T11 target structure exerts effector function by activating immune cells in CNS against glioma where cytokine modulation provide favorable microenvironment

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Glycoprotein T 11 target structure (T11TS), derived from sheep erythrocyte membrane, directly interacts with T cells to activate them to enter in the brain. When untreated, glioma exerts an immune-suppressive environment in its vicinity by secreting prostaglandin E2 (PGE₂), IL-10, tumor growth factor β, gangliosides etc. to dampen the immune attack. But exogenous administration of T11TS reverses the situation to pro-inflammatory immune active state by expressing enhanced IL-12 and tumor necrosis factor α (TNF-α) production and suppression of IL-4 and IL-10 levels. The T11TS activated lymphocytic accumulation along the capillary endothelium in brain and their penetration in the matrix was evident from histological sections. IL-6 with TNF-α facilitates leukocyte migration to glioma site to exert cytotoxic effector function. Brain infiltrated lymphocytes offer cytotoxic proximity to neoplastic glial cells, which lead them to apoptosis. In the Th1 dominated microenvironment microglial cells was found with enhanced phagocytic functions. Initially infiltrated lymphocytes with microglia showed increased production of TNF-α, interferon γ (IFN-γ) to facilitate their effector actions. Repeated dosing of T11TS shows glioma abrogation in rat model, but also a resurgence of anti-inflammatory cytokine environment found with increased IL-4, IL-10 and decreased IL-12, IL-6, TNF-α. This is a unique homeostatic regulation of total immune system after T11TS mediated carnage of glioma. The resultant balance of cytokines between interacting glioma cells, T cells and microglia in T11TS induced condition determines the success of its immunotherapeutic effect in glioma.

Keywords: Cytokines, Homeostasis, Lymphocytes, Microglia, Pro-inflammatory, T11TS

Glioma accounts for more than half of the primary brain tumor. The unique anatomy and biology of glioma, invasive nature, expression of radio and chemo resistant genes, multi drug resistance (MDR) family proteins, hurdles of blood-brain-barrier (BBB) render surgery, radiotherapy and chemotherapeutic treatments still mostly ineffective. As a result the higher grade (III and IV) gliomas show the life expectancy of maximum 9-18 months¹,². So a supplementary modality has been attempted to contest with this deadly killer by using the immune defense arsenal of brain.

T 11 target structure (T11TS) has been isolated from sheep erythrocyte membrane and administered in chemical carcinogen induced rat bearing glioma. It reactivates the glioma suppressed cell mediated immune system, both systemic and intracranial³. T11TS ligands with CD2 receptor present on leukocytes and assist to form the immunological synapse to recognize glioma antigens, upregulate co-stimulatory molecules and exert effector functions⁴. Increase in cytotoxicity of brain infiltrated lymphocytes and increase of oxidative burst and release of nitric oxide (NO) by both macrophage and intracranial microglia after T11TS administration have been detected⁵. The resulting killing of glioma cells by apoptosis, cell cycle arrest and controlled proliferation of microglia and infiltrated lymphocytes and related molecular mechanistic pathways have now being exposed to a large extent⁶,⁷. An intricate communication is required to effectively build this well-controlled immune attack against glioma in central nervous system (CNS). Cytokines are those intracellular signaling mediators those correspond and thereby control the overall

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direction of immune functions to restrict and destroy glioma. However, one of the major hurdles in this process is the immunosuppressive micro-environment contributed by the glioma cell by producing several suppressive factors, along with the production of anti-inflammatory cytokines like IL-10, tumor growth factor β (TGF-β) etc\(^9\). To make a conducive platform for the effective functioning of the immunocytes, a shift of balance towards the proinflammatory cytokines is necessary. The infiltrated activated lymphocytes from periphery is a potential source of the cytokines, but most importantly the resident microglia act as a major source and target of different cytokines and in turn capable of regulating the micro-environment\(^9\). How T11TS directs the cellular functions and fates by controlling the cytokine network among interacting cells has been investigated in the present study. Defining the balanced interplay of cytokines between these cells is essential to predict the direction of immune action in glioma environment. This will improve the understanding of T11TS action against glioma and its feasibility as a possible therapeutic mediator.

**Materials and Methods**

**Isolation of T11TS**—T 11 target structure (T11TS)/sheep form of leukocyte function antigen 3 (SLFA3) was isolated from sheep erythrocyte (SRBC) membrane as described previously\(^10,11\). Briefly, SRBC was trypsinized, treated with TCA and neutralized. The glycoprotein was separated by ion exchange chromatography on a DEAE-Cellulose column (LPLC system) and eluted with a five-chambered gradient system. Elute fraction III was selected as the fraction of choice by complete rosette inhibition. The elute fraction III from LPLC was further subjected to ion exchange chromatography in HPLC system to purify T11TS more precisely. The fractions obtained from HPLC were then analyzed by ELISA by using anti-Human LFA-3 (having cross-reactivity with SLFA-3). The activity curve obtained from ELISA will detect the fraction of choice from the elutes containing T11TS in a definitive manner.

**Animals**—Healthy newborn (5 days old) Druckray rats supplied by Central Drug Laboratory, Kolkata, India and maintained in the laboratory were used. Chemical carcinogen, N-N′-ethyl nitrosourea (ENU) was injected (80 mg/kg body weight) intraperitoneally (ip) to 7 days old rats\(^3,10\). Animals were grouped as follows: (i) normal control [N], (ii) 5 months old ENU administered animals [E] (optimal period for glioma development), (iii) ENU animals treated with 1\(^{\text{st}}\) dose of T11TS [ET1], (iv) 1\(^{\text{st}}\) and 2\(^{\text{nd}}\) dose of T11TS [ET2], (v) 1\(^{\text{st}}\), 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) dose of T11TS [ET3]. Rats (5-6 months old) averaging 125 g body weight, received 1ml of T11TS (0.4 mg/kg) intraperitoneally (ip).

**Histological examinations**—Specific portions of brain tissue from different groups of animals were collected after dissecting the skulls and were prepared for routine histological studies. Collected tissues were fixed in 10% formal-buffer overnight, dehydrated and finally embedded in paraffin through histokinet processing. Tissue sections were cut at 5 µm thickness and finally stained with routine haematoxyline/eosine.

**Isolation of intracranial microglia**—The intracranial immune reactive microglial cells were also isolated from each animal group as described previously\(^5,12\). Briefly, single cell suspension of whole brain was incubated with collagenase (250 µg/ml) and DNaseI (250 µg/ml) (Sigma, USA) at 37°C for 45 min each. Then cell suspensions were layered on 30/60% Percoll gradients at 1000 g for 25 min and brain mononuclear cells were collected from the interface. The mononuclear cell suspensions were then laid on a plastic petri dish (Corning, USA), incubated for 30 min in a CO\(_2\) incubator, adherent cells washed out with PBS-EDTA and layered on a Nycodenz gradient (1.068 specific gravity) (Nycomed, Oslo, Norway) to centrifuge for 20 min at 400 g. Cells removed from the interface washed thrice with PBS and finally resuspended in RPMI-1640 media (Sigma, USA). The microglial cells were characterized depending on their surface receptor expression (CD11b\(^+,\) MHC class II\(^+,\) CD4\(^+,\) GFAP\(^+)\) as described earlier\(^5\) and subjected to assess surface receptor expression.

**Isolation of brain infiltrating lymphocytes (BIL)**—To prepare the single cell suspension of brain, the whole brain was minced and passed through a 80 gauge wire mesh and then the cells were incubated with collagenase I and IV (0.05 mg/ml), hyaluronidase (0.025 mg/ml), soyabin trypsin inhibitor (0.2 TIU/ml), DNase I (0.01 mg/ml) dissolved in PBS for 15 min at 37°C\(^13\). The cell suspension (~ 5 × 10\(^7\) cells/ml) was then applied over a differential Percoll density gradient of 60/50/40/30% and subjected to centrifuge for 30 min. A thin band was observed in between the interface of 40/30% gradient of Percoll. This band was collected
carefully and washed thrice with PBS and resuspended in RPMI-1640 for further functional analysis. These isolated cells from all the experimental animal groups were characterized by anti CD3 monoclonal antibody to ascertain the purity of the lymphocytes in the isolated cell populations.

Analysis of intracellular cytokine profile (TNF-α, IFN-γ, IL-4, IL-6, IL-10 and IL-12) of microglia, BIL and glioma cells by flowcytometry—Respective cell populations of \(-1 \times 10^7\) cells/ml were treated with paraformaldehyde (3% in PBS) at 4°C for an hour. Then the cell suspension was washed and collected to permeabilize the sample with Triton-X (0.5%) for 1 hr at 4°C. After washing, the treated cell preparation was collected for mAb tagging against respective cytokines. mAb of TNF-α, IFN-γ, IL-4, IL-6, IL-10 and IL-12 (BD PharMingen, USA) and FITC or PE conjugated secondary antibody (BD PharMingen, USA) were used. Glioma, brain infiltrating lymphocytes (BIL) and microglia at a concentration of 1-2 \(\times 10^7\) cells/ml were first tagged with 50 µl of diluted (1:100) mAb against cytokines. After incubating at 4°C for 30 min in dark, the cells were washed twice with PBS, tagged with secondary antibody and incubated at 4°C for 30 min in dark. All the cytokines were analyzed separately except IL-10 and IL-12, when dual tagged cells (IL-10 with PE conjugate and IL-12 with FITC conjugate) were prepared. The cells were subjected to flowcytometric analysis in ‘FACS Caliber’ Flowcytometer of BD Bioscience, USA (Argon Laser) by using Cell Quest Software. A total of 10,000 events were acquired and analyzed to obtain the results and respective isotype controls were maintained.

Results

Events of cellular interactions was depicted in the tissue sections showing lymphocytes invading, attacking and killing neoplastic cells when aided with T11TS

The normal assembly of neurons, astrocytes and oligodendrocytes mostly was observed in the brain parenchyma with adequate intracellular spacing (Fig. 1a). But this cellular assembly was destroyed in ENU induced rat of 5 months. It showed dense assemblage of oligodendrocytic cells minimizing the intracellular spacing and packed around proliferating centers in a honeycomb like fashion (Fig. 1b) indicating the incidence of oligodendroglia by chemo-carcinogen ENU.

The interesting feature observed after 1st and 2nd dose of T11TS administration was the movement of lymphocytes and their margination in the brain capillary, tethering and then penetration through the blood brain barrier (BBB) (Fig. 1c and 1d). These penetrated lymphocytes navigate to the proliferating oligodendroglial cells. One oligodendrocyte was attacked by three lymphocytes (Fig. 1e) and tissue portions observed in Fig. 1f and 1g demonstrated the rupture and damage of oligodendroglial neoplastic cells due to the ‘kiss of death’ offered by cytotoxic lymphocytes. Lastly in Fig. 1h, the diffused cellular architecture with nuclear blebbing, breakage of nuclear structure and disappearance of cells show the signs of apoptotic clearance of neoplastic cells. These are observed predominantly in brain parenchyma after the 3rd dose of T11TS.

Cytokines explained the cellular activities - nature of cytokine dominance determined the direction of immune reaction and its effectiveness to reduce glioma

Intracellular TNF-α cytokine profile of glioma cells, microglia and BIL—Cytokine TNF-α was found nearly similar in normal (31.28% ± 5.5) and glioma state (33.37% ± 7.2) in brain tissue cells/glioma cells isolated from CNS, and showed no significant changes during treatment phase. But for microglia, a huge burst of TNF-α production was observed with 93.02% ± 6.6 expression in ENU induced glioma state (E). During T11TS mediated treatment phase it retreated to 54.8% ± 9.8 in 1st dose, slightly uplifted in 2nd dose to 70.03% ± 10.2 and then held back to 40.38% ± 6.6 in the final dose (ET3). Brain infiltrating lymphocytes showed merely normal level of the cytokine production till the treatment began, but a sudden expansion of TNF-α production was found in 2nd (82.25% ± 10.2) and last (79.55% ± 8.9) dose of T11TS (Fig. 2).

Intracellular IFN-γ cytokine profile of glioma cells, microglia and BIL—For normal brain tissue cells IFN-γ production was minimal (2.48% ± 0.7), but in glioma it increased to 9.75% ± 1.6 (E). After T11TS administration it further increased to 13.23% ± 2.1 and 13.88% ± 1.9 in two respective doses. In final dose the level came down to 7.65% ± 1.1 (ET3). For microglia, the cells slightly increased the production of this cytokine (23.95% ± 4.66) in glioma (E) that gradually neutralized around the normal level by T11TS treatment. Infiltrated lymphocytes showed two-fold increase of this cytokine intracellular assembly to 34.17% ± 4.8 in ENU induced...
Fig. 1 — (a) normal brain tissue shows few astrocytes, oligodendrocytes and neurons scattered in the tissue matrix with considerable intracellular spacing. (b) but ENU induced oligodendroglioma shows dense cellular assembly without intracellular spacing consisting of mitotic figures and typical honeycomb like appearance. (c) With T11TS administration lymphocyte margination has been observed on the endothelium in the blood vessels in CNS, and (d) the characteristic penetration and migration of lymphocytes from capillary lumen in observed during 1st and 2nd dose of T11TS administration. In (e), (f), (g) and (h) the cytotoxic effector function of lymphocytes is observed when they attaches to the neoplastic cells offering the ‘kiss of death’ and the cells showing degenerative changes with deformed cytoplasm, disrupted cellular architecture and nuclear blabbing and their diffused appearance. These are the tissue pictures featuring 2nd and 3rd dose of T11TS treatment showing carnage of glioma.
glioma (E) than normal (16.72% ± 2.5). Though T11TS decreased the level to considerable extent (ET1= 20.4% ± 2.8), it never reached to normal (Fig. 3).

Intracellular IL-10 and IL-12 Cytokine Profile of Glioma Cells, Microglia and BIL—IL-10 production in the glioma cells was found highest in neoplastic condition (E=5.98% ± 1.1) and with T11TS application gradually decreased to 1.4% ± 0.3 in the final dose (ET3) as depicted in Fig. 4a. However, IL-12 production gradually improved from normal (1.45% ± 0.21) and ENU condition (3.1% ± 0.76) with the application of T11TS and reached its highest level to 7.3% ± 0.78 in the 3rd dose. But the double positive cell population producing both IL-10 and IL-12, after initial increase in 1st dose of T11TS (9.41% ± 1.2), again decreased in last two doses (ET2=6.6% ± 0.7 and ET3=6.1% ± 0.92) (Fig. 4a). In case of microglial cells, IL-10 producing population showed very low level of activity with slight increase in tumor condition (2.4% ± 0.52), which was decreased by T11TS application. IL-12 producing population showed decrease of production in tumor bearing condition (3.6% ± 0.8) than normal (5.98% ± 0.95), which was again recovered with T11TS (5.99% ± 1.02 in 2nd dose) and final dose limit IL-12 production to 3.97% ± 0.69. Dual positive population of microglia produce both the cytokines in higher amount in ENU condition (14.5% ± 1.2) compared to normal (5.82% ± 0.66) and reached its highest in 2nd dose of T11TS (25.8% ± 2.1), but sharply decreased in final dose (9.85% ± 0.7) (Fig. 4b). The low level of production of IL-10 was found in brain infiltrating lymphocytes, which increased in last two doses (2.21% ± 0.41 and 2.15% ± 0.56 respectively). In double positive population, a static level of production was observed up to 1st dose, which sharply increased in 2nd dose (18.4% ± 2.22)
and decreased in 3rd dose (11.13% ± 1.3) (Fig. 4c). Final dose of T11TS had shown a limiting property in cytokine production and balance in all cases.

**Intracellular IL-4 Cytokine Profile of Glioma Cells, Microglia and BIL**—In normal brain tissue the overall production of IL-4 was only 1.1 ± 0.3%, which in glioma condition sharply shot up to 42.5 ± 5.2%. But with application of T11TS it dropped down to basal level (1.4 ± 0.24) and more or less this level of IL-4 expression continued for the next two doses (Fig. 5). Microglia showed 11 ± 1.85% intracellular production of IL-4 and that increased in glioma. But T11TS application significantly decreased its expression to 9.46 ± 2%, and further reduced significantly to 3.9 ± 0.86%. The final T11TS dose again increased IL-4 production significantly to 20.4 ± 3.1% probably to regulate immune activity to homeostasis (Fig. 5). For infiltrating lymphocytes (BIL), the normal production of IL-4 was 6.1 ± 1.04%, which increased to 11.2 ± 2.3% in glioma. But with T11TS it sharply dropped to only 0.58 ± 0.1% and then increased in the last two doses to 5.69 ± 1.1% and 7.9 ± 1.21% respectively (Fig. 5).

**Intracellular IL-6 Cytokine Profile of Glioma Cells, Microglia and BIL**—A wide increase of IL-6 production was found in glioma cells with the 2nd dose of T11TS (41.67% ± 2.86) and decreased sharply to 1.27% ± 0.51 in the final dose. However, microglia showed increase of IL-6 production in ENU condition (7.82% ± 1.4), which decreased remarkably in 1st dose (1.5% ± 0.4) and then gradually increased to near normal value in 3rd dose (4.74% ± 0.69). In lymphocytes, the production of this cytokines was quite high, and gradually increased from normal to ENU and 1st dose of T11TS, reached its maximum value in 2nd dose (20.62% ± 3.1) and dropped to lowest level (5.81% ± 1.3) in the final dose (Fig. 6).
Fig. 4 — (a) Intracytoplasmic production of cytokines IL-10 and IL-12 in glioma cells showed the increased IL-10 in ENU condition which decreased gradually with T11TS treatment, when IL-12 production increased in consecutive doses. But cells producing dual cytokines were also decreasing in final two doses of T11TS. (b) Production of IL-10 slightly increased in ENU animals and decreased in initial doses of T11TS, but again increased slightly in last doses in microglial cells. But IL-12 production, which was decreased in tumorigenic state, maximally increased in 2nd T11TS dose with the double positive cell population, however dampened in final dose. (c) Brain infiltrating lymphocytes (BIL) showed high IL-12 production with T11TS treatment viz. in 2nd dose, whereas it was regulated in 3rd dose. However IL-10 production, which was decreased after 1st dose of T11TS gradually, increased again in final dose.
Fig. 5 — Glioma cells secret maximum level of IL-4 in full grown glioma condition, but drops to near normal level with the treatment of T11TS. Microglia shows decrease of IL-4 in 2nd dose which increases in last dose, BIL also shows decrease with T11TS administration.

Discussion

In previous studies the antineoplastic action of T11TS was elucidated in dose and time dependent fashion. Findings elaborated various cellular and molecular procedures of its action to clear glioma mass. Many of those events have been visualized in the histological elaborations furnished in the present work. These immune effector actions have been verified in respect to the cytokine milieu.

T11TS application initiated lymphocyte activation and anchorage to vascular endothelium of capillary lumen (Fig. 1c). The penetration occurs with repeated doses (Fig. 1d) when lymphocytes entered in brain parenchyma. Previous studies supported this visualization with increased record of both CD4+ and CD8+ lymphocytes in brain. The increased level of TNFα and IL-6 in brain microenvironment and immunocytes facilitate this migration. The
leukocyte recruiting action of TNFα in repeated T11TS doses increased their level (Fig. 2). On the contrary, repeated TNFα production by microglia in glioma prompted their accumulation in glioma site. Also the capacity of providing death signals by TNFα18, their increase in final doses and death of glioma cells by lymphocytes (Fig. 1e-h) indicated the clear correlation. IL-6 in combination with TNFα possess wide functional range including adhesion molecule expression, leukocyte movement, NO production, B cell differentiation etc16,17,19. High level of IL-6 in last two doses in glioma microenvironment possibly enhanced NO production in microglia and in turn, facilitated leukocyte entry to destroy neoplastic cells. The increased NO production of microglia, its increase in phagocytic activity and CD11b expression after T11TS administration had already been documented5.

Microglia acts as a major cytokine source in brain20. But the balance of local cytokine environment is determined by interacting cells. IFNγ, in association with IL-12, controls both innate and adaptive arms of immune attack; enhance MHC upregulation and co-activating molecule expression; also helps in glioma antigen recognition and CTL functions21,22. But the opposite actions of IL-10 and IL-4 dampen the immune attack and intensifies the pre-existing immune suppressive glioma environment23,24,8. It was found that more IL-10 production by lymphocytes and microglia during glioma was initially decreased by T11TS administration, but regained it in final dose (Fig. 4). But the opposing IL-12 was produced maximum in 2nd dose and then overall decrease was observed in final dose. It clearly demonstrated that the initial immune suppressive environment in glioma had shifted towards pro-inflammation with initial doses of T11TS, highest in 2nd dose. But the balance swung to anti-inflammation in final dose. The same trend was found previously with the receptor modulations of microglia8. IFNγ showed lesser variation than other cytokines, but strikingly microglia showed a higher production of IFNγ in glioma (Fig. 3). The increased TNFα and IL-6 helped microglial accumulation in glioma vicinity and this increased IFNγ would signify their effort of glioma antigen presentation25. But lymphocytes were then crippled in immunosuppressive glioma environment6. High level of IL-4 in glioma also facilitated the process (Fig. 5). T11TS induction altered the situation by activating and pushing lymphocytes to glioma, which produced pro-inflammatory cytokines TNFα, IFNγ etc. and effectively deliver the effector functions. With application of T11TS a sharp decrease of IL-4 was observed. But in final dose microglia showed an increase in IL-4 production, which was intended to restrain the active phase of immune action to homeostasis.

From this study, the complex cytokine network interplay has been found between interacting brain infiltrated lymphocytes, microglia and glioma cells. But the core of this network is to introduce or provide an inflammatory environment overcoming the immunosuppressing glioma condition. Initially T11TS mediated signaling increases the overall balance towards the production of proinflammatory cytokines and activates the lymphocytes and microglia, but after final dose it helps to maintain the homeostasis by augmenting the anti-inflammatory mediators. This resultant effect proves T11TS as a good therapeutic mediator that can prevent the damage of the system by controlling the immune activation state after the destruction of glioma. The histological findings strengthen these findings of cellular activities of the lymphocytes and microglia during glioma reduction. Also infiltrations of lymphocytes, microglial activation, their immune functions, glioma cell apoptosis events are being realized in connection with their cytokine action6,11. Thus the findings provide another explanatory note about the therapeutic efficacy of T11TS against glioma.

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Reference


