Glycoconjugate Journal 20, 515–523, 2004

Documentation of immune profile of microglia through cell surface marker study in glioma model primed by a novel cell surface glycopeptide T11TS/SLFA-3

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Statement of the Problem: **The sheep erythrocyte membrane glycoprotein T11TS/SLFA-3 can form a ligand-receptor complex with CD2 present on immunocyte and exert stimuli for activation and proliferation. Regression of brain tumor with the application of T11TS indicates the probable role of microglia, the chief immunomodulatory cell within the brain compartment. In the present study microglial activation and immunophenotypic modulation were assessed in T11TS treated brain tumor-bearing animal models. Rat glioma models induced by chemical carcinogen ENU were treated with three consecutive doses of T11TS. Microglial cells from brain were isolated and assessed through E-rosette formation, SEM and FACS for CD2, MHC class II, CD25, and CD4. The preliminary indication of presence of CD2 on microglia through E-rosette formation was confirmed by SEM and FACS. MHC class II and CD2 single and double positive subpopulations exist, and their expression is also modulated in different doses of T11TS. A general trend of highest receptor saturation and microglial activation, measured through the activation marker CD25 and CD4 expression, was observed in 2nd dose of T11TS administration, which was then dampened via a complex immune feedback mechanism in the 3rd dose.**
Published in 2004.

Keywords: **T11TS, brain tumor, microglia, CD2, CD25, immunophenotyping**

Introduction

The T11 sheep erythrocytes binding glycoprotein, also known as CD2/E-rosette receptor/LFA-2 (molecular weight 50 Kd), is expressed throughout human T-lymphocyte ontogeny [1]. CD-2, a member of immunoglobulin supergene family, is a single chain type-I transmembrane molecule consisting of 351 amino acids, two Ig like domains and three potential glycosylation sites in the extracellular region. This CD2/T11/E-rosette receptor forms erythrocyte rosetting with the sheep erythrocyte (SRBC) through a receptor-ligand complex with transmembrane glycopeptide T11 target structure (T11TS), also known as Sheep form of LFA-3 (SLFA-3) or S-CD58, present on the SRBC cell surface [2]. This T11TS/SLFA3 also belongs to immunoglobulin supergene family members estimated to have a carbohydrate content of nearly two-third of the glycoprotein. Galactose, sialic acid, and acetylgalactosamine are major carbohydrate contents whereas major amino acids are lysine and glycine [3]. Electrostatic potential maps reveal the binding surface of T11TS/SLFA3 with CD2 on immunocytes to be nearly identical with human CD58 and supports a 'hand-shake' model of CD2-CD58 interaction [4]. This ligand receptor complex plays a critical role in alternative antigen independent polyclonal T-cell activation, proliferation, and lymphokine secretion through an antigen independent cytolytic machinery [5,6]. Our previous studies demonstrated that T11TS/SLFA-3, isolated from SRBC membrane, when applied on experimentally induced brain tumor-bearing rats exhibits tumor regression by augmenting cell mediated immune (CMI) reactivity primarily at the peripheral level and subsequently activating immunocompetent cells to infiltrate through the blood-brain-barrier (BBB) into the brain [7].

Brain, the immune privileged compartment, allows only the entry of the activated lymphocytes. Non-specifically activated

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lymphocytes by T11TS/SLFA3 enter the brain parenchyma crossing the BBB and interact with the intrinsic immune effector cells of brain, the microglia (CR3/CD11b^{+} and CD45^{low}) [8]. Recent evidences demonstrated that microglia become activated to perform several immune functions including induction of inflammation, cytotoxicity, regulation of T-cell response, and antigen presentation in response to various neuro-pathogenic conditions [9]. In addition to lymphocytes, significant microglial infiltration is observed in the site of brain tumor development [10]. Reactive microgliosis and proliferation is directly dependent upon the dialogue between microglia and T cells, which in turn regulates the Th1/Th2 response within the brain compartment [11,12]. Therefore, activation of microglia directly or indirectly via T cells by T11TS/SLFA-3 provides a better option to regulate tumor growth within the brain compartment. In the present study microglial cell surface molecular expression, as modulated with T11TS administration in animal models with or without brain tumors, were assessed through flowcytometry. Activation of microglial cells was documented by modulation of CD25 markers and confirmed by CD4 expression in the different groups of microglial cells. Since, T11TS was found to modulate microglial activity, the presence of its ligand CD2 was searched for on the cell surface. Presence of rosette formation with SRBC revealing the bridges formed between the cell membranes of both cells were determined under scanning electron microscopy. Moreover, attempts have been made to investigate whether the differentiation of microglia into a functional APC is achieved in tumor-bearing animals through MHC class II expression.

Materials and methods

Preparation of glycopeptide T11TS/SLFA-3

The glycopeptide T11TS/SLFA-3 was isolated from sheep red blood cells (SRBC) as described previously [7]. Briefly, trypsin-digested and trichloroacetic acid (TCA) treated SRBC were subjected to ion-exchange chromatography on a DEAE-Cellulose column previously equilibrated with 0.05 M formate buffer, pH 6.8. The acidic glycopeptide was then eluted with a five chambered gradient system containing (1) water, (2) 0.05 M, (3) 0.2 M, (4) 0.4 M formic acid, and (5) 0.4 M formic acid in 0.3 M sodium chloride. The third elute fraction contained the desired glycopeptide T11TS as determined previously [7]. The glycopeptide having glycosylated chains was confirmed by staining the SDS-PAGE with PAS stain.

Induction of brain tumors

N′-N′-ethylnitrosourea (ENU) was freshly prepared by desolving 10 mg/ml in sterile saline and adjusted the pH to 4.5 with crystalline ascorbic acid. ENU was injected into newborn (3–5 days old) rat intraperitonially (i.p) with an acute dose of 80 mg/Kg body weight [13].

Animals

Healthy newborn Druckray rats of both sexes, 2–3 days old, supplied by Central Drug Laboratory, Kolkata, India, and subsequently maintained in our laboratory were used as experimental animals. They were grouped as follows: (1) Normal control [N], (2) 7 months old ENU-administered animals [E] (optimal period of glioma development), (3) ENU animals treated with 1st dose of T11TS [ET1], (4) ENU animals treated with 1st and 2nd doses of T11TS [ET2], (5) ENU animals treated with 1st, 2nd and 3rd doses of T11TS [ET3]. An interval of 6 days was maintained between the consecutive doses of T11TS administration.

Isolation of microglial cells

Single cell suspension of the whole brain was passed through an 80-gauge wire mesh and digested by incubation with collagenase (250 μg/ml) and DNaseI(250 μg/ml) (Sigma,USA) at 37°C for 45 min each. The resulting cell suspensions were layered on 30/60% Percoll gradients at 1000 × g for 25 min [14] and brain mononuclear cells were collected from the interface. The mononuclear cell suspensions were then laid on a plastic petridish (Corning, USA), and incubated for 30 min in a CO_2 incubator. The adherent cells were washed out with PBS-EDTA and laid on a Nycodenz gradient (1.068 specific gravity) (Nycomed, Oslo, Norway) for 20 min at 400 g for the density gradient centrifugation. The cells were removed from the interface and washed thrice with PBS [15]. Isolated microglia cells were characterized immunophenotypically with CD11b^{high}, GFAP$^{(-)}$, CD4$^{(+)}$, RT1B$^{(+)}$ [16].

E-rosetting of microglia

Microglial cells isolated from the brain of different groups of animals were assayed for their E-rosette formation capacity. An aliquot of cells (3–4 × 10^6 cells per 0.25 ml) was mixed with 0.25 ml of 1% (PCV/Vol) SRBC and incubated at 37°C for 15 min. Following centrifugation for 5 min at 400 g, the preparation was kept at 4°C overnight. Number of rosettes formed were counted per 200 Microglial cells and expressed as rosette % [17].

Scanning electron microscopy (SEM) of E-rosette forming microglia

E-rosette-forming microglial cell preparations were processed for SEM by glutaraldehyde fixation, serial dehydration in graded alcohol and finally in acetone. The preparations were critical-point dried and then coated with gold for 2 min (35 mA, 1 KV) in a Diode sputtering system and viewed under Jeol JSM 5200 Scanning Electron Microscope at 25 KV beam voltage. Each specimen was scanned and photographed at direct magnification at 1500× to 7500×.

Receptor modulation of microglial cells by fluorescence activated cell sorting

The cell surface receptor modulations from different groups of animals were assayed by the following monoclonal antibodies: (a) FITC-labeled anti-rat CD-2, (b) PE labeled anti-rat MHC class-II, (c) FITC labeled anti-rat CD-25 and (d) FITC-labeled anti-rat CD-4 (Becton Dickinson, USA). The cells were prepared and analysed on FACS Caliber (Argon Laser, excitation at 488 nm, 515-band pan filter) (Becton Dickinson, USA) by using Cell Quest Software (Becton Dickinson, USA). Adjacent isotype controls were also maintained in all groups.

Results

E-rosette formation of microglia

Microglia separated from normal [N], tumor-bearing [E], and treated [ET1, ET2 and ET3] animals showed different degrees of rosette formation when counted as rosettes per 200 microglia. Significant increase of rosette forming capacity (% rosette) was significantly higher, compared to normal and ENU counter parts, in first and second doses of T11TS with depression in ET3 group (Table 1).

Scanning electron microscopy (SEM) of E-rosette forming microglia

SEM study of E-rosette-forming microglia revealed interesting features. Microglia form rosettes and super-rosettes with multiple SRBCs anchored with the cell membrane. Definite cytoplasmic projections of microglia and attachment with the SRBC indicate the intermembranous anchorage system between microglia and SRBC through CD2-T11TS interaction (Figure 1).

Receptor modulation of microglial cells assessed by FACS

CD2 and MHC class II

FACS analysis showed the expression and modulation of CD2 and MHC class II receptors on the microglial cells before and after T11TS administration. The results indicate the existence of different subtypes of microglia according to their phenotypic distribution. In all groups double positive CD2-MHC class II microglial subpopulation was found with differential expressions. The double positive subpopulation in the normal [N] group (16.16%) was increased in tumorogenic condition [E] (31.66%), but slightly decreased in the first dose of T11TS administration [ET1] (30.50%). However, with the administration of a second dose of T11TS [ET2], the CD2-MHC class II expression was increased significantly upto 39.11% which was again downregulated to 12.25% in the third dose [ET3]. Another single positive subtype of microglia expressing MHC class II in PE quadrant had shown a gradual increase of their antigen presenting capacity in normal [N] (1.89%), tumor-bearing condition [E] (2.45%), first dose of T11TS administration [ET1] (4.26%) with no significant downregulation in second dose [ET2] (3.57%). The highest modulation of MHC class II marker was found in third dose [ET3] with a significant increase upto 12.39%. The third subtype of microglia expressing single positive CD2 marker on their surface represented themselves in FITC quadrant CD2 receptors were downregulated in tumorigenic condition [E] (5.83%) when compared with normal [N] (7.93%) animals CD2 expression on microglia gradually increased with three consecutive doses of T11TS [ET1, ET2 &ET3] on microglial surface to 11.55%, 14.31% and 18.63% respectively. Therefore, with the identification of still unreported receptor CD2 on microglial surface, FACS analysis also indicates the presence of different subtypes of microglia according to their immunophenotype (Figure 2).

Table 1. Percentage of E-rosette formation in different groups of microglia

Groups	*% Rosette*
[N]	21.33 ± 3.01
[E]	3.5 ± 0.5
[ET1]	51.55 ± 2.45
[ET2]	78.33 ± 1.04
[ET3]	28.75 ± 0.6

CD25

To evaluate the immunepotentiation of microglia the most important activation marker of peripheral immunocytes, *i.e.* IL-2R/CD25 [18], were searched for in microglial cells within the brain compartment. The existence of CD25 on microglia was observed with their differential expression in different groups. In normal [N] and tumerogenic condition [E], the values were similar (9.05% and 10.11% respectively). But in 1st [ET1] and 2nd [ET2] doses, CD25 upregulation occurred up to 20.87% and 33.35% respectively, whereas in 3rd [ET3] dose no further upregulation was observed (17.93%) (Figure 3).

CD4

Another cell surface activation marker for microglial cells, viz. CD4 [19], was evaluated. Flowcytometric data demonstrated normal [N] expression of the receptor (24.75%) was downregulated in ENU animals [E] (13.75%). This downregulated expression is restored with the first dose of T11TS [ET1] (27.07%) and became highest in the second dose [ET2] (53.52%). Further dosage [ET3] regulated the enhanced CD4 expression (19.23%) (Figure 4).

Discussion

Present study indicates that the minor glycopeptide of sheep erythrocyte membrane viz. T11TS/SLFA-3, having nonreducing terminal galactose residues in the N-linked sugar chain

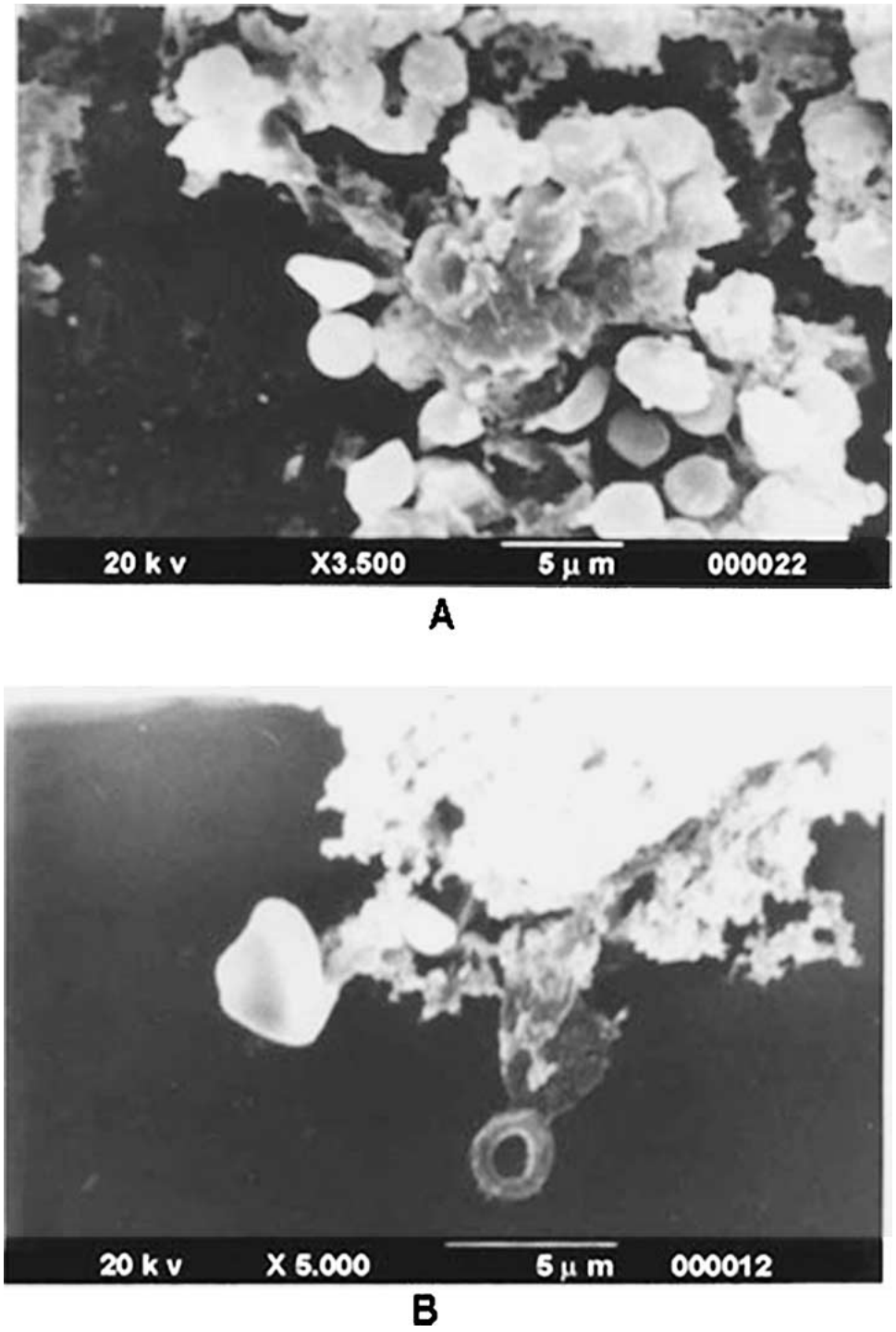

Figure 1. (A) Microglial rosette formation with sheep red blood cells (SRBC). (B) Rosette forming microglia showing cell membrane protrusions of both microglia and SRBC forming bridges.

[20], have the potential to modulate the immune system within the brain compartment. The transmembrane or GPI anchorage of the glycopeptide (destined through alternative splicing) is dissociated after trypsin digestion and selectively isolated by ion-exchange chromatography in DEAE-Cellulose column (modified from Kitao *et al*. [3]). As described previously, rosette inhibition assay and different immune-functional assay confirm the presence of T11TS in the third elute fraction [EF III] [7]. The isolation procedure of T11TS/SLFA-3 mostly followed the method of Kitao *et al*. (1976) [3] and also positive staining with Schiff's reagent (unpublished data). The comassie blue stained single banding of the protein on SDS-PAGE [21] corresponds

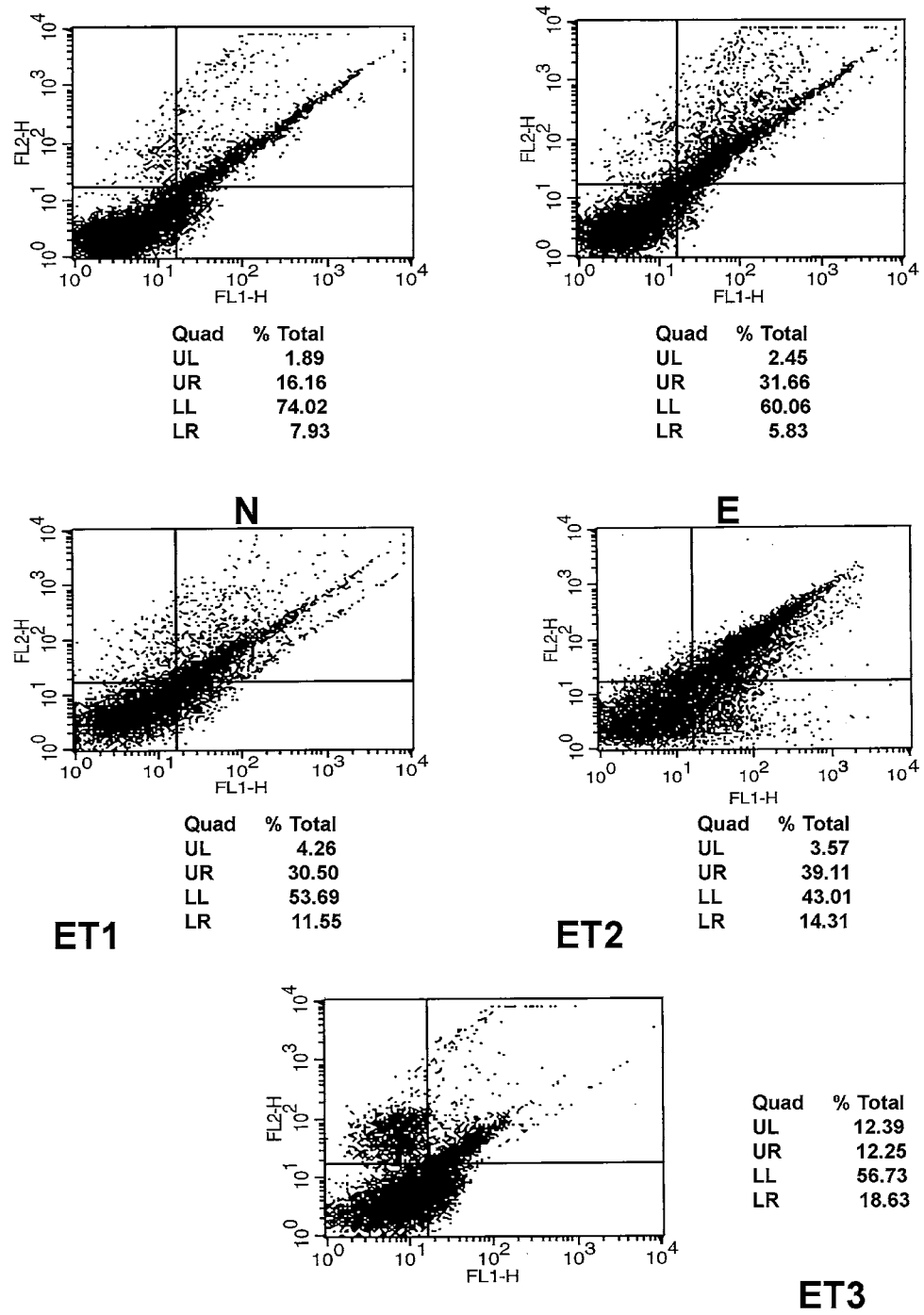

Figure 2. CD2 and MHC class II expression and their modulation in normal (N), tumor-bearing animals (E) and T11TS treated animals of three consecutive doses (ET1, ET2, ET3).

exactly with the PAS staining of EF-III elute on the gel confirmed its glycoprotein nature. The glycosylated portions consist of acetylglucosamine, sialic acid, acetylgalactosamine and galactose [3]. However existing data showed that immunoreactivity of T11TS/SLFA-3 depends mostly on its interaction with CD2 through its binding surface [2,4]. Immunomodulatory property of T11TS had previously been demonstrated by several workers. Augmentation of antigen independent activation of T cells and NK cells, IL-2R expression as well as alternative pathway of T cell activation via CD2-T11TS interaction were well documented [5,6,22]. Further investigation revealed that T11TS exerts a strong mitogenic stimulus through T11 (CD2)-associated *Src*-like protein tyrosine kinase from the surface of T lymphocytes [15]. However any disease relevance of the glycopeptide was surprisingly lacking [23] until our previous studies demonstrated its immunotherapeutic and antitumor

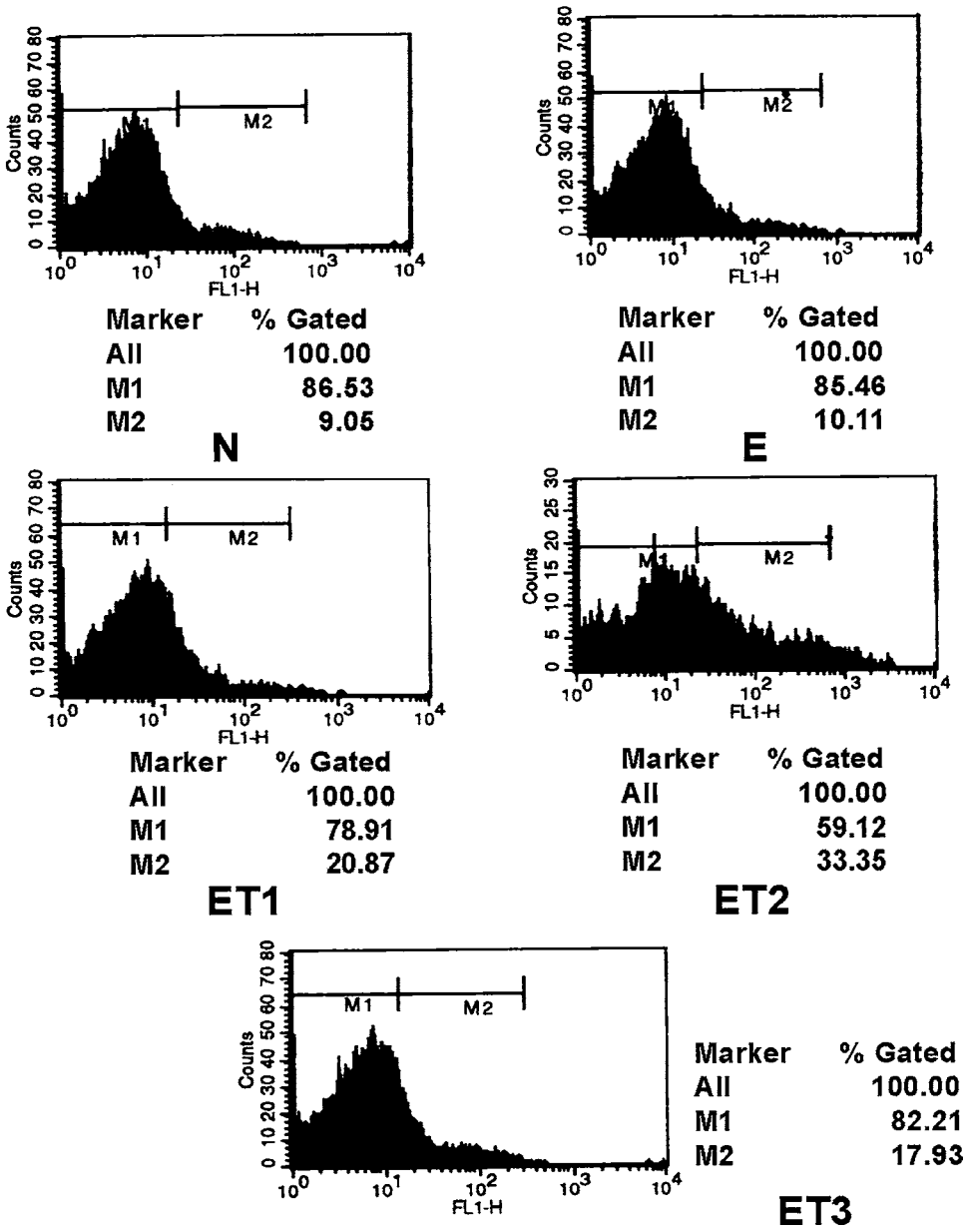

Figure 3. CD25 expression and their modulation in normal (N), tumor-bearing animals (E) and T11TS treated animals of three consecutive doses (ET1, ET2, ET3).

potential in brain tumor animal model [7,24]. In the present study the involvement of microglia, the chief immune-effector cell of the brain, is evaluated through assessing the surface molecular expression of the cell membrane *i.e.* immunophenotyping the cell in brain tumor bearing-animals treated with T11TS.

During the controversial years of identifying and establishing microglia in neuroimmunology, a successful attempt of microglial rosette formation with SRBC was carried out by Morantz *et al.* [17] in an enzyme digested glioma sample. During rosette formation in lymphocytes, a critical interaction occurs between T11TS/SLFA-3 and the CD2/T11 receptor (also known as E-rosette receptor) expressed on T cells and NK cells [2]. Ligand-receptor interaction of T11TS (a sheep homologue of human CD58) with CD2 expressed on human lymphocytes is possible, due to the nearly identical electrostatic potential map of T11TS in comparison to the binding surface of human CD58 [4]. In the present study, microglial rosette formation observed in varied percentages in different groups of animals indicates a possibility of existence of an unreported receptor CD2 on microglia (Table 1). The SEM study confirmed that SRBC cytoskeletal reorientations resulting in cell membrane protrusions of both SRBC and microglia with the formation of cytoplasmic bridges (Figure 1). FACS studies with monoclonal anti-CD2 antibody established the fact that CD2 exists on microglial cell surface and its expression can be modulated with

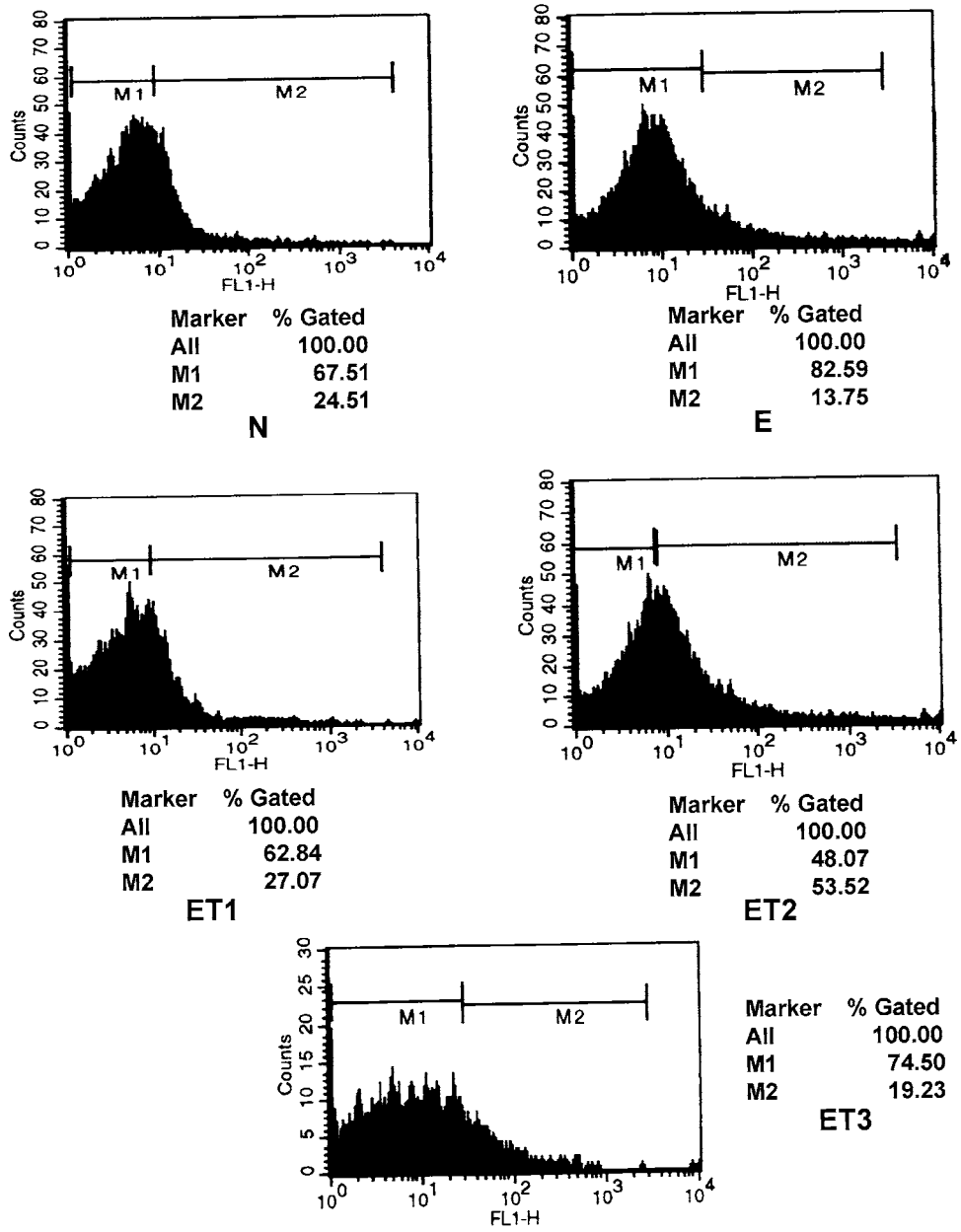

Figure 4. CD4 expression and their modulation in normal (N), tumor-bearing animals (E) and T11TS treated animals of three consecutive doses (ET1, ET2, ET3).

consecutive doses of T11TS (Figure 2). The results corroborate those of rosette formations (Table 1 and Figure 1).

When mAb against MHC class II and CD2 were administered together on the isolated microglial population, a distinct distribution of the microglial cell populations according to their receptor positivity was observed in the three quadrants of the FACS analysis result. In the PE quadrant a subpopulation of microglial cells expressed MHC class II single positivity. A slight increase of MHC class II positivity in ENU group when compared to the normal increases to much higher level in ET1 with a slight decrease in ET2 and significant increase in ET3 indicating that T11TS induces the expression of MHC class II, thereby causing antigen presentation in the tumor-bearing subjects. These findings are in accordance with our previous study of the effect of SRBC and other specific BRMs on microglial cells with highest antigen presenting capacity seen in SRBC-induced tumor-bearing group, which corroborated with the functional antigen-presenting capacity of PLN assay [16]. Expression of a single positive receptor in another subpopulation of microglia observed in FITC quadrant showed the downregulation of the receptors in ENU compared to normal, and then, with consecutive doses of T11TS administration, showed a steep increase in the CD2 expression in ET1, ET2, and ET3 groups. Probably, the ligand T11TS

induces CD2 expression in microglial population with repeated doses. The third population of microglia exhibited double positivity of MHC class II and CD2 receptors, where both receptors were significantly upregulated from normal to ENU group, and though in ET1 group not much difference was observed, another significant upregulation was seen in ET2 group with a significant downregulation in ET3 group, probably via a complex immune feedback cascade [12]. Though the conventional concept of morphological subset of microglia, which is amoeboid and ramified form [11,25] existed, immunophenotype depending on surface molecular expression offered a better approach to divide microglial population according to their immune functions.

Since, IL-2R (CD25) is the activation marker of lymphocytes [18], a search was made for the detection of this marker on the activated microglial cells. In the present study (Figure 3) IL-2R was detected on microglia and the density levels varied in different groups. With insignificant increases in the ENU group, compared to normal, the consecutive doses of T11TS in the tumor-bearing animals showed saturation of IL-2R being highest in ET2 group (33.35%) while compared to ET1 (20.87%) and ET3 (17.93%) groups. In the first phase of activation, due to the cross talk between microglia and brain infiltrating lymphocytes (BIL), the Th1 lymphocyte are stimulated liberating the proactivation cytokines showing highest upregulation of the activated marker in ET2 group, and in the second phase of the crosstalk as the Th2 cells are stimulated, they dampen the receptor saturation to a lower level. In another study by our group MHC class II and IL-2R receptors showed double positivity with upregulation of single positive MHC class II in sheep erythrocyte stimulated groups [16].

The CD4 marker is designated to be another activation marker of microglia as these markers showed modulatory effect in different neuropathogenic conditions [19]. Modulation of CD4 marker indicates that their downregulatory response in ENU group compared to normal was again upregulated with T11TS administration in ET1 group and very significant saturation of the marker was found in ET2 group followed by downregulation in ET3. Both the activation marker studies (IL-2R and CD4) confirms that activation of microglial cells start with the highest degree of activation showed in a second dose of T11TS [ET2].

Several evidences confirm that microglia represent a highly responsive glial population having potential to engage in recognizing and eliminating invading pathogens, regulating adoptive immunity, and participating in pro- and anti-inflammatory mechanisms [9]. Th1 cytokines provide signals for microglia to mature, present antigen, and amplify locally the proinflammatory immune response. Conversely, Th2 inducing capacity of microglia and astrocytes together with their ability to produce anti-inflammatory mediators could play a role in providing counter regulatory signals limiting CNS inflammation. Therefore, modulation of this master regulator of the neuroimmune system can offer an answer against different neuropathogenic condition including brain tumor. Activation of T cells by T11TS via their CD2 receptor already have demonstrated to have profound immunostimulatory effect with their greater presence in the CNS crossing the BBB [7]. But identification of CD2 on microglia has opened up a possibility to regulate their response directly by T11TS. Hence, microglia presenting CD2 can be modulated by T11TS and thereby express their CD25 (IL-2R), MHC class II, and CD4 molecules which in turn regulate the immune profile within cranium through interaction with brain infiltrating lymphocytes. Similar upregulatory trends of antigen presentation (MHC class II) and activation (IL-2R/CD25 and CD4) of microglia in first two doses of T11TS and then a decline of the properties in the third dose hint toward a pro- and anti-inflammatory cascade of reactions between microglia and brain infiltrating T cells which is yet to be confirmed by assessing the cytokine profile. The present study pointed out the existence of new immunophenotypic subsets of microglia and hint towards the role of microglia in the mechanism of brain tumor regression by T11TS.

Conclusion

Presence of CD2 and CD25 receptors have been documented on microglial cells. CD2 receptor expression was modulated with its ligand T11TS/SLFA-3, thereby upregulating the microglial activation marker CD4 and CD25. Activation was related with antigen presentation through MHC class II expression with repeated doses of T11TS. The receptor modulation of microglia reflected their functional alterations suggesting T11TS to be an immunotherapeutic agent for brain tumor abrogation.

Acknowledgement

The present work is financially supported by the Department of Science and Technology, Government of India and University Grant Commission (UGC), Government of India.

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