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# T11TS Induced Modulation of Macrophages Associated Cytokines TNF-α,VEGF and Apoptototic Protein Bax, Bcl2 Abrogates Tumor Cells in *In vitro* Grade I,II Human Glioma

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## Abstract

T11 target structure (T11TS), a membrane glycoprotein has been documented with anti-neoplastic activity in glioma bearing animal model in our lab. In this study, we have evaluated the phagocytic potential, expression of VEGF, TNF-α in T11TS treated and untreated macrophages in all four grades of glioma. The data indicates the significant enhancement of phagocytosis in T11TS treated macrophages of Grade I and II glioma. There was significant up-regulation in TNF-α and significant down-regulationin VEGF expression in T11TS treated macrophages in grade I and II glioma.We alsoattempted to know any possible apoptotic role of T11TS in tumor cell by comparing Bax and Bcl2 in treated and untreated tumour cell of all four grades. We found significant up-regulation in Bax expression and down-regulation in Bcl2 expression of grade I and II glioma. The outcome may help in pushing this molecule into pharmaceutical domain.

**Keywords:** Macrophages; T11TS; Immunomodulation; Cytokine; Phagocytosis

**Abbreviations:** T11TS: T11 Target Structure; VEGF: Vascular Endothelial Growth Factor; TNF: Tumor Necrosis Factor; HIF: Hypoxia Inducible Factor

## Introduction

Despite significant multimodality in innovative therapeutic intervention, glioma continues to show its worst prognosis and restricts the victim's life to 12-18 months after diagnosis. The efficiency of glioma antigen presentation in brain confers an important way out for immunotherapeutic approaches where macrophages supposedly acts as the key player [1,2].

Macrophages are considered as sentinels of the immune system. These cells participate in innate immunity and act as the first line of defence in immune response to foreign invaders. They also participate in initiation of the acquired immune response by ingesting foreign particles and presenting them on their surface with major histocompatibility (MHC) complex. In their resting stage, macrophages are relatively quiescent, showing low levels of oxygen consumption, MHC class II gene expression, and cytokine secretion. However, once activated, they exhibit maximal secretion of factors like IL-1, IL-6, TNF-α, reactive oxygen species and nitric oxide produced by inducible nitric oxide synthase (iNOS) [3].

It was first deciphered in our laboratory that T11TS/S-LFA3 is a very potent anti-neoplastic agent which can act by reversing the immunesuppressed state of glioma bearing rats and by way of immune-stimulation it kills the glioma cells by apoptosis. T11TS has been isolated in our laboratory from sheep red blood cells membrane and also has been characterised as a glycoprotein [4-8]. The T11 sheep erythrocytes binding glycoprotein, also known as CD2/E-rossette receptor/LFA-2 is expressed throughout human T-lymphocyte ontogeny [9]. 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 [7,8]. Our group for the first time successfully established the role of T11TS as catalyzing cell cycle arrest [5] and also specific apoptotic inducer [6] of the brain tumour cells in animals. T11TS has been documented as orchestrator of cytotoxic T lymphocyte mediated killing of target tumor cell in *in vitro* studies of human glioma model [10]. In addition, favourable immunomodulation in cytotoxic proteins and other phenotypic markers in grade I, II glioma model have been well documented.

The present study has been designed to assess the immunomodulatory role of T11TS in all four grades of human glioma model. In the present study we have shown that there is an enhancement in phagocytic activity in T11TS treated macrophages of Grade I,II glioma. The significant increase in the expression of associated cytokine TNF-α in T11TS treated macrophages of grade I,II support our observation related to up regulation of phagocytic activity. Down-regulation of VEGF in T11TS treated macrophages of grade I,II further strengthen hope for its potential therapeutic intervention as an anti-angiogenic agent. We also attempted to investigate the effect of T11S on apoptotic protein Bax and Bcl2 in tumor cells.

The sequential analysis related to T11TS effect over major immunological entities in the four grades of glioma remains the cardinal feature of the study. These preliminary findings may help us in

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initiation of *in vivo* human study of T11TS, a prerequisite for clinical trial.

## Materials and Methods

### Procurement of glioma sample and patient blood

Procurement of excised and biopsied human glioma tissue from patients along with 5 ml 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 tissues were dissected stereo tactically by neurosurgeons during the actual operation and have been examined by expert Neuropathologist at the above Institute and glioma diagnosis was confirmed and graded accordingly [11,12]. They characterized the cell for tumor markers by immunohistopathology 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 [13]. Moreover, all norms for research with human subjects were done as per the specification and methodologies described in *Good Clinical Practices* [13,14]. Twenty random samples were collected from each of all four grades of glioma were obtained during 3 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. Five samples of each grade were used in the beginning for dose estimation study of TIITS.

### Tumor grouping for *in vitro* studies

The patients were selected from those who did not receive radiotherapy or chemotherapy. Three groups were maintained in culture by serial passage namely group I benign (meningioma) tumor (as disease control), group II glioma control and group III same glioma tissue treated with T11TS. Groups II and III were subdivided into 4 groups each based on the four grades of glioma samples that were obtained [11,12].

**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 10$^{th}$ passage, when just small rounded cells were seen in the culture, a slow growth rate was observed (data not shown). At 11$^{th}$ 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 1). The high growth rate was observed for the successive passages (Figure 1).

| Grades and types of Tumor specimen | No. of samples/ specimen | Sex (M=Male; F=Female) | Age (in years) |
|---|---|---|---|
| Meningioma | 6 | M=4, F=2 | Between 6- 56 yrs. |
| Grade I<br>Polycystic Astrocytoma<br>Choroid plexus<br>Papilloma | 20<br>5<br>7<br>8 | F=8, M=12 | Between 11-36 yrs |
| Grade II<br>Fibrillary astrocytoma<br>Protoplasmic astrocytoma<br>Gemistocytic Astrocytoma<br>Oligodendroglioma<br>Ependymoma | 20<br>4<br>4<br>6<br>1<br>5 | F=6, M=14 | Between 17-56 yrs. |
| Grade III<br>Anaplastic Astrocytoma<br>Anaplastic Oligodendroglioma<br>Anaplastic Ependymoma | 20<br>8<br>7<br>5 | F=5, M=15 | Between 16-64 yrs. |
| Grade IV<br>Glioblastoma Multiformes<br>Glioblastoma Sarcomastum<br>Medulloblastoma | 20<br>6<br>9<br>5 | F=5, M=15 | Between 26-64 yrs |

**Table 1:** Showing details of grades and types of glioma specimen.

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 [4-8].

### Isolation of macrophages

Macrophages of patient blood were separated by single cell preparation as in the following: blood was laid in corresponding marked Petri dishes. After 30 minutes incubation at 37°C in humidified atmosphere of a $CO_2$ incubator, non adherent cells were washed off with Phosphate Buffer Saline (PBS) and adherent cells were collected by washing with Phosphate Buffer Saline-Ethylenediaminetetraacetic acid(PBS-EDTA) and then washed with PBS and finally suspended in 1 ml media [7,8].

### Dose estimation study of TIITS

The protein concentration of the 3$^{rd}$ elute fraction of T11TS was determined by Bradford assay (Bio rad, USA) and diluted to 1:10, 1:100, 1:1000. To establish most effective dose these dilutions were separately incubated with the peripheral macrophages of five glioma

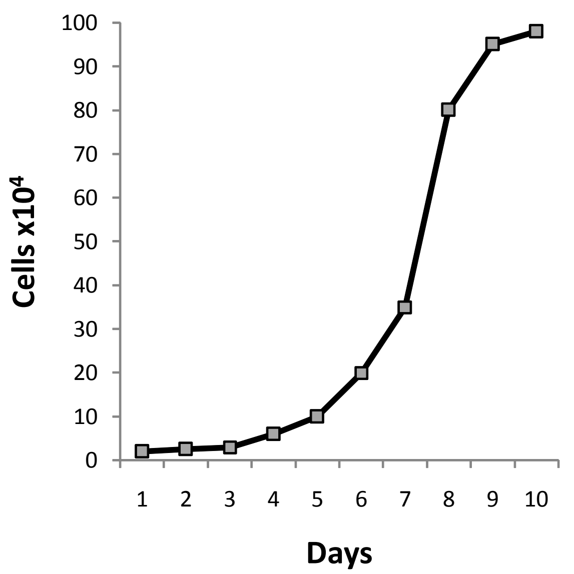



**Figure 1:** Growth curve showing tumor cell culture maintenance.

samples of each grade. Effective dose of T11TS was determined by estimating phagocytic activity of macrophages using standard nitro blue tetrazolium reduction assay. Serial dilution 1:10, 1:100 and 1:1000 of T11TS were separately incubated with peripheral macrophages for 24 hrs. The macrophage harvested ($1\times10^6$ cells/ml) were allowed to phagocytose the target brain tumor cells in presence of nitro blue tetrazolium (NBT) chloride keeping the effector : target ratio which has been described in the later section [7,8].

### Estimation of phagocytic activity of macrophages (by nitro blue tetrazolium reduction assay)

Macrophages of each grade of glioma incubated with T11TS for 24 hrs. The macrophage harvested ($1\times10^6$ cells/ml) were allowed to phagocytose the target brain tumor cells in presence of nitro blue tetrazolium (NBT) chloride keeping the effector : target ratio as 100:1. Reduction of yellow NBT to blue formazan indicated the extent of phagocytic burst by effectors concerned. The preparation was incubated for 18 hrs at 37°C in 4% $CO_2$ humidified atmosphere. Finally, the reaction was stopped by adding 0.1 N chilled HCl and pellet was extracted with boiling pyridine for the reduced blue formazan. The intensity of color assayed spectrophotometrically provided a direct measure of the extent of phagocytosis at 530 nm [7,8].

### TNF-α, VEGF analysis by sandwich ELISA

For vascular endothelial growth factor (VEGF) and Tumor necrosis Factor (TNF-α) measurements, macrophages from each grade of glioma were divided into 2 groups. One group is incubated with T11TS and other without T11TS for 24 hrs. Cells were grown in serum-free media and added to the ELISA plate (#453210, Neogen Corporation, USA for TNF-α and #-452610, Neogen Corporation, USA for VEGF) directly as per manufacturer's directions.

Method adopted, strictly follows the ELISA kit manufacturer's Neogen, USA standard protocol.

### Estimation of apoptotic protein Bax and $Bcl_2$

To study the apoptotic protein Bax and Bcl2 the different groups of tumor cells of both glioma and meningioma group were maintained. Tumor cells were incubated with T11TS for 24hrs. Then the cell suspension was washed with cold PBS and collected to permeabilize the sample with Triton X-100 (0.5%) for 1 h at 4°C. After these cells were incubated with 5 µl of respective FITC conjugated anti human monoclonal antibodies Bax and $Bcl_2$ (manufacturer BD Bioscience, USA). After 30 min washed cells were subjected to flow cytometric analysis in FACS Caliber instrument. The percent expression of each protein was flowcytometrically assessed in FACS-Calibur Instrument (BD Biosciences, USA) using CellQuest Pro software. For each sample, 10,000 events were acquired and analyzed [5,6].

### Statistical analysis

The results of spectrophotometer, 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 equalled 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). P values less than 0.05 are used as a cutoff point.

## Results

### Results of dose estimation study of T11TS

Protein content of T11TS as estimated by Bradford method in the 3rd elute was 50 µg/ml. The 1:100 dilution of T11TS (0.5 µg/ml) produced most significant (Figure 2) and consistent phagocytosis of autologous tumor by peripheral macrophages. Thus, this dilution of T11TS was opted for further estimations.

### Results of analysis of phagocytic activity by macrophages in all four grades of glioma

Compared to phagocytic activity in benign (control) tumor (0.134 ± 0.0081) (Figure 3) the value of grade I T11TS untreated glioma cells phagocytosis was raised to 0.137 ± 0.0089 and values in T11TS treated glioma group activation was significantly increased ($p<0.0001$) (0.162 ± 0.0098). In grade II T11TS untreated glioma cells the phagocytic values increased significantly (0.146 ± 0.022) when compared to grade

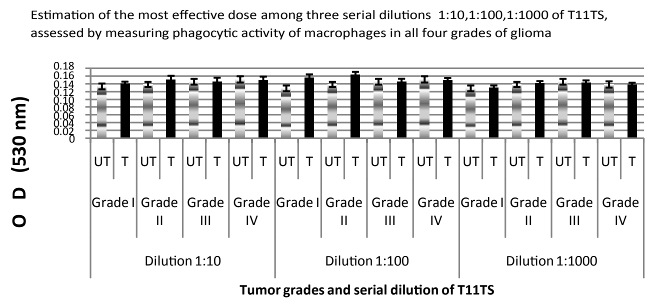
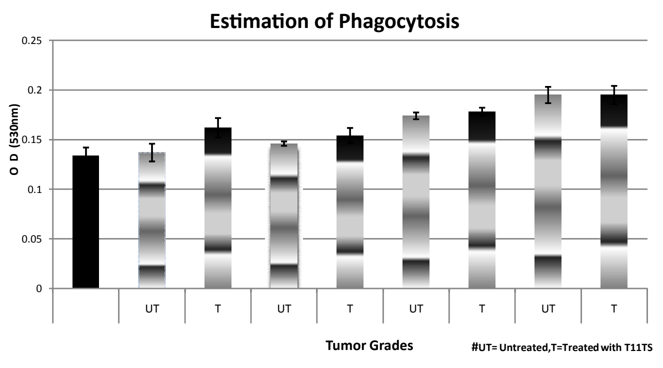



**Figure 2:** Estimation of the most effective dose among three serial dilutions 1:10, 1:100, 1:1000 of T11TS, assessed by measuring phagocytic activity of macrophages in all four grades of glioma.This comparison was made by measuring phagocytic potential between treated macrophages with serial dilution of T11TS and untreated macrophages in all four grades of glioma.




**Stastical analysis**

(I) Meningioma (Disease control)-M=0.134, SD=0.0081, p<0.0001
(II) Grade I (Untreated)-M=0.137, SD=0.0089, (Treated) M=0.162, SD=0.0098, p<0.0001
(III) Grade II (Untreated)-M=0.146, SD=0.0022, (Treated) M=0.154, SD=0.0078, p<0.0001
(IV) Grade III (Untreated)-M=0.174, SD=0.0034, (Treated) M=0.178, SD=0.0092, p>0.05
(V) Grade IV (Untreated)-M=0.195, SD=0.0082, (Treated) M=0.195, SD=0.0092, p>0.05
*M: Mean; SD: Standard Deviation.

**Figure 3:** Estimation of phagocytosis by peripheral macrophages against autologous tumor cells by nitro blue tetrazolium reduction assay in all four grades of human glioma. Comparative study were made between untreated and T11TS treated peripheral macrophages in all four grades of glioma. Mean ± SD is based upon six meningioma, twenty each of grade I to 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.

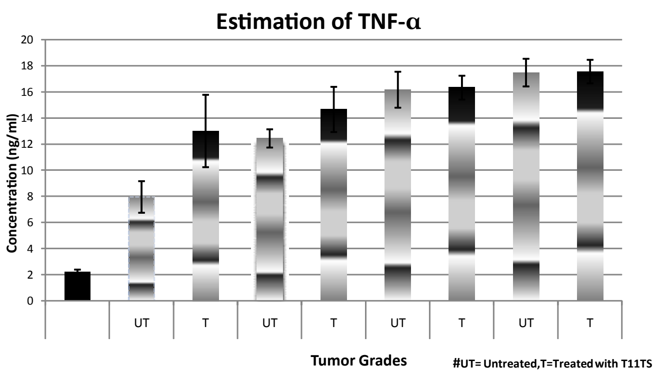
I T11Ts untreated glioma control and in T11TS treated glioma group the value significantly increased ($p<0.0001$) (0.154 ± 0.0078). There were no significant changes in grade III where control value was 0.174 ± 0.0034 and with T11TS activation 0.178 ± 0.0092, in grade IV phagocytosis without T11TS was 0.195 ± 0.0082, and with T11TS, value was 0.195 ± 0.0092.

## Results of analysis of TNF-α

Expression of *TNF*-α in benign (control) tumor's macrophage was 2.26 ± 0.1386 (Figure 4). There was increase in the expression of macrophage secreted *TNF*-α of grade I glioma to 7.959 ± 1.21. This expression further rose significantly to 13.01 ± 2.77 ($p<0.0001$) in T11TS treated macrophage of grade I glioma. In grade II glioma macrophages the expression of TNF-α showed further up regulation (12.44 ± 0.69) which showed significant ($p<0.0001$) change in T11TS treated macrophages (14.66 ± 1.731). In grade III glioma the expression of TNF-α was higher in control non activated macrophage (16.17 ± 1.377), which remain insignificant ($p>0.05$) (16.33 ± 0.9125) in T11TS activated macrophages. Again, in untreated macrophages of grade IV glioma showed higher expression of TNF-α (17.48 ± 1.06) but remain insignificant ($p>0.05$) change in T11TS treated macrophages (17.56 ± 0.90).

## Results of analysis of VEGF

Expression of VEGF was 2.283 ± 0.4629 in benign (control) tumor (Figure 5). But this value increased in grade T11TS untreated glioma to 11.296 ± 0.9685 but significant ($p<0.0001$) down regulation (7.685 ± 2.364) was found with T11TS. Significant down regulation of VEGF was reported in grade II ($p<0.0001$) (14.076 ± 0.6462) with T11TS activation, compared to the value of 16.127 ± 1.6231 in glioma T11TS untreated group. Insignificant changes were observed in grade III (19.491 ± 1.812) and grade IV (29.562 ± 0.965) activated with T11TS in comparison to glioma control values of grade III (19.763 ± 1.676) and grade IV (29.682 ± 1.124) respectively.




**Stastical analysis**

(I) Meningioma (Disease control) – M=2.26, SD=0.1386, p<0.0001

(II) Grade I (Untreated)–M=7.959, SD=1.21, (Treated) M=13.01, SD=2.77, p<0.0001

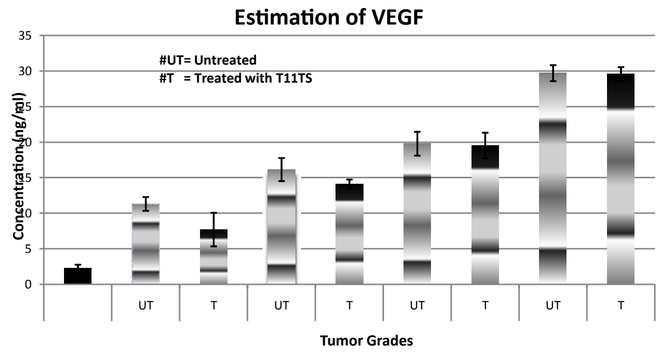
(III) Grade II (Untreated)–M=12.44, SD=0.69, (Treated) M=14.66, SD=1.731, p<0.0001

(IV) Grade III (Untreated)–M=16.17, SD=1.377, (Treated) M=16.33, SD=0.9125, p>0.05

(V) Grade IV (Untreated)–M=17.48, SD=1.06, (Treated) M=17.56, SD=0.90, p>0.05

**Figure 4:** Estimation of phagocytosis by peripheral macrophages against autologous tumor cells by nitro blue tetrazolium reduction assay in all four grades of human glioma. Comparative study were made between untreated and T11TS treated peripheral macrophages in all four grades of glioma. Mean ± SD is based upon six meningioma, twenty each of grade I to 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.




**Stastical analysis**

(I) Meningioma (Disease control) – M=2.283, SD=0.4629, p<0.0001

(II) Grade I (Untreated)-M=11.296, SD=0.9685, (Treated) M=7.685, SD=2.364, p<0.0001

(III) Grade II (Untreated)-M=16.127, SD=1.6231, (Treated) M=14.076, SD=0.6462, p<0.0001

(IV) Grade III (Untreated)-M=19.763, SD=1.676, (Treated) M=19.491, SD=1.812, p>0.05

(V) Grade IV (Untreated)-M=29.682, SD=1.124, (Treated) M=29.562, SD=0.9653, p>0.05

**Figure 5:** Estimation of VEGF expression in peripheral macrophages by Sandwich ELISA method in all four grades of human glioma. Comparative study were made between untreated and T11TS treated peripheral macrophages in all four grades of glioma. Mean ± SD is based upon six meningioma, twenty each of grade I to IV glioma samples.

Meningioma were used as disease control. Significant downregulation 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.

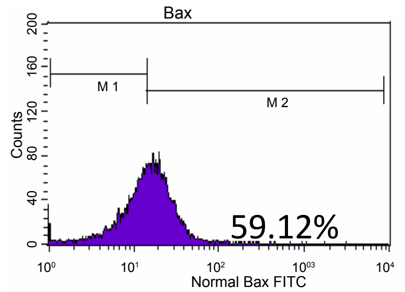



**Figure 6a (i):** Bax expression in meningioma (benign tumor).

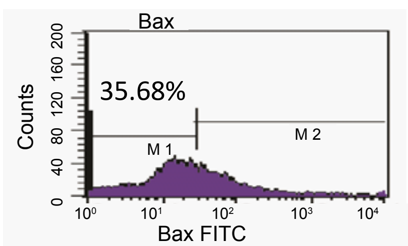



**Figure 6a (ii):** Bax expression in Grade I (disease control) .

## Results of analysis of apoptotic protein Bax by Flocytometry

Estimated value of Bax expression (Figure 6b) in benign (control)

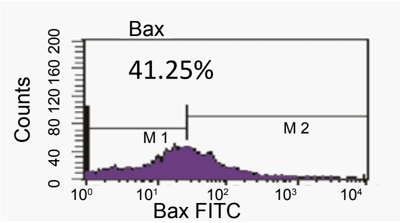



**Figure 6a (iii):** Bax expression in Grade I (T11TS treated).

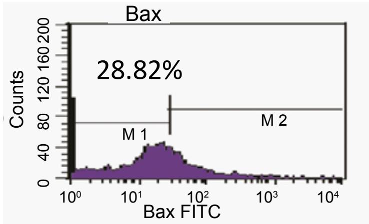



**Figure 6a (iv):** Bax expression in Grade II (T11TS untreated).

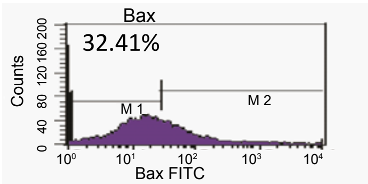



**Figure 6a (v):** Bax expression in Grade II (T11TS treated).

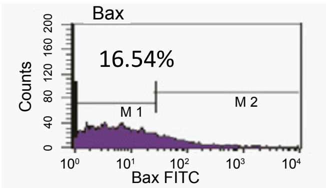



**Figure 6a (vi):** Bax expression in Grade III (T11TS untreated).

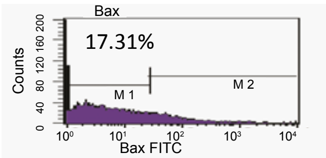



**Figure 6a (vii):** Bax expression in Grade III (T11TS treated).

tumor was 78.66 ± 3.724 (Figure 6a (i)) which down regulates in Grade I & II in T11Ts untreated glioma group to 34.545 ± 6.727 (Figure 6a (ii)) and 29.51 ± 1.33 (Figure 6a (iv)) respectively. However, T11TS activated tumor cell of grade I glioma showed significant ($p<0.0001$) up regulation of this expression value (41.669 ± 8.512) (Figure 6a (iii)). Grade II T11TS activated tumor cell showed significant expression ($p<0.0001$, 32.718 ± 2.78) as compared to untreated Grade II glioma cells (Figure 6a (v)). Expression of Bax in tumor cell of T11TS untreated grade III and IV glioma cells down regulates to 27.278 ± 2.878 (Figure 6a (vi)) and 26.254 ± 2.701 (Figure 6a (viii)) respectively. Even T11TS activated tumor cell showed insignificant change in grade III ($p>0.05$, 27.858 ± 2.802) (Figure 6a (vii)) and IV ($p>0.05$, 26.309 ± 2.611) (Figure 6a (ix)) as compared to the T11TS untreated glioma cells of grades III and IV.

## Results of analysis of $Bcl_2$ expression estimated by flocytometry

Estimated expression (Figure 7b) of $Bcl_2$ in benign (control) tumor was 38.382 ± 6.287 (Figure 7a (i)) which upregulates in T11TS untreated glioma of grade I & II to 57.498 ± 3.677 (Figure 7a (ii)) and

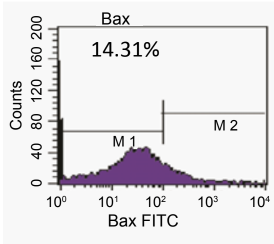



**Figure 6a (viii):** Bax expression in Grade IV (T11TS untreated).

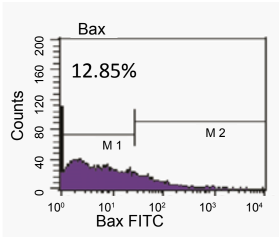
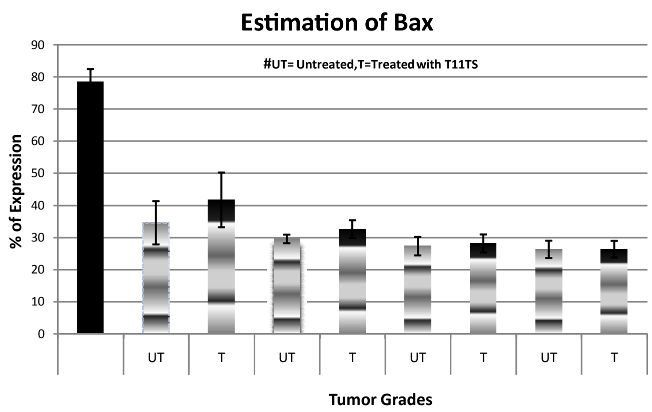



**Figure 6a (ix):** Bax expression in Grade IV (T11TS treated).




**Stastical analysis**

(I) Meningioma (Disease control) – M=78.66, SD=3.724, $p<0.0001$.

(II) Grade I (Untreated)–M=34.545, SD=6. 727, (Treated) M=41.669, SD=8.512, $p<0.0001$.

(III) Grade II (Untreated)–M=29.51, SD=1.33, (Treated) M=32.56, SD=2.78, $p<0.0001$

(IV) Grade III (Untreated)–M=27.278, SD=2.878, (Treated) M=27.12, SD=2.802, $p>0.05$

(V) Grade IV (Untreated)–M=26.254, SD=2.701, (Treated) M=26.309, SD=2.611, $p>0.05$

* M: Mean; SD: Standard Deviation

**Figure 6b:** Estimation of Bax expression in tumor cell by Flocytometry in all four grades of human glioma. Comparative study were made between untreated and T11TS treated peripheral macrophages in all four grades of glioma. Mean ± SD is based upon six meningioma, twenty each of grade I to IV glioma samples.

Meningioma were used as disease control. Significant enhancement in Bax expression 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.

**Citation:** Kumar P, Chatterjee S, Ghosh SN, Acharya S, Kumari A, et al. (2013) T11TS Induced Modulation of Macrophages Associated Cytokines TNF-α, VEGF and Apoptototic Protein Bax, Bcl2 Abrogates Tumor Cells in *In vitro* Grade I,II Human Glioma. J Clin Cell Immunol S1: 006. doi:10.4172/2155-9899.S1-006



60.473 ± 2.834 (Figure 7a (iv)) respectively as compared to T11TS untreated glioma of the same groups. T11TS incubated tumor cell of grade I and II showed significant down regulation ($p<0.0001$, 41.847 ± 5.468) (Figure 7a (iii)) and ($p<0.0001$, 55.157 ± 0.8067) (Figure 7a (v)) respectively. Expression of $Bcl_2$ in tumor cell of grade III (69.690 ± 3.763) (Figure 7a (vi)) and IV (72.788 ± 4.063) (Figure 7a (viii)) which showed insignificant change after T11TS incubation. As compared to T11TS untreated glioma groups there were insignificant changes in grades III and IV of T11TS treated glioma groups (68.302 ± 3.358) (Figure 7a (vii)) and (70.472 ±3.520) (Figure 7a (ix)) respectively.

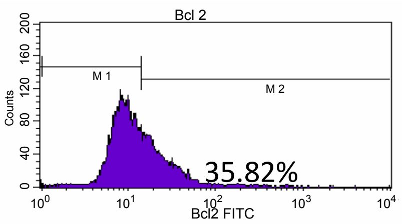



**Figure 7a (i):** Bcl-2 expression in meningioma ((benign tumor).

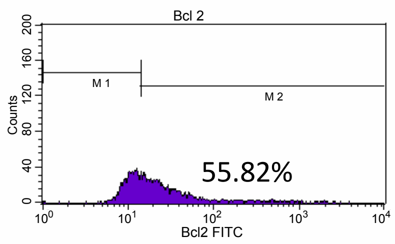



**Figure 7a (ii):** Bcl-2 expression in Grade I ((T11TS untreated).

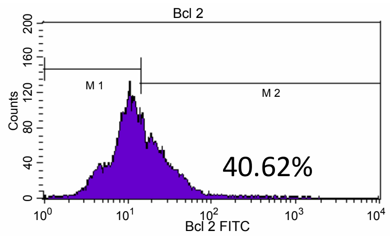



**Figure 7a (iii):** Bcl-2 expression in Grade I (T11TS treated).

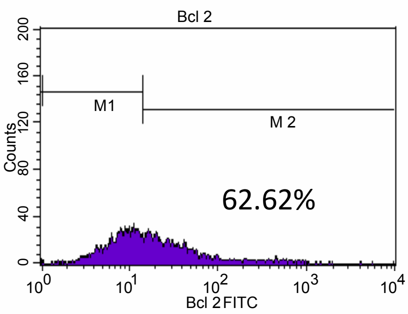



**Figure 7a (iv):** Bcl-2 expression in GradeII ((T11TS untreated).

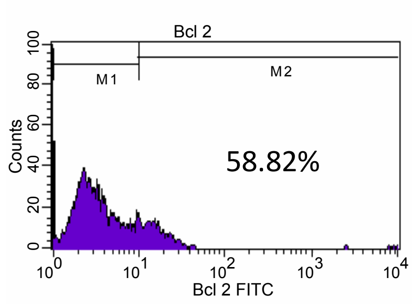



**Figure 7a (v):** Bcl-2 expression in GradeII (T11TS treated).

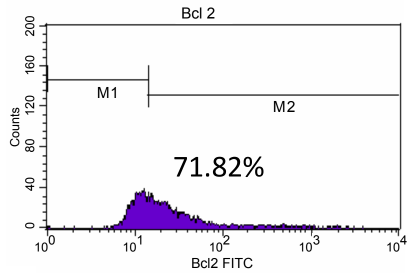



**Figure 7a (vi):** Bcl-2 expression in GradeIII (T11TS untreated).

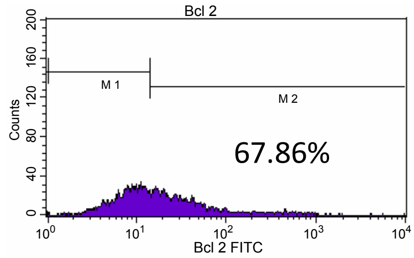



**Figure 7a (vii):** Bcl-2 expression in GradeIII (T11TS treated).

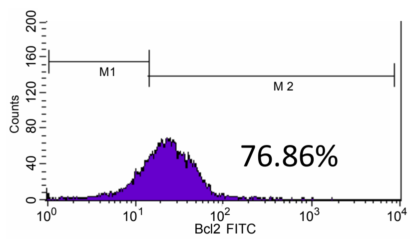



**Figure 7a (viii)** Bcl-2 expression in GradeIV (T11TS untreated).

## Discussion

Tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process [15]. The macrophage is the pivotal member of inflammatory cells within the tumor stroma. Upon activation, macrophages can produce a number of growth stimulators and inhibitors, proteolytic enzymes, inflammatory mediators, and cytokines [16-18]. Therefore, macrophages are regarded as key regulators of the link between inflammation and tumor [19].

We found gradual quantitative increase in expression of macrophage numbers from grade I to grade IV glioma. Macrophages

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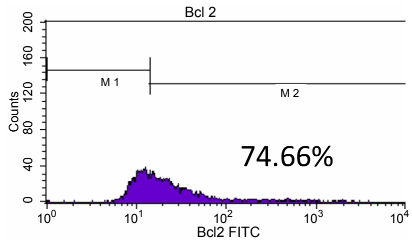
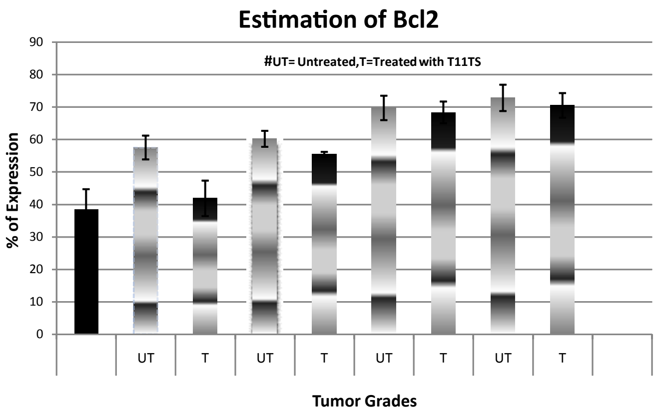



**Figure 7a (ix):** Bcl-2 expression in GradeIV (T11TS treated).




**Stastical analysis**

(I) Meningioma (Disease control) – M=38.382, SD=6.287, p<0.0001

(II) Grade I (Untreated)–M=57.498, SD=3.677(Treated), M=41.847, SD=5.468, p<0.0001

(III) Grade II (Untreated)–M=60.17, SD=2.46, (Treated) M=55.34, SD=0.8067, p<0.0001

(IV) Grade III (Untreated)–M=69.690, SD=3.763, (Treated) M=68.302, SD=3.358, p>0.05.

(V) Grade IV (Untreated)–M=72.788, SD=4.063, (Treated) M=70.472, SD=3.520, p>0.05.

*M: Mean; SD: Standard Deviation

**Figure 7b:** Estimation of Bcl2 expression in tumor cell by Flocytometry in all four grades of human glioma. Comparative study were made between untreated and T11TS treated peripheral macrophages in all four grades of glioma. Mean ± SD is based upon six meningioma, twenty each of grade I to IV glioma samples.

Meningioma were used as disease control. Significant enhancement in Bax expression 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.

activated by T11TS of grade I and II showed significant increase in phagocytosis in comparison to glioma control. Interestingly, no alteration in phagocytosis was visible between T11TS activated and T11TS untreated glioma control macrophages of grade III & IV. This indicates and supports our and others previous finding which states the presence of immunosuppressive micro milieu, mainly consisting of TGF-β, IL-10, PGE215 [20]. Interestingly, cytokines in the brain and other stressors can also generate systemic immunodepression at the monocyte level. Thus mediators of the CNS are implicated in the regulation of immune functions and may play a role in both conditioning the host's response to endogenous or exogenous stimuli and generating a "brain-mediated" immunodepression [21]. This hampers effective immune responses and promotes tumor development by suppressing T cell activation and inducing Th2 responses [18]. Thus, it might have stopped the activation of macrophages by T11TS.

We compared the T11TS induced VEGF expression of macrophages associated VEGF with glioma control in all four grade. It has been suggested that hypoxia, which is present in most tumors, acts as a trigger for macrophage recruitment and expression of increased levels of Hypoxia inducible factor (HIF) by macrophages. The latter in turn stimulates the production of VEGF [22,23]. VEGF secretion in macrophages of grade I showed mild increase in comparison to normal control. However, macrophages of grade II, III & IV showed more increase in VEGF expression. This observation in grade III & IV glioma is in accordance to previous findings which states VEGF as a key component of the angiogenic process and its elevated levels are a common feature of many human tumors and diseased tissues [24,25]. Macrophages incubated with T11TS of grade I glioma showed significant down-regulation of VEGF. But no change was observed between glioma control and T11TS treated macrophage of grade II, III & IV glioma. These findings suggest the antiangiogenic role of T11TS in grade I. No significant change in T11TS treated macrophages with respect to meningioma control in grade II, III & IV glioma is an indication towards presence of immunosuppressive barrier which needs to overcome.

Tumor cell lysis occurs through cell–cell contact or via release of soluble lytic factors, such as TNF-α and NO [26]. Assessment of TNF-α was done to see its intermediate role in cytotoxic activity of macrophages in glioma control and treated tumor of all four grades. TNF-α can induce tumor cell lysis directly, but also upregulates release of IL-1 by macrophages, which has both cytotoxic and growth inhibitory effects on tumor cells [24]. Estimation of TNF-α in macrophages of glioma showed linear increase from grade I to IV. T11TS activated macrophages of grade I and II demonstrated significant enhancement in release of TNF-α. Interestingly, no significant alteration in release of TNF-α was observed between T11TS activated and non T11TS activated macrophages of grade III and IV glioma. TNF-α triggers apoptotic cascade manifested by release of cytochrome c from mitochondria in cytosol [26]. This activity probably seized in treated macrophages of grade III and IV glioma. Induction of TNF-α expression and phagocytic activity in treated macrophages of grade I glioma prompted us to assess any possible proapototic role of T11TS.

T11TS have already been documented as pro apoptotic *in vivo* animal model in our lab [6,27]. Apoptosis is pivotal in reducing the pathological consequences associated with disturbed balance between cell division and cell death [28]. The apoptotic cascade can be initiated via two major pathways, involving either the release of cytochrome c from the mitochondria (mitochondria pathway), or activation of death receptors in response to ligand binding (death receptor pathway) [28-30]. Upon triggering of either pathway, a specific family of cysteine proteases, the caspases, is activated to execute the cell's fate in a programmed fashion, leading to the typical morphologic changes [27]. Resistance to induction of apoptosis, a form of cell death critically regulated by members of the proto-oncogene $Bcl_2$ family, constitutes one major obstacle to radiotherapy and chemotherapy in many cancer cells [31]. The expressions of $Bcl_2$ and its homologue Bcl-xL are elevated in human glioblastoma tumors compared with non-neoplastic glial cells, although, more specifically, an increase in the expression of $Bcl_2$ itself from low-grade astrocytoma (WHO grade II) [32,33]. A significant part of the survival function of $Bcl_2$ in cancer cells seems to reside in its ability to counterbalance the detrimental effects of its proapoptotic counterpart Bax, a multidomain protein that, on its activation by an apoptotic stimulus, acquires the ability to directly perturb mitochondrial membrane permeability and to promote the release of apoptogenic proteins from this organelle [31-34].

To find out any possible proapoptotic role of TIITS, we compared the ratio of apoptotic protein Bax and $Bcl_2$ in all four grades of glioma. The ratio between anti- and pro-apoptotic proteins is said to be a determinant for tissue homeostasis, essentially, by influencing the sensitivity of cells to inducers of apoptosis [7,35]. *In vitro* studies in tumoral glial or neuronal cell lines have shown that increases in $Bcl_2$ protein induced a greater resistance to chemotherapeutic drug and radiotherapy [36]. On the other hand, over expression of bax sensitized cells to apoptosis induced by various agents [33]. We found low expression of $Bcl_2$ in meningioma control. But it increases in linear fashion from Grade I to Grade IV glioma. T11TS treated tumor cell of grade I and II glioma showed significant down regulation of $Bcl_2$. There was no change in $Bcl_2$ expression observed between meningioma control and T11TS treated tumor cell of grade III and IV glioma. Estimation of Bax expression showed higher expression in disease control tumor cell of grade I & II in comparison to grade III & IV. Treated with T11TS, tumor cell of grade I and II showed significant enhancement in the expression in comparison to disease control. No such alteration was observed in treated tumor cell of grade III and IV glioma. The above observation is a hint for the possible role of T11TS as apoptotic inducer in grade I and 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 immunepotentiation in the first two doses the highest being in the second dose. The third dose brought back this level to the normal level [5-8,27]. But in the present in *in vitro* study of human glioma patients a single dose which was used could not probably booster up the immunepotentiation in grades III and IV. Being inspired by the animal studies conducted by our group with the administration of T11TS in glioma bearing rats [5-8,27]. Clinical trials will be proposed for three doses of intravenous T11TS administration in the glioma patients. The outcome of the study is a prerequisite for clinical trial, which would be pivotal in pushing this molecule TIITS into pharmaceutical domain.

## Highlight of Study

- Increased phagocytic activity in T11TS treated macrophages of Grade I & II glioma
- Significant enhancement of TNF-α in T11TS treated macrophages of grade I & II glioma
- Downregulation of VEGF in T11TS treated macrophages of grade I & II glioma
- T11TS induced modulation of protein Bax and Bcl2 in tumor cell of grade I & II glioma

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