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The novel-molecule T11TS facilitated arousal of glioma-mediated dormancy of bone-marrow hematopoietic stem-cells

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Abstract

Aim: T11TS, a potent anti-gliomagenic glycoprotein, stimulates both peripheral and intracranial immune response. The status of bone marrow hematopoietic stem cells (BMHSCs), the cradle of regeneration of all blood cells, during gliomagenic global immune devastations has not yet been investigated. Therefore, we aimed to delineate the effects of T11TS on immature and mature compartments of hematopoietic machinery.

Methods: Flowcytometric analysis of cultured BMHSCs was evaluated for assessing the expression pattern of early hematopoietic stem cells (HSCs) markers such as CD34⁺, Sca-1⁺, c-kit⁺ and also Angiopoietin-1 and Tie-2 both in normal, glioma, and in T11TS treated glioma-bearing animals. Immunofluorescence imaging and western blot analyses of BMHSCs were also carried out.

Results: There was significant downregulation of HSCs-markers CD34⁺, Sca-1⁺, c-kit⁺ in ethyl nitrosourea-induced glioma-bearing animals followed by an increase in the expression level of Ang-1 and Tie-2 that determines the quiescence and self-renewability of stem cells. T11TS administration reversed the gliomagenic transformation of expression of the above mentioned markers. The results flowcytometric-analysis was also well corroborated with immunofluorescence imaging and western blot analysis.



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Conclusion: Collectively, the above experimental evidence hints towards gliomagenic maneuver of receptor expression of HSCs to derange the systemic immunity and T11TS mediated manipulation towards revival/rejuvenation of the same.

Keywords: Glioma, immunosuppression, T11TS, immunotherapy, hematopoietic stem cells, immunophenotyping, immunomodulation

INTRODUCTION

Systemic depression of cellular immunity and subsequent global immune suppression is the classical poor prognostic fact of gliomagenesis^[1]. Debilitating gliomas evade the host immune surveillance either by adopting immune-editing strategies such as impairment of antigen presenting machinery^[2], activation of negative co-stimulator signals for example cytotoxic T-lymphocyte-associated protein 4 (CTLA4), B7 homolog 1/programmed death 1 (PD1) and Programmed death-ligand 1 (PDL1)^[3-5] and recruitment of proapoptotic pathway [e.g., Fas receptor, Fas ligand (FasL)]^[6], expansion of regulatory T cell population^[7] and down regulation or absence of tumor specific antigen^[8] or by secreting cytokines such as interleukin 10 (IL-10), interleukin 6 (IL-6), transforming growth factor beta 1 (TGF- β), prostaglandin E2 (PGE2), and various gangliosides which have been implicated for their direct immune suppressive role to diminish the antitumor immune response during glioma progression^[9-11].

Interestingly, gliomagenic generation of potent immune suppressors in addition acts as intense inhibitory regulators of hematopoiesis by modulating the function of bone marrow hematopoietic stem cells (BMHSCs)^[12,13]. TGF- β , a key negative regulator of hematopoiesis not only suppresses *in vitro* proliferation of both progenitors and mature stem cells but also induces quiescent state of functional hematopoietic stem cells (HSCs)^[14,15]. Malignant brain tumor derived gangliosides inhibited hematopoiesis at different stages of differentiation and resulted in bone marrow hypoplasia^[16,17]. PGE2 regulates hematopoiesis in a dose and time-dependent manner and modulates hematopoiesis as negative and positive feedback control^[18,19]. The immunosuppressive, IL-10 is found to contribute its inhibitory impact in hematolymphopoiesis during various pathological conditions^[20,21].

Viewing HSCs as the foundation for the immune response^[22] we hypothesize that global immune suppression during glioma progression might have an important deleterious bearing on synchronized internal signaling network of hematopoietic machinery that regulates HSCs homeostasis, proliferation, and migration. However, there is an iota of literature about the status of bone marrow-derived HSCs during glioma condition and/or immunotherapeutic modulation of HSCs. There has been little success in preventing destructive nature of glioma either by modulating glioma stem cells or by using genetically engineered stem cells in glioma therapy^[23,24].

HSCs are delineated by their propensity for self-renewal and differentiation into entire committed hematopoietic lineages. With few exceptions, proper functioning of early hematopoietic stem and progenitor cells are dependent on c-kit, stem cell antigen-1 (Sca-1) and CD34 mediated signalling system for their self-renewal, proliferation, and survival^[25-27]. On the other hand, Angiopoietin-1 (Ang-1) and tyrosine-protein kinase receptor (Tie-2), the two important cell surface markers maintain homeostasis of HSCs by regulating molecular crosstalk between HSCs and bone marrow niche and protect HSCs from pathological conditions^[28,29].

The novel immunomodulatory glycopeptide, T11 target structure/Sheep-Lymphocyte function-associated antigen-3/CD58 (T11TS/SLFA-3/S-CD58)^[30], has been delineated in our lab for its multipotent anticancer activities against N-ethyl-N-nitrosourea (ENU) induced glioma-bearing rat model and also *in vitro* human glioma samples^[31]. Typically T11TS mediated eradication of glioma is due to the simultaneous rejuvenation of peripheral and intracranial immune system which was profoundly suppressed due to gliomagenic secretion of immunosuppressive cytokines such as IL-10, TGF- β , IL-4, and PGE2, *etc.* T11TS imparts immune-potentiality by activating T-lymphocytes^[32], NK cells^[33], macrophage (M Φ)^[34], Neutrophils^[30]

and microglial cells^[35]. A recent finding by our lab also documented that T11TS favors T cells survival not only by inhibiting glioma mediated apoptogenic death of T cells^[36] but also by repairing gliomagenic impairment of phosphatidylinositol 3-kinase/AKT (PI3K-AKT) signaling cascades^[37]. Further study in our lab also deciphered that T11TS immunotherapy, mediate functional activation of T cells by rectifying gliomagenic anti-proliferative action on T-cell by correcting CD2-mediated nuclear factor of activated T-cell calcineurin pathway^[38]. Some of our previous publications also delineated that T11TS reduces glioma mass simultaneously by accelerating the apoptotic death of brain tumor cells and by decreasing the number of dividing glioma-bearing cells^[39,40].

The above immune rejuvenation and induced increased potentiality during T11TS treatment might have an important bearing on the concomitant production of activated immunocompetent cells through the hematopoietic machinery in the bone marrow. The query for such regenerative immunocompetence remains unanswered on the hematopoietic level. Interestingly, in our recent publication, we have elucidated for the first time that T11TS also protects the BMHSCs by inhibiting the premature apoptogenic death by counteracting gliomagenic stimulation of intrinsic, extrinsic apoptogenic pathway and also by inhibiting Granzyme-B mediated apoptotic fate of HSCs within bone marrow milieu^[41].

Hence, the present study emphasized on possible modulations of key elements of the early phases of hematopoiesis on bone marrow HSCs during glioma growth and following T11TS therapy. Our finding shows modulatory effects of T11TS therapy towards differentiation, proliferation, activation of HSCs against gliomagenic shock. This entirely new finding not only illuminates the role of HSCs in glioma and also the relevance of T11TS therapy against this dreadful disease, but it may also drive us towards an important new target for basic investigation and, potentially, therapeutic intervention against many more hematological malignancies.

METHODS

Animals

Healthy Swiss albino rat pups of both sexes (4.5-6 g) were maintained in our Institutional animal facility as per Institutional Ethical Committee guidelines monitored by Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India regulations^[41]. Six animals in each group were weaned at 1 month of age and housed in individual cages at 22 °C in a 12 h light/dark cycle. Animals were fed with standard autoclaved food pellets along with water ad libitum.

The experimental animals were grouped into the following 5 groups: (1) age matched normal healthy control [N]; (2) 3-5 days-old neonatal animals intraperitoneally (i.p.) injected with ENU and reared for 5 months (optimal period for glioma development) [ENU]; (3) ENU animals (5 months of age) treated with single dose of (i.p.) T11TS [ET1]; (4) ENU animals treated with 2 doses of (i.p.) T11TS [ET2] at an interval 6 days for each dose; (5) ENU animals treated with 3 doses of (i.p.) T11TS [ET3] at an interval 6 days for each dose.

Brain tumor induction with ethyl nitrosourea

ENU the engineered alkylating compound is a strong mutagen and is observed to be the most intense of neurocarcinogens engendering brain tumors with architectural and physiological likenesses to typically ensuing neural neoplasms in mankind^[42]. ENU does not require any metabolic computation for its enactment^[43]. ENU can enter into brain in spite of an intact blood brain barrier and the alkylated products of the DNA are readily formed within the brain tissue^[44].

ENU was freshly prepared by dissolving 10 mg/mL in sterile saline and adjusting the pH to 4.5 with crystalline ascorbic acid. ENU was injected i.p. to newborn rats (3-5 days old) with a dose of 80 mg/kg body weight^[36]. Maintenance of age-matched control was done by rearing healthy rat pups up to 5 months of age.

Isolation of T11TS

The glycopeptide T11TS/SLFA-3 was isolated from sheep red blood cell (SRBC) membrane. Briefly, SRBC was trypsinized, and after nonspecific protein precipitation by TCA, was subjected to ion exchange chromatography on a DEAE cellulose column, with a five-chambered gradient system. Finally, elute fraction III was selected as the fraction of choice.

Administration of T11TS in animals

The first dose of 1 mL of T11TS was administered in rats intraperitoneally (i.p.) from the third elute fraction (EF III), which was followed by a second booster dose on the sixth day and the third booster dose on the day 12, making a dose schedule of 1, 2 and 3 mL to the ET1, ET2 and ET3 animals, respectively^[36,45].

Isolation of HSC from bone marrow

From the long bones (femur, tibia, and fibula) bone marrow was isolated as described previously^[41,46]. A single cell suspension in an aseptic condition was prepared from the bone marrow. The cells were subjected to centrifugation at 1000 rpm for 20 min on a bi-layered Percoll density gradient namely 1.077 at the bottom and 1.050 at the top. Cells obtained from the top [i.e. low-density compartment (LDC)] and bottom [i.e. high-density compartment (HDC)] layers were collected separately and washed thrice in PBS followed by culturing in RPMI media as described and characterized by Chatterjee *et al.*^[46], 2010.

Short-term stem cell culture

The LDC and HDC cells were cultured and maintained for 5 days in a 75 mm culture dish (Corning, USA) containing 4 mL of RPMI-1640 supplemented with 10% FBS, 100 ng/mL of SCF, 20 ng/mL of IL3 at 37 °C in an atmosphere of 5% CO₂ as described by Mondal *et al.*^[41], 2018.

Effect of T11TS on the phenotypic markers of HSCs

Bone marrow-derived isolated LDC and HDC cells of all groups were taken from 5-day cultures on the 6th day and were subjected to flowcytometric analysis. Percentage of extracellular CD34, Sca-1 and c-kit, Ang-1 and Tie-2 population in LDC and HDC cells were evaluated using primary antibodies against CD34 (BD Biosciences, USA), Sca-1 (Abcam Inc., USA), c-kit, Ang-1 and Tie-2 (Santa Cruz Biotechnology Inc, USA) respectively followed by PE-conjugated anti-rat respective monoclonal antibodies for 30 min as described by Mondal *et al.*^[41], 2018 with little modification. FACS Calibur (BD Biosciences) with Cell Quest Pro software was used for acquisition and analysis of percent cellular expression of each protein as quantified. A total of 10,000 events were acquired and analyzed for each sample. Gating was performed with respect to the individual group of the unstained control sample. Values indicated in “section3” are the mean of six individual studies with S.D. calculated on each mean value.

Immunofluorescence imaging of cells

Short term cultured HSCs grown overnight on the poly L-lysine coated sterile cover slips at 37 °C were fixed with 4% paraformaldehyde in PBS for 20 min. After blocking, the cells were incubated overnight with anti-primary Ang-1, Tie-2, CD34, and c-kit antibody [1:250 dilutions in PBS with 1% bovine serum albumin (BSA)] at 4 °C. After washing with PBS, CD34 and c-kit tagged cells were incubated with FITC secondary antibody and anti-Ang-1 and Tie-2 tagged cells were incubated with TRITC conjugated secondary antibody (1:500 dilutions in PBS with 1% BSA). The rest of the procedures such as staining of the nucleus with DAPI, visualization, capturing of images and quantification and representation of data were done as described^[41,47]. Briefly, Nis-Elements D3.00 were used for capturing images and quantification. Each condition was observed in triplicate and six images were taken for each sample. Figures are representative of the group. Results were expressed as mean fluorescence intensity of number total cells positively stained by the desired fluorescence tagged protein out of the total number of cells counted for each experimental group. Figures are representative of the group. As a negative control, primary antibody was omitted.

Statistical analysis

Data shown are representative of six independent experiments ($n = 6$) and values are expressed as mean \pm SD unless otherwise stated. For statistical comparison, the one-way analysis of variance was performed followed by application of the Tukey's *post-hoc* test. A P value < 0.05 was considered to be statistically significant.

RESULTS

Bone marrow-derived CD34⁺ cell, Sca-1⁺ cell and c-kit⁺ cell, Ang-1 cell and Tie-2 cell: density specific compartmentalization

During our phenotyping characterization of HSCs from the bone marrow, densitometric centrifugation technique resulted in two well distinct categories of cells. Cells at LDC (1.050) indicate immature HSCs and the other at HDC (1.077) denote mature HSCs^[48], which will lead to the generation of the progenitors. The harvested cells were subjected to CD34, Sca-1, c-kit, Ang-1, and Tie-2 positivity in a cell sorter.

Effect of T11TS on the phenotypic markers CD34 of BMHSCs

Bone marrow-derived CD34⁺-enriched cell population claimed to be one of the most critical markers for HSCs^[49] and their expression is down-regulated as they differentiate into mature cells^[50]. Single staining was done for CD34. Flowcytometric analysis [Figure 1A and B] showed a higher enrichment of CD34⁺ cells in the LDC level than in the HDC. In normal rats, there was a higher level of expression of CD34 both in the LDC and HDC in comparison to the ENU treated glioma-bearing rat where BMHSCs showed a significantly ($P < 0.001$) decreased level of expression both in the LDC and HDC. However, there was a very significant ($P < 0.001$) increase in expression levels of both in the LDC and HDC in all three dose levels, i.e., ET1, ET2 and ET3 in T11TS treated glioma-bearing rats.

In situ immunofluorescence imaging studies [Figure 1C] further confirmed the CD34 FACS findings. In the ENU group, the majority of CD34⁺ cells showed a significant ($P < 0.001$) decrease in their expression of fluorescence intensity both in the LDC and (1.692 \pm 0.439) in the HDC as compared to the normal group in both compartments. The intensity gradually increased following T11TS therapy in dose dependent manner ET1, ET2 and a significant up-regulation were noted at the third dose, i.e., ET3 in both the LDC and HDC compartments.

Effect of T11TS on the phenotypic markers Sca-1 of BMHSCs

Sca-1, an important marker of emerging HSCs, regulates the overall developmental program of HSCs towards self-renewal, lineage fate and c-kit expression^[25,51].

Flowcytometric studies [Figure 2A and B] showed that there was significant down regulation ($P < 0.001$) of Sca-1 expression both in the LDC and HDC (in glioma-bearing rats as compared to the normal groups in both the LDC and HDC. Following T11TS therapy, there was significant up-regulation ($P < 0.001$) of the expression of Sca-1 at all dose levels (ET1, ET2 and ET3) both in the immature and mature compartments compared to glioma-bearing groups. Immunoblot data [Figure 2C and D] corroborated the flowcytometric finding and confirmed that the expression of Sca-1 increased significantly ($P < 0.0001$) in dose-dependent manner in glioma associated BMHSCs of T11TS treated groups both in the LDC and HDC compared to that in HSCs of glioma-bearing ENU group both in LDC and HDC.

Changes in the expression pattern of c-kit following T11TS therapy

C-Kit, a tyrosine kinase receptor for stem cell factor, acts as a unique marker for HSCs^[52] and regulates the fate of HSCs^[53]. Even small changes in the expression pattern of c-kit resulted in dramatic phenotypes and profoundly altered the developmental rhythm of hematopoiesis^[54].

Flowcytometric studies showed [Figure 3A and B] that following glioma induction, expression of c-kit in BMHSCs of ENU group, there was a remarkable ($P < 0.001$) decrease in the expression level, both in LDC

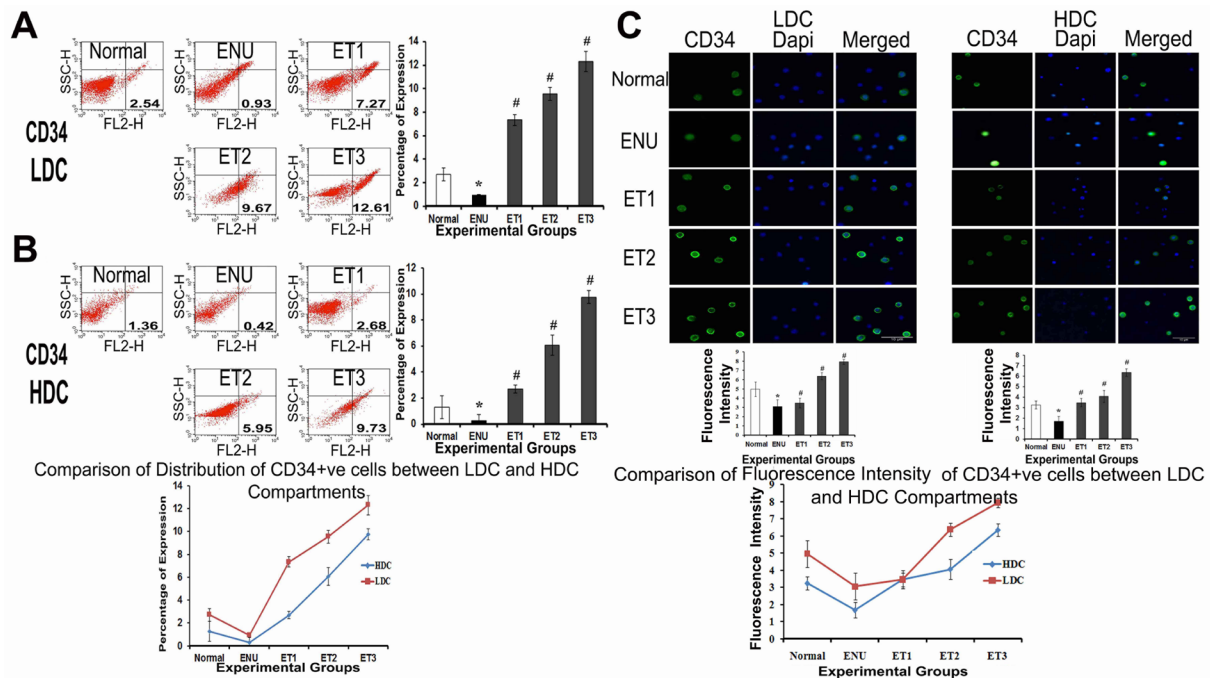


Figure 1. Comparative study of expression of CD34 in HSCs of five experimental animal groups, viz., normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of CD34 in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of CD34 percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of the expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group); B: Flowcytometric analysis of CD34 percent positive cells in the HDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group). Comparison of Distribution of CD34⁺ Cells between LDC and HDC: The line diagram shows both the LDC and HDC HSCs there is a steep high rise in their expression level of CD34⁺ cells following T11TS administration, but compared to HDC the LDC cells show the high level of proliferation as compared to ENU and normal groups probably hinting arousal of HSCs from niche as CD34 expressed mainly in primitive HSCs; C: representative images (100 \times magnification) showing immunofluorescent staining of cell surface CD34 expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3) in both the LDC and HDC compartments. Each condition was observed in triplicate and six images were taken for each sample. Figures are representative of the group. Results were expressed as the percent CD34-positive cells out of the total number of cells counted for each experimental group. Column CD34: FITC-stained CD34 expression in bone marrow derived hematopoietic stem cells green in color due to the presence of CD34. Column DAPI: nuclei appear blue due to staining with DAPI. Column merged: FITC-stained fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean fluorescence intensity \pm SD of the respective group. At the bottom the comparison of the mean fluorescence intensity of CD34 positive cells between LDC and HDC has been also depicted. *Significant ($P < 0.001$) decrease in ENU compared with that of normal control group. #Significant ($P < 0.001$) increase, when individually comparing T11TS treated groups with glioma-bearing ENU group in both the compartments. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

and HDC. T11TS administration in glioma-bearing animal significantly ($P < 0.001$) up-regulated c-kit expression in glioma associated BMHSCs of both the groups in all three dose levels both in the mature and immature compartments as compared to the significantly low expression in the ENU induced glioma-bearing group.

Immunofluorescence imaging studies also confirmed our findings. Immunofluorescence staining and imaging [Figure 3C] of cytoplasmic c-kit expression of bone marrow-derived hematopoietic stem cells were evident from robust ($P < 0.001$) decrease in cytoplasmic mean fluorescence intensity observed in bone marrow-derived hematopoietic stem cells of ENU group both in the LDC and HDC. Normal bone marrow-derived hematopoietic stem cells showed moderate fluorescence staining and hence medium

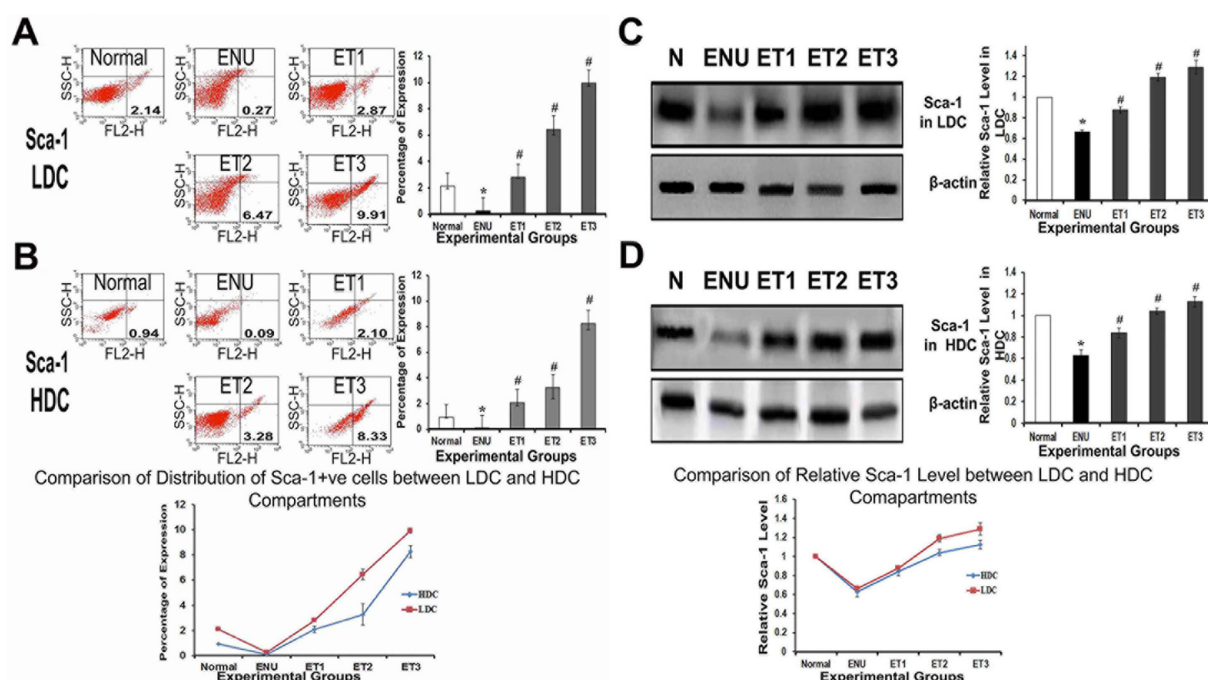


Figure 2. Comparative study of expression of Sca-1 in HSCs of five experimental animal groups, viz., normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of Sca-1 in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of Sca-1 percent positive cells in the low density compartment using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group); B: Flowcytometric analysis of Sca-1 percent positive cells in the high density compartment (HDC) using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group). Comparison of Distribution of Sca-1⁺ cells between LDC and HDC: The line diagram shows both the LDC and HDC HSCs there are a steep high rise in their expression level of Sca-1 cells after T11TS administration, but compared to HDC the LDC cells show the high level of proliferation as compared to ENU and Normal groups probably hinting activated state of HSCs towards further maturity since Sca-1 expressed in both primitive and mature cells; C: Expression of Sca-1 in LDC was analyzed by immunoblotting using anti-Sca-1 specific antibody. The immunoblot shows band intensities for the Sca-1. β -actin was used as loading control and blots were reprobred with anti- β actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams; D: expression of Sca-1 in HDC was analyzed by immunoblotting using anti-Sca-1 specific antibody. The immunoblot shows band intensities for the Sca-1. β -actin was used as loading control and blots were reprobred with anti- β actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams. *Significant ($P < 0.001$) decrease in ENU compared with that of normal control group. #Significant ($P < 0.001$) increase, when individually comparing T11TS treated groups with glioma-bearing ENU group. At the bottom, the comparison of expression of relative pixel intensities of Sca-1⁺ cells between LDC and HDC. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

mean intensity both in the LDC and HDC. T11TS treatment caused a significant ($P < 0.001$) increase in cytoplasmic c-kit expression in glioma-associated BMHSCs in all ET1, ET2 and ET3 groups, in dose dependent manner both in the LDC and HDC compared to that in hematopoietic stem cells of glioma-bearing ENU group in both the compartments.

Modulation of Tie-2/Ang-1 signaling of bone marrow-derived hematopoietic stem cells

Regulatory role of Ang-1/Tie-2 signaling pathway during maturation of HSCs under various pathological stresses and correlation of activation of this pathway with quiescence state of HSCs within the bone marrow niche is gradually emerging. The status of maintenance of HSC at quiescence not only determines the lifelong self-renewing ability of HSC but also enhances their survivability by optimizing cell division^[28]. In addition, up regulated Ang-1/Tie-2 signaling is believed to impart in the long-term repopulating ability of HSCs^[55].

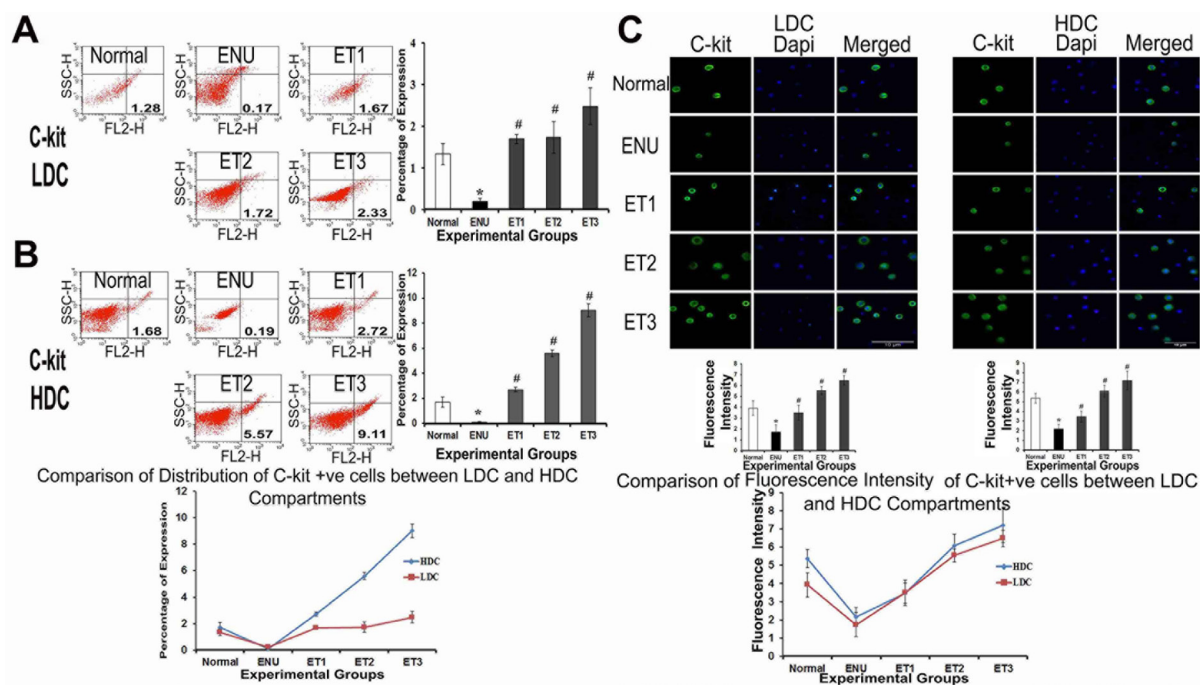
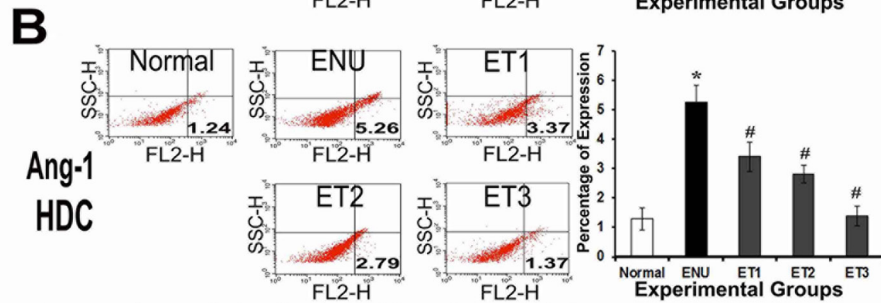
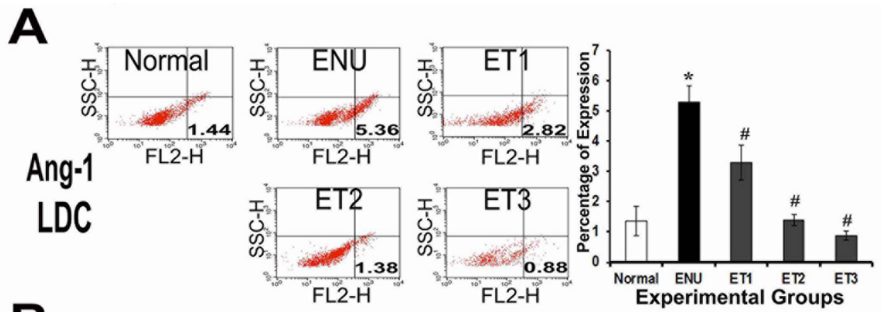


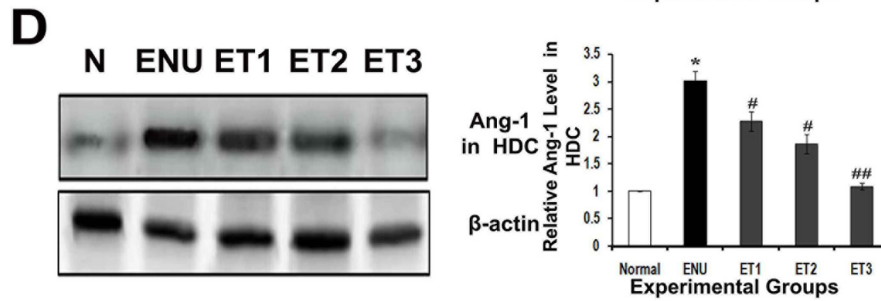
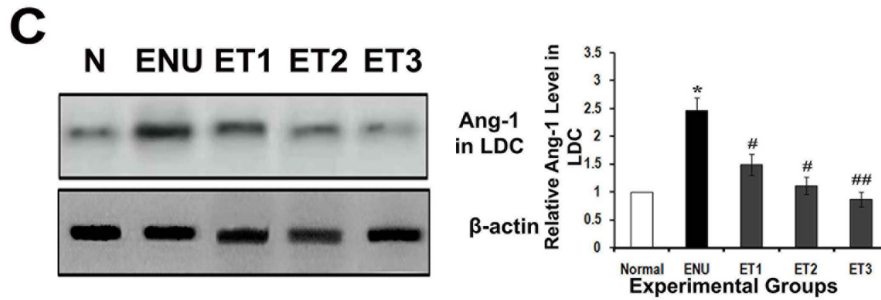
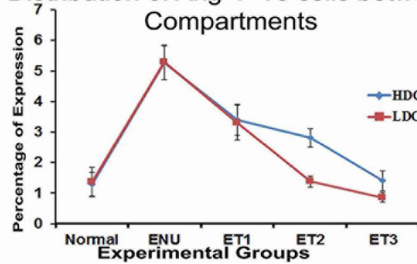
Figure 3. Comparative study of expression of c-kit in HSCs of five experimental animal groups, viz., the normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of c-kit in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of c-kit percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group); B: Flowcytometric analysis of c-kit percent positive cells in the high density compartments (HDC) using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group). Comparison of Distribution of c-kit⁺ Cells between LDC and HDC. The line diagram shows both the LDC and HDC HSCs there is a steep high rise in their expression level of c-kit cells after T11TS administration, but compared to HDC the LDC cells shows the high level of proliferation as compared to ENU and normal since c-kit ligation opens up SCF signaling cascade which leads to lineage commitment of HSCs, so the results obtained here with also hints driving of both the group of cells towards lineage commitment; C: representative images (100 \times magnification) showing immunofluorescent staining of cell surface c-kit expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3) in both the LDC and HDC compartment. Each condition was observed in triplicate and six images were taken for each sample. Figures are representative of the group. Results were expressed as the percent c-kit-positive cells out of the total number of cells counted for each experimental group. Column C-kit: FITC-stained c-kit expression in bone marrow derived hematopoietic stem cells green in color due to the presence of c-kit. Column Dapi: Nuclei appear blue due to staining with DAPI. Column Merge: Merged image FITC-stained fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity \pm SD of the respective group. At the bottom the comparison of the mean intensity of c-kit positive cells between LDC and HDC has been also depicted. *Significant ($P < 0.001$) decrease in ENU compared with that of normal control group. #Significant ($P < 0.001$) increase, when individually comparing T11TS treated groups with glioma-bearing ENU group in both the compartments. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

Flowcytometric studies showed [Figure 4A and B] that following glioma induction Ang-1 expression BMHSCs of the ENU group in both the LDC and HDC compartments was remarkably ($P < 0.001$) elevated compared to low basal expression in normal BMHSCs in both the LDC and HDC. T11TS treatment in glioma model, significantly ($P < 0.001$) inhibited Ang-1 expression in glioma associated BMHSCs of LDC and HDC at all three (ET1, ET2 and ET3) dose levels in both the compartments as compared to the remarkably high expression in ENU group both in the LDC and HDC.

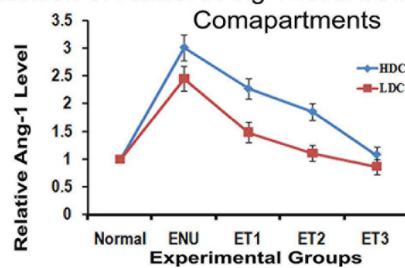
Relative band intensities of immunoblot experiment showed [Figure 4C and D] that expression of Ang-1 decreases significantly in BMHSCs both in the LDC and HDC in T11TS treated glioma associated BMHSCs in dose-dependent manner as compared to the significantly high level of expression in ENU induced glioma-



Comparison of Distribution of Ang-1+ve cells between LDC and HDC



Comparison of Relative Ang-1 Level between LDC and HDC



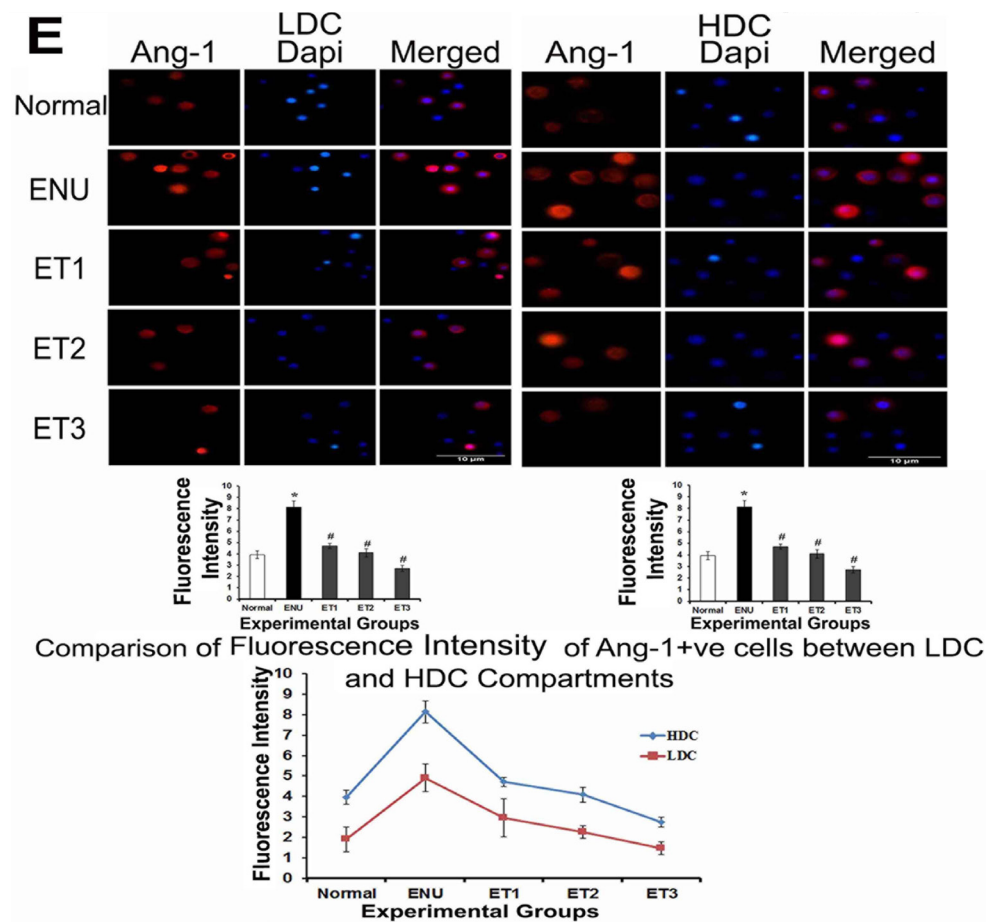


Figure 4. Comparative study of expression of Ang-1 in HSCs of five experimental animal groups, viz., the normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of Ang-1 in HSCs cells isolated from bone marrow of normal controls and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of Ang-1 percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group); B: Flowcytometric analysis of Ang-1 percent positive cells in the HDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form represented in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group). Comparison of distribution of Ang-1⁺ cells between LDC and HDC: Certain groups of stem cells, which probably attach to the osteoblastic niche show higher expression of Ang-1⁺ cells, indicating that quiescent state is being maintained in the Ang-1⁺ cells to protect them from pathological insults and fine tuning the self-renewal capacity. Here high level of expression in the glioma group might be due to the attempt to save the stem cell pool from the devastating effect of a chemical carcinogen ENU. The subsequent treatment with three doses of T11TS brings back both the HDC and LDC cells to bear normal levels of Ang-1; C: expression of Ang-1 in LDC was analyzed by immunoblotting using anti-Ang-1 specific antibody. The immunoblot shows band intensities for the Ang-1. β -actin was used as loading control and blots were reprobated with anti- β actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams; D: expression of Ang-1 in HDC was analyzed by immunoblotting using anti-Ang-1 specific antibody. The immunoblot shows band intensities for the Ang-1. β -actin was used as loading control and blots were reprobated with anti- β actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams; E: representative images (100 \times magnification) showing immunofluorescent staining of Ang-1 expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3 groups) in both the groups LDC and HDC. Each condition was observed in triplicate and six images were taken for each sample. Column Ang-1: TRITC stained Ang-1 expression in isolated BMHSC cells which appears red in color. Column Dapi: DAPI-stained nuclei appear blue. Column Merge: Merged picture TRITC fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity \pm SD of the respective group. *Significant ($P < 0.001$) increase in ENU compared with that of normal control group. #Significant ($P < 0.001$) decrease, when individually comparing T11TS treated groups with glioma-bearing ENU group. At the bottom the comparison of the mean intensity of Ang-1 positive cells between LDC and HDC has been also depicted. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

bearing group both in the LDC and HDC. Further, *in situ* immunofluorescence imaging studies [Figure 4E] of isolated BMHSCs showed a sharp ($P < 0.001$) increase in mean fluorescence intensity of cytoplasmic Ang-1 in HSCs of ENU induced glioma-bearing group compared to that in normal BMHSCs in both the immature and mature compartments which showed moderate fluorescence staining and hence the moderate mean intensity of fluorescence. However, T11TS treatment was able to bring down the fluorescence intensity of cytoplasmic Ang-1 in dose dependent manner ($P < 0.001$) in glioma-associated HSCs of ET1, ET2, and ET3 groups compared to that in HSCs of the glioma-bearing ENU group.

T11TS therapy inhibits Tie-2 expression

Flowcytometric studies showed [Figure 5A and B] that there was simultaneous remarkable ($P < 0.001$) elevated expression of Tie-2 in BMHSCs following glioma induction of the ENU group in both the compartments LDC and HDC compared to low basal expression in normal BMHSCs in both the LDC and HDC. T11TS therapy in glioma model, which significantly ($P < 0.001$) decreased Tie-2 expression in glioma associated BMHSCs of both LDC and HDC compartment in all the three ET1, ET2 and ET3 dose levels in dose dependent manner as compared to the remarkably high expression in ENU group both in the LDC and HDC.

In situ immunofluorescence staining of isolated BMHSCs of rats from different experimental groups showed that glioma induction in the ENU group caused a remarkable increase in Tie-2 expression [Figure 5C] in HSCs within bone marrow both in the LDC and HDC when compared to that in isolated HSCs from a normal control group in both the compartments. T11TS therapy caused a significant ($P < 0.001$) dose-dependent down regulation of Tie-2 expression in glioma-bearing HSCs within the bone marrow of T11TS treated groups both in the LDC and HDC compartments.

Tie-2 expression pattern in glioma-associated BMHSCs as observed from flowcytometric studies concurred with the *in-situ* immunofluorescence imaging results.

DISCUSSION

There is global immune suppression during glioma growth. Intraperitoneal administration of ENU in 5-6 days old neonatal rats produce grade IV glioma after 5-6 months of age. The salient features of ENU induced full-blown grade IV glioma, have been elaborately elucidated histologically^[56], by Glial fibrillary acidic protein (GFAP) staining^[57], by cell proliferation index studies^[58], and by *in situ* immunofluorescences^[59]. The published literature has also conclusively documented the gliomagenic global immune suppression on glioma-bearing animals and the beneficial immunomodulatory effect of T11TS immunotherapy^[60-63]. Following T11TS administration, there is profound immune stimulation which in turn results in the proliferation of the immunocytes and rejuvenates immune suppressive state of the malignant glioma^[61,62,64-67]. Therefore, the novelty of our work is that this is the first reporting of the status of HSCs during glioma occurrence and thereafter following immunotherapy, which has not been explored so far. Both, in HDC comprised of mature cells and LDC composed of relatively immature cells, the suppressive effect on HSCs during a chemical carcinogen-induced glioma condition has been documented and reversal of this suppressive situation after T11TS administration has also been noted.

The Sialomucin CD34 is a critical marker for primitive HSCs, the expression pattern of which appears to be responsible for most of the early phases of hematopoietic activity^[50,68]. Our study demonstrated that T11TS therapy significantly stimulates the level of expressions of CD34 both at the LDC and HDC in BMHSCs of glioma-bearing rats. The diminished level of CD34⁺ on HSCs of glioma-bearing animals indicates a diminution of primitive HSCs and suppression of normal hematopoiesis during glioma progression, which might be associated with the accelerated pre-mature apoptosis of the hematopoietic machinery due to the invading glioma^[41]. From our experiments, it was delineated that LDC in all the experimental groups contains

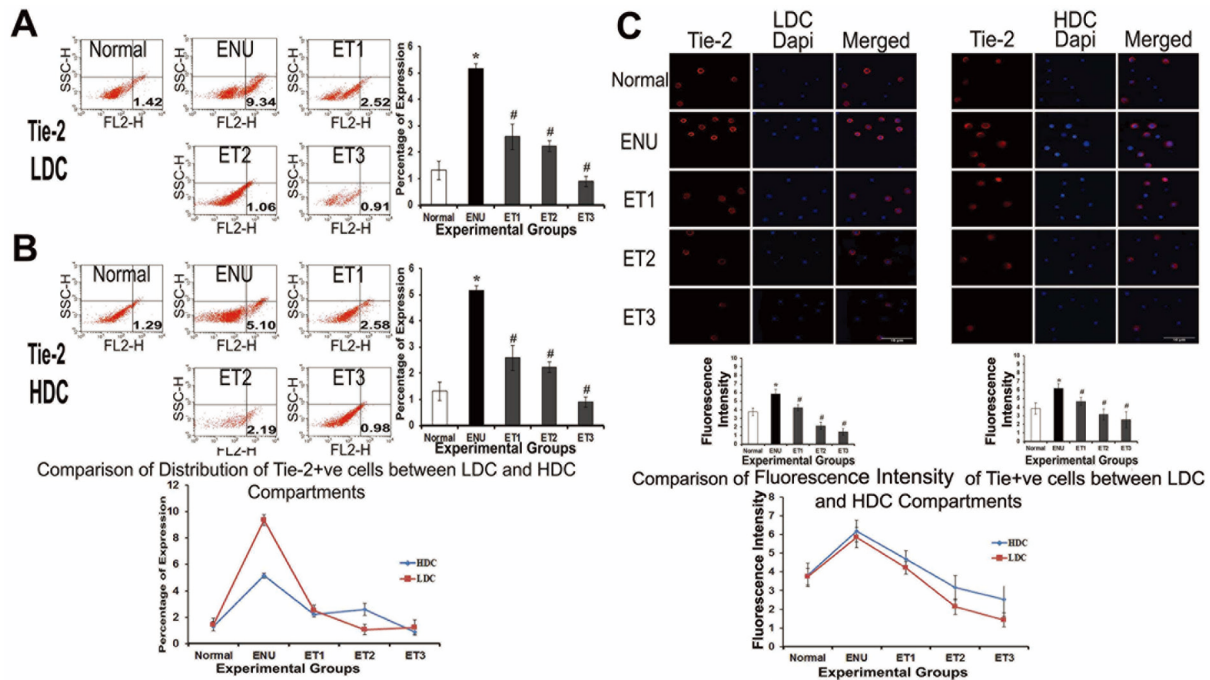


Figure 5. Comparative study of expression of Tie-2 in HSCs of five experimental animal groups, viz., normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of Tie-2 in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of Tie-2 percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group); B: Flowcytometric analysis of Tie-2 percent positive cells in the HDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean \pm SD ($n = 6$ animals per group). Comparison of Distribution of Tie-2⁺ Cells between LDC and HDC: Tie-2 which is ligand to Ang-1 and is expressed on HSCs is highly expressed in the LDC group compared to the HDC in ENU treated groups, though both groups show up-regulated expression compared to the normal group. This shows that the immature HSCs are more protected than the mature cells at HDC during the ENU insult. T11TS down regulates the higher expression of both the groups below normal levels, indicating that T11TS revive the quiescent state of HSCs driving them towards differentiation and self-renewal properties; C: representative images (100 \times magnification) showing immunofluorescent staining of Tie-2 expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3 groups) in both the groups LDC and HDC. Each condition was observed in triplicate and six images were taken for each sample. Column Tie-2: TRITC stained Tie-2 expression in isolated BMHSC cells which appears red in color. Column Dapi: DAPI-stained nuclei appear blue. Column Merge: Merged picture TRITC fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity \pm SD of the respective group. *Significant ($P < 0.001$) increase in ENU compared with that of normal control group. #Significant ($P < 0.001$) decrease, when individually comparing T11TS treated groups with glioma-bearing ENU group. At the bottom the comparison of the mean intensity of Tie-2 positive cells between LDC and HDC has been also depicted. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

more immature CD34⁺ stem cells than the HDC [Figure 1A and B] which is perhaps due to migration and spontaneous differentiation of CD34⁺ stem cells from the HDC compartment towards further maturity. Moreover, compartmental mobilization of bone marrow cells under various pathological/physiological conditions is a routine phenomenon^[69]. The result suggests that T11TS is able to stimulate glioma-associated HSC's revival from the niche with the regeneration of the CD34⁺ cells^[41].

With the aim to delineate mechanistic insights of T11TS immunotherapy on hematopoietic signaling system during gliomagenesis, we have further investigated another two important markers of HSCs, Sca-1 and c-kit known for their long-term stem cell activity^[70].

Sca-1 or lymphocyte activation protein-6A (Ly-6 A/E)^[71] not only marks various developmental stages of HSCs^[25] but also plays an indispensable regulatory role during self-renewal for normal hematopoiesis^[25,72]. In our experiment [Figure 2A and B], it has been observed that compared to the normal group, glioma induction causes a significant decrease of the level of Sca-1 both at LDC and HDC. Evaluation with normal counterparts, HDC population was shown to be lower than the population at LDC which might be due to impairment of normal transition of immature HSCs from LDC to HDC during glioma growth, ultimately resulting in a decrease in the maturity of Sca-1⁺ HSCs due to a negative feedback effect. During T11TS administration, there was a gradual increase in expressions of Sca-1 at all dose levels, both at LDC and HDC and a sharp increase in Sca-1⁺ cells after third dose of T11TS with higher level of expression in the LDC group as pointed out in the line diagram [Figure 2]. T11TS therapy not only promotes self-renewal of both the population of Sca-1⁺ cells but also hints towards further maturity. Phenotypic expression of Sca-1 distinguishes the stem cell compartment from committed myeloid and lymphoid progenitors, as its rapid down regulation was observed during differentiation^[73].

The proto-oncogene c-kit, a cell surface receptor tyrosine kinase and its ligand stem cell factor (SCF) play an essential role in intra-marrow hematopoiesis during adulthood^[74]. C-kit is exclusively expressed in primitive HSCs to impart essential regulatory function during the early stages of hematopoiesis^[75-77]. *In vivo* blocking of interaction between c-kit and SCF demote HSCs self-renewal by hindering the release of HSCs from the bone marrow. In our experiment [Figure 3A and B], ENU mediated glioma induction causes a significant decrease of the expression level of c-kit both in the LDC and HDC as compared to the normal group, but after treatment with T11TS, the level gradually increased with the consecutive doses (ET1 and ET2), and the level of receptor expression is significantly higher than the glioma group and even higher than the normal group at the third dose (ET3) both in the LDC and HDC. In glioma-bearing animals, the lower expression level in the HDC as compared to LDC might be due to the preferential killing of comparatively mature cells that are expressing constitutively active c-kit in terms of the active form of the cell cycle in the HDC sparing immature more primitive dormant progenitors^[78]. Further, the comparative line diagram [Figure 3] shows simultaneous up-regulation of c-kit⁺ cells in the HDC as compared to LDC of T11TS treated groups, indicating that the primitive immature progenitors have a lower c-kit expression as compared to their mature descendants in the HDC having higher c-kit expression. The observation further confirms the notion that as immature primitive cells enter the phase of maturation, divide actively and initiate the phase of differentiation, the expression of c-kit intensifies^[79,80]. This is perhaps due to the fact that the high expression pattern of c-kit opens up SCF signaling cascade to guide the HSCs towards lineage commitment where SCF may influence the migration of hematopoietic stem cells to their ultimate destinations of development.

Both short-term and long-term repopulating HSCs reside within the CD34⁺, Sca-1⁺ and c-kit⁺ bone marrow population^[81]. During normal physiological condition, a fine tune between self-renewal and differentiation is able to maintain the constant number of HSCs. However, disease conditions such as anemia, cancer or during myelotoxic chemotherapy have been evidence of alteration of total stem cell number indicating that the counterbalance between self-renewal and differentiation can reconcile as per physiological needs^[82,83]. This pliability is most likely achieved by molecular crosstalk between stem cells and their specific microenvironment called the niche. This reciprocal intracellular interaction between HSCs and bone marrow niche also regulates the status of HSCs and influences their surface phenotypes. On the other hand, the quiescent state of hematopoietic stem cells is thought to be critical in sustaining a self-renewing HSC compartment for life and to shield the stem cell pool from premature exhaustion during various pathological conditions. Indeed, the maintenance of quiescent state and slow cell-cycle progression of HSCs could directly be linked to the robust reconstitution ability and long-term sustainability^[84]. In addition, quiescent HSC populations are resistant to 5-fluorouracil-induced

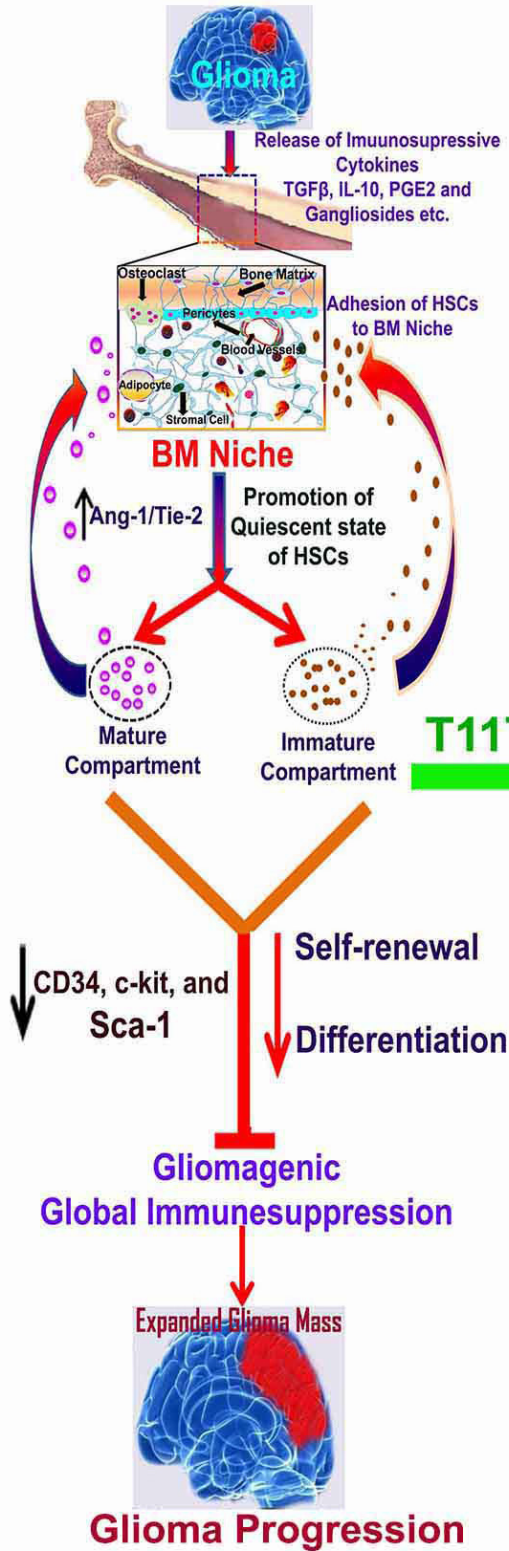
myelosuppression^[28], suggesting that the quiescence of HSCs is closely associated with the protection of the HSC pool from the various stresses induced by myelotoxic insults. However, precise regulatory mechanisms of niche cell-HSCs remain elusive.

Ang-1 expressed by niche cell is one of the indispensable interacting molecules which function as an autocrine activating factor for Tie-2 signaling in HSCs and their interaction activates β 1-integrin and N-cadherin, enhances quiescent state and facilitates long-term repopulating activity of HSCs by facilitating induced adhesion to bone and homing during the physiological crisis. The critical regulatory role of Ang-1/Tie-2 signaling in the maintenance of HSCs quiescence and promoting their adhesion to bone marrow niche, resulting in protection of HSCs compartment from myelosuppressive insults has been documented before^[28]. Furthermore, the mechanistic insight of this protective interaction against apoptosis by activating the PI3K pathway has also been addressed^[85]. We have also documented the inhibitory role of T11TS on glioma mediated pro-angiogenic Ang-1/Tie-2 signaling within brain endothelial cells ultimately restraining pro-tumorigenic angiogenesis in rodent glioma model^[86]. Although the mechanism through which glioma modulated activation of Ang-1/Tie-2 signaling within bone marrow niche and their correlation with gliomagenic global immune suppression remain to be elucidated, we hypothesize that gliomagenic up regulation of Ang-1/Tie-2 interaction disrupts the normal hematopoietic synchrony and ultimately affect the lymphohematopoiesis.

Over expression of Ang-1⁺ cells at osteoblastic niche signifying the quiescent state of HSCs as a protective barrier during pathological conditions to fine tune the self-renewal capacity. Here [Figure 4A and B], high level of expression in the glioma group might be due to the attempt to save the stem cell pool from the devastating effect of the chemical carcinogen ENU. The subsequent treatment with three doses of T11TS brings back both the HDC and LDC cells to near normal levels of Ang-1. The above mentioned effect of Ang-1 on HSCs in the glioma-bearing state is definitely mediated by over expression of Tie-2 as Ang-1 is believed to be the dominant ligand for Tie-2 receptor in LT-BMHSCs and contributed in sustenance of stem cell activities within the bone marrow niche^[87]. In our finding [Figure 5A and B] higher expression of Tie-2 at LDC compared to HDC in ENU treated groups as compared to normal group hint to their protective effect on the immature HSCs than the mature cells at HDC during ENU insult. T11TS down regulates the higher expression of Tie-2 from both the mature and immature hematopoietic cells below normal levels, indicating that T11TS revive the quiescent state of HSCs driving them towards normal hematopoiesis of reconstitution and self-renewal.

In summary, our current investigation clearly demonstrated for the first time, that during glioma condition, there was significant disruption in early phases of hematopoietic cell signaling. Our phenotypic studies of the two compartments of HSCs comprising immature LDC and mature HDC cells indicate that during glioma development all the receptors in both the compartments are drastically reduced due to reversion to the quiescent state of the particular population and also cell loss due to apoptosis as hinted in our recent publication^[41]. Their regenerative capacities were all renewed by T11TS treatment rendering “Therapeutically equipped HSC”. Primarily the immature cells had been stimulated from the quiescent state as shown by CD34 up-regulation. Next, they were driven towards maturity as denoted by Sca-1 up-regulation and lastly c-kit up regulations indicating the signaling involvements towards progenitor cell development. Up-regulation of the niche receptor Ang-1 and its counter-receptors Tie-2 of HSCs in the glioma group and drastic down regulation of both the stem cell pool by T11TS confirms the protective effect of T11TS to facilitate revival from the quiescent state of HSCs driving them towards differentiation and self-renewal properties [Figure 6]. Our findings should provide helpful guidance for the therapeutic utilization of T11TS during glioma and will also facilitate the understanding of how HSCs behave during gliomagenic immunological shock.

Glioma mediated Hibernation of HSC and Global Immune Suppression



T11TS mediated rejuvenation of HSC leading to immune potentiation

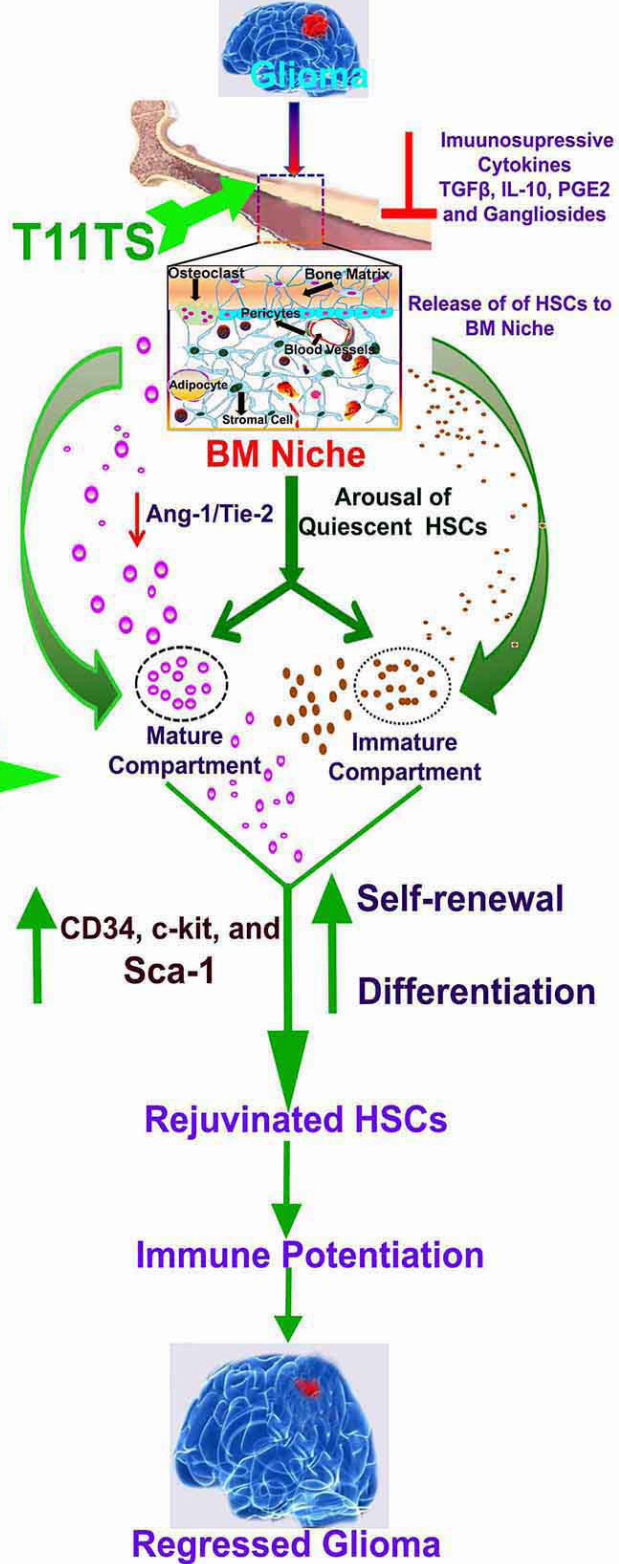


Figure 6. Proposed pathway of gliomagenic alteration of expression pattern of important early phase phenotypic markers in ENU induced glioma-bearing rats and their modulation following T11TS therapy hints towards immunocompetence against immune suppressive glioma. ENU: N-ethyl-N-nitrosourea

DECLARATIONS

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Authors' contributions

Conducted experiments and provided results: Mondal S, Datta A, Hazra I, Omar Faruk SM, Moitra S, Chaudhuri S, Nath L, Das PK, Basu AK

Co-wrote the first draft: Mondal S

Edited the manuscript: Chaudhuri S

Co-supervised aspects of the project: Tripathi SK

Provided overall supervision of the project, edited and finalized the manuscript: Chaudhuri S

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All studies involving animals were reviewed and approved by Institutional Ethical Committee at Calcutta School of Tropical Medicine, Kolkata, West Bengal, India, monitored by Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India (CPCSEA) regulations.

Consent for publication

Not applicable.

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