated lymphocytes of grade III,IV glioma.

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