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Article

A Novel Role of *Tinospora Cordifolia* in Amelioration of Cancer-Induced Systemic Deterioration By Taming Neutrophil Infiltration and Hyperactivation

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Abstract: Cancer has emerged as a systemic disease which targets various organs thus challenging the overall physiology of the host. Recently, we have shown that hyperactive neutrophils infiltrate various organs of tumor bearing host and contribute significantly to gradual systemic deterioration. Therefore, taming neutrophils via potent immunomodulators could be an appropriate therapeutic approach in regulating systemic damage. *Tinospora cordifolia* (TC), an Ayurvedic panacea, is known for its immense medicinal values in traditional literature and recent reports have also documented its strong immunomodulatory potential. However, whether TC can regulate neutrophils to exert its therapeutic effectiveness has not been deciphered so far. To discern this, we utilized murine model of Dalton's Lymphoma (DL) wherein, we have earlier reported heightened infiltration of neutrophils and their hyperactivation. Our findings showed that TC treatment significantly reduced neutrophil count in peripheral blood and their infiltration in vital organs of tumor bearing host. Further, it ameliorated neutrophil hyperactivation by down regulating the expression of its key cargoes including neutrophil elastase (NE), myeloperoxidase (MPO), MMP-8, MMP-9 and cathepsin G (CSTG) at early and mid stage of tumor growth. In addition, TC treatment prevented histopathological alterations and restored the normal serum enzyme levels at different stages of tumor growth. Importantly, TC treatment also showed significant reduction in tumor burden which was accompanied by a remarkable increase in survival of the tumor-bearing mice. We conclude that *Tinospora cordifolia* could limit systemic damage via regulating neutrophil infiltration and hyperactivation which can further lead to cancer control at both prophylactic and therapeutic level.

Keywords: *Tinospora cordifolia*; Neutrophils; Systemic deterioration; Hyperactivation; Granular cargoes; Immunomodulation; Cancer

1. Introduction

Neutrophils are the key effector cells of the innate immune system and being the professional phagocytes, they are the first one to migrate at the site of infection, thus constitute the first line of defense [1]. Neutrophils are highly efficient in sensing and eradicating invading pathogens via release of an array of proteins stored in their cytosolic granules into the extracellular spaces [2]. Therefore, the effector functions of neutrophils largely depend upon the release of these granular cargoes which include antimicrobial proteins such as lactoferrin, lysozyme, serine proteases which include neutrophil elastase, cathepsin G, matrix metalloproteinases (MMP-8, MMP-9) and myeloperoxidase [3]. At the site of infection, once neutrophil function is over, their clearance is essential for resolution of inflammation to maintain tissue homeostasis. However, overwhelming activation of neutrophils, inappropriate recruitment and unregulated release of their effector molecules can damage the host tissue thus reflecting a state of chronic inflammation [4]. A plethora of studies has correlated chronic inflammation with pathogenesis of cancer wherein neutrophils represent a crucial component of this process [5, 6]. An elevated

peripheral blood neutrophil count as well as its infiltration within the tumor microenvironment has been observed in patients with different cancer types [7-9]. Importantly, neutrophil-to-lymphocyte ratio (NLR) has emerged as a potential biomarker of cancer prognosis [10, 11].

The crucial role of neutrophils in facilitating tumor progression has generated significant research interest around neutrophils in the tumor microenvironment [12]. However, these enigmatic cells have now expanded their horizon beyond the tumor microenvironment i.e. at systemic level which is evident by our recent study [13]. We have reported a crucial role of neutrophils and their derived mediators in mediating systemic deterioration in cancer. Besides their high count in peripheral blood, we observed a correlation between increased neutrophil infiltrations in peripheral organs with progressive stages of tumor. Additionally, neutrophils displayed hyperactive functions via release of toxic granular cargoes which concomitantly resulted in gradual tissue damage with tumor progression. As cancer is a systemic disease [14] therefore targeting neutrophils could be a better approach to prevent systemic damage which would thereby lead to increased survival of cancer patients.

Neutrophils are crucial for host defense; hence, their elimination or complete inhibition of the effector functions could not be the appropriate therapeutic strategy. In view of this, use of potent immunomodulators which can effectively regulate neutrophil hyperactivation can be the most appropriate approach in cancer therapeutics. A number of chemically synthesized compounds and monoclonal antibodies are currently being used as immunomodulatory agents [15]. However, due to the occurrence of adverse effects, natural immunomodulators are considered to be the potential agents to be used in therapeutic regimens [16]. Several plant-derived compounds have been identified over the years for their immunomodulatory characteristics [17]. Particularly phytoconstituents, such as alkaloids, flavonoids, lactones, polysaccharides, diterpenoids and glycosides, have been reported to be responsible for the immunomodulation properties of plants [18]. Among the vast library of medicinal plants, *Tinospora cordifolia* is well known for its valuable phytoconstituents with therapeutic efficacy [19]. Commonly called as Guduchi, it is a natural herb that belongs to the family of Menispermaceae. It is usually found in Asian countries like India, Myanmar, Sri Lanka, and China. In the traditional Ayurvedic literature, *Tinospora cordifolia* is known for its huge applications in the treatment of various diseases such as jaundice, rheumatoid arthritis, urinary disorders, skin diseases, diabetes, anemia, inflammation, and allergic conditions [20]. It is considered to be a potent 'rasayana' which boosts the immune system, restores standard white blood cell structure, counts and functions [21]. Importantly, in India during COVID-19 crisis, the Ministry of AYUSH (Ayurveda, Yoga and Naturopathy, Unani, Siddha & Homeopathy) released an advisory that incorporated simple, household measures to boost immunity wherein they suggested *Tinospora cordifolia* as one of the major prophylactic to improve the immunity. It was also suggested as an add-on intervention to conventional treatment and seems to be beneficial in symptomatic management in COVID-19 [22, 23].

Various existing studies have reported the strong immunomodulatory potential of *Tinospora cordifolia* on macrophages [24]. However, its effect on neutrophils, which are the most dominant immune cells, and crucial players in chronic inflammatory diseases, has not been deciphered so far. Therefore, in the present study we aimed to evaluate the immunomodulatory potential of *Tinospora cordifolia* in ameliorating neutrophil-mediated systemic effects of cancer. To address this, we used a well-accepted Dalton's lymphoma (DL) mice model which is a non-Hodgkin's T-cell lymphoma and represents an excellent model to study various parameters of cancer development, signaling mechanisms, and also for therapeutic drug screening [25]. *Tinospora cordifolia* extract (TCE) treatment was scheduled at early, mid and advanced stages of tumor growth at a dose of 400 mg/kg b.wt for 30 consecutive days. We first examined the effect of TCE on neutrophil number and function in the tumor bearing host. Interestingly, we observed significant reduction in neutrophil count in peripheral blood and their infiltration in vital organs of tumor bearing

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mice at different stages of tumor growth. In parallel, TCE treatment ameliorated hyperactive neutrophil function in tumor bearing mice by regulating the elevated levels of neutrophil-derived granular cargoes, including neutrophil elastase (NE), myeloperoxidase (MPO), MMP-8, MMP-9 and cathepsin G. We also showed that TCE treatment suppressed the high oxidative stress in the vital organs of tumor bearing mice. Further, we observed that TCE treatment also maintained the tissue histoarchitecture and serum enzyme levels at different stages of tumor growth. In addition, TCE treatment restored the body weight, girth size and morphology of lymphoid organs, with a remarkable increase in survival of the tumor-bearing mice at different stages of tumor growth. All together, these results suggest that *Tinospora cordifoli*a is a potential immunomodulator which can regulate neutrophil hyperactivation and ameliorates cancer-induced systemic deterioration at different stages of tumor growth.

2. Material and Methods

2.1. Chemicals and reagents

DAB (3,3'-diaminobenzidine), RNA Later (AM7020), Revert-Aid first strand cDNA synthesis kit (K1621) were purchased from Thermo Scientific. Paraformaldehyde, methanol, glycerol, hematoxylin and eosin were from SRL India. Poly-L-lysine, H_2O_2 and citrate buffer were purchased from Sigma-Aldrich. The Elastase kit (E-EL-M0444) was from Elabscience (Houston, Texas). DHE (dihydroethidium) dye was from Invitrogen Molecular Probes (D11347). Anti-Ly6G (Ab25377) was purchased from Abcam (Cambridge, USA) whereas Anti-MPO (PAA601Mu01) and anti-elastase (PAA181Mu01) were from Cloud Clone Corp. Alexa-Fluor 568 goat anti-rabbit secondary (A11036) was from Invitrogen and goat anti-rabbit FITC (Sc2012) was from Santa-Cruz Biotechnology. Xylene was purchased from Fisher Scientific and absolute alcohol was from Merck. RNeasy Micro Kit (74104) was from Qiagen. Guduchi (*Tinospora cordifolia*) powder was purchased from Nirogam India Pvt. Ltd. DPX, slides, cover slips and other reagents were of the highest analytical grade and were obtained from the common source**.**

2.2. Experimental animals

Inbred strains of pathogen free BALB/c mice (22-25g) of either sex were obtained from the animal house facility of Department of Zoology, University of Delhi. Animals were housed in propylene cages, fresh and clean drinking water was supplied ad libitum with a standard pellet diet. Throughout the period of the experiment, animals were kept at a constant environment and diet conditions. Temperature was maintained at 18-26°C with light/dark cycles of 12h interval. The study was performed in accordance with the guidance for the care and use of laboratory animals with approval of the University of Delhi and Committee for the Purpose of Control and Suppression of Experiments on Animals (CPCSEA), India.

2.3. Tumor induction and maintenance

Dalton's lymphoma (DL) cells were obtained from the Department of Biotechnology, Banaras Hindu University. The cells were maintained in the peritoneum of BALB/c mice by serial intraperitoneal transplantation as described earlier [26]. For the experiment purpose DL cells were collected from the donor mice and were immediately suspended in sterile isotonic saline (PBS). The viability of DL cells was confirmed by the trypan blue assay and total number of cells /ml. was counted. The total number of cells was adjusted to 1×10^6cells/ml. and then injected in the peritoneal cavity of healthy 3-4 months old BALB/c mice.

2.4. Extract preparation

5g of *Tinospora cordifolia* powder was weighed and dissolved in 100ml of 50% methanol (molecular grade) in a conical flask. The mixture was stirred overnight on a

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magnetic stirrer for two days and then filtered through Whatman filter paper twice to obtain more clear extract as it would help in making the sample more concentrated in Rotary evaporation technique. The filtrate was centrifuged at 5000rpm and the supernatant was evaporated in a Rotary evaporator (bucchi) at 40°C and then finally dried in a lyophilizer (Macflow). The extract was redissolved in PBS at a concentration of 400mg/ml (stock). The extract was then finally filtered through a 0.22µm filter and stored at -20°C until use.

2.5. Experimental Groups

BALB/c mice of either sex, each weighing 22-25g and aged 8-10 weeks were divided into five groups, with each group consisting of nine mice. Group I served as control and in rest four groups, Dalton's lymphoma was induced by injecting 1×10^6 cell/ml of tumor cell suspension i.p. *Tinospora cordifolia* extract (TCE) was administered at different time points. In the early treatment group (day 0), extract and DL cells were injected on the same day. To study the effect on the mid stage of tumor growth (day 7), TCE was administered after 7 day of tumor establishment. A third parameter was also studied in which TCE was administered after 14 days of tumor growth depicting the advanced stage (day 14). The treatment continued for 30 days in all the groups at a dose of 400mg/kg body weight.

2.6. Physiological parameters

Body weight and girth size was recorded in all the groups at the beginning of the experiment and then sequentially with treatment. Animals were sacrificed after 30 days of TCE treatment. Lymphoid organs such as spleen, thymus and lymph nodes were excised to evaluate the difference in the morphology. Effect of TCE on survival of tumor bearing mice was also analyzed using the Kaplan-Meier survival method.

2.7. Hematological and biochemical parameters

Blood samples were collected by cardiac puncture into test tubes with and without EDTA (anti-coagulant). EDTA containing test tubes were used for analysis of polymorphs, lymphocytes and total leukocyte count (TLC). Hematological analysis was performed using automatic hematology analyzer (Clindiag India). The blood in non EDTA test tube was allowed to stand for one hour in the room temperature, then centrifuged at 3000 rpm for five minutes and clear serum was obtained which was further used for the determination of various biochemical parameters like total protein, urea, albumin, ALT, AST, and creatinine using commercially available kits (Erba diagnostic kits)

2.8. Histological Examination

Hematoxylin-eosin (H&E) staining was performed for the histopathological analysis of liver, lungs, spleen and peritoneum. Tissues were dissected out and immediately rinsed in PBS. Subsequently, every tissue specimen was divided into two parts. One part of the tissue specimen was stored in liquid nitrogen for ELISA assay and another part was fixed in NBA (neutral buffer formalin) and then embedded in paraffin for histopathological examination. The thin sections of 5µm were cut, transferred on clean slides, dewaxed in xylene and stained with hematoxylin and eosin. Finally slides were observed under light microscope (Nikon) using NIS Element software at 100X and 400X magnifications.

2.9. Immunohistochemical analysis

Immunohistochemistry was performed to detect the expression of Ly6G protein in liver, lungs, spleen and peritoneum. Samples were fixed in 4% PFA, embedded in paraffin and were cut into 5µm sections. To remove the paraffin, slides were immersed in xylene 2 times, for 10 minutes each, rehydrated with graded ethanol, 100%, 95%, 80%, 70% and 50%, for 5 minutes each, and transferred to tap water. The endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 for 20 min. Slides were heated in

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sodium citrate buffer (pH 6.0) solution at 95°C for 20 minutes for antigen retrieval. Non-specific reactivity was blocked by incubating the slides with 5% normal goat serum for 1 hour. The slides were washed three times in PBST and incubated with anti-Ly6G primary antibody (1:100) at 4°C overnight in a humidified chamber. Sections were washed three times in PBST (0.2% Tween-20) and incubated with goat anti-rat secondary antibody (1:200) for 2 hour at room temperature. Sections were then stained with DAB for 5 minutes and subsequently stained with hematoxylin and observed under light microscope using NIS Element software.

2.10. Immunofluorescence for Ly6G, NE and MPO

EDTA anticoagulated peripheral blood samples were smeared on glass slides, air dried and fixed in methanol. Slides were permeabilized with 4% PFA for 20 minutes at 4°C. Non-specific reactivity was blocked by incubating the slides with 5% normal goat serum for 1h. Slides were then incubated with anti-Ly6G primary antibody (1:200) at 4°C overnight. Slides were washed three times with PBST (0.2% Tween-20) and incubated with Alexa Fluor 488 goat anti-rat secondary antibody for 2h in dark at room temperature. For NE expression in blood, slides were incubated with anti-elastase (1:200) overnight, and FITC-labeled goat anti-rabbit secondary (1:200) for 2h. Similarly, to analyze MPO expression, slides were incubated with anti-MPO (1:200) overnight and Alexa Fluor 568 anti-rabbit secondary (1:200) for 2h. The sections were mounted in Prolong Vectashield® mounting medium with DAPI, covered with a cover slip and sealed. Samples were then observed under a fluorescence microscope (Nikon).

2.11. Preparation of tissue sections for O_2 detection

Tissues were rinsed immediately in chilled PBS once excised and snap frozen in liquid nitrogen. 20µm thick cryosections of unfixed sections were obtained using cryostat and placed on a clean poly L lysine coated slide. DHE was suspended in DMSO at a stock concentration of 10mM and diluted for a final working concentration of 10µm in PBS. DHE was topically applied to each tissue section and slides were incubated in a light protected humidified incubator at 37°C for 15 minutes. Slides were then mounted with 5% glycerol and immediately analyzed under fluorescence microscope (Nikon). Minimum six slides per condition were analyzed for ROS quantification.

2.12. Detection of neutrophil elastase by ELISA

Blood samples were collected in microcentrifuge tubes under sterile conditions. Samples were allowed to clot and centrifuged at 1000g for 10 min. Serum was separated and stored in aliquots at -80°C. Ascitic fluid was also collected and stored. Tissues such as lungs, liver, peritoneum, and spleen were excised, rinsed in sterile PBS, blotted on tissue paper (to remove excess buffer) and weighed. 600µl of RIPA lysis buffer was added to 5mg tissue and homogenized using electric homogenizer. After homogenization, samples were left on ice for 30 minutes before transfer into pre-labeled micro centrifuge tubes. Samples were centrifuged (4°C, 10000 x g, 10 mins). Supernatants from each sample were removed, aliquoted and stored at -80°C pending analysis. The levels of elastase in serum, ascites and tissue specimens were detected by a multi detection microplate reader using a sandwich Elisa kit (Elabscience) according to the manufacturer's protocol.

2.13. RNA extraction and real-time quantitative PCR

In order to evaluate the expression of genes, total RNA from liver, lungs, spleen and peritoneum tissue was extracted using RNeasy Micro Kit (Qiagen) as per the manufacturer's protocol. The quality of RNA was checked by Nanodrop; 260/280 nm absorbance ratio close to 2.0 was accepted as 'pure' RNA. 1ug RNA was treated with RNase free DNaseI (Thermo scientific) and reverse transcribed using Revert-Aid first strand cDNA synthesis kit (Thermo scientific, K1621). The mRNA expression of NE, MPO,

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MMP-8, MMP-9, CSTG and β-actin genes was measured by the Real time PCR (qPCR) run on Applied Biosystems ViiA7 Thermal cycler. Both sample and reference (*β-actin*) genes were run in duplicates in a reaction volume of 6 µl (1 µl of cDNA +1 µl each of forward and reverse primer +3 µl of Power SYBR green master mix). Conditions for the PCR reaction were 3 min at 95 °C and then 40 cycles, each consisting of 15 s at 95 °C and 45s at 60 °C. The relative mRNA expression was calculated as fold change, the $2^{-(\Delta\Delta Ct)}$ value and data were normalized by *β-actin.* Primers were designed using NCBI primer blast. The primer sequences for semi-quantitative PCR experiments are as listed below:

Gene	***Forward***	***Reverse***
NE	AATTTCCGGTCAGTGCAGGT	TGGCGTTAATGGTAGCGGAG
MPO	AGACCCTCGAATCGCCAATG	AATGCCACCTTCCAACACGA
MMP-8	CCACACACAGCTTGCCAATG	GCTTCTCTGCAACCATCGTG
MMP-9	GCTCTGCTGCCCCTTACCA	GGTGTTCGAATGGCCTTTAGTG
CSTG	GATCTGTGTGGGAAACCCGA	CATAGGAGACGATGCCCTGG
β-ACTIN	CTTCTTGGGTATGGAATCCTG	GTAATCTCCTTCTGCATCCTG

2.14. Statistical analysis

Data were analyzed using GraphPad Prism (version 6.0, GraphPad Software). All experiments were performed in biological triplicates and the data are presented as means ± standard deviation. The statistical significance of differences between was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test for multiple intervention experiments. A *p* value of 0.05 or less was considered to represent a statistically significant difference.

3. Results

3.1. TCE regulates neutrophil count in peripheral blood and their infiltration in vital organs

Our previous study showed an increase in neutrophil count in peripheral blood and their infiltration in the vital organs of tumor bearing host [13]. Additionally, their high infiltration and function was correlated with cancer-induced systemic damage. Therefore, we were interested to investigate whether *Tinospora cordifolia,* a potent immunomodulatory phytochemical, can regulate neutrophils in cancer conditions or not. Firstly, we assayed the hematological parameters such as total leukocyte count (TLC), lymphocytes and polymorph count in peripheral blood of different treatment groups. We observed an increase in TLC in the tumor bearing group; whereas, groups receiving TCE treatment in early (day 0), mid (day7) and advanced stage (day14) of tumor growth

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showed a significant ($p<0.05$) restoration in TLC (fig.1B). On the contrary, we observed a reduction in lymphocyte count in the tumor bearing group which showed a significant increase in the early, mid and late treatment group (fig.1B). Next, we looked for the number of polymorphs in tumor conditions. Interestingly, we observed upregulated polymorphs count in the tumor bearing group which showed a significant reduction upon TCE treatment in early (day 0) and mid stage (day7) groups; however, in the late treatment group, the reduction was insignificant (fig.1B). Next, we confirmed the presence of neutrophils by characterizing them using anti-Ly6G antibody (neutrophil-specific marker) wherein we observed increased $Ly6G^+$ cells in peripheral blood of tumor-bearing mice. Interestingly, we observed a significant reduction in the number of $Ly6G^+$ cells in the early and mid stage treatment group while the reduction was insignificant in the late treatment group (fig.1C). This result is in line with reduction in polymorphs count with treatment and thereby corroborated our investigation. Moreover, we examined the effect of TCE on neutrophil infiltration in vital organs, which include liver, lungs, spleen and peritoneum, of tumor bearing mice. We observed that TCE administration showed a pronounced effect showing no to low neutrophil infiltration in early and mid stage treatment groups (fig.1D). Importantly, late treatment group showed significant reduction in the neutrophil infiltration, particularly, in liver and peritoneum compared to the tumor bearing group.

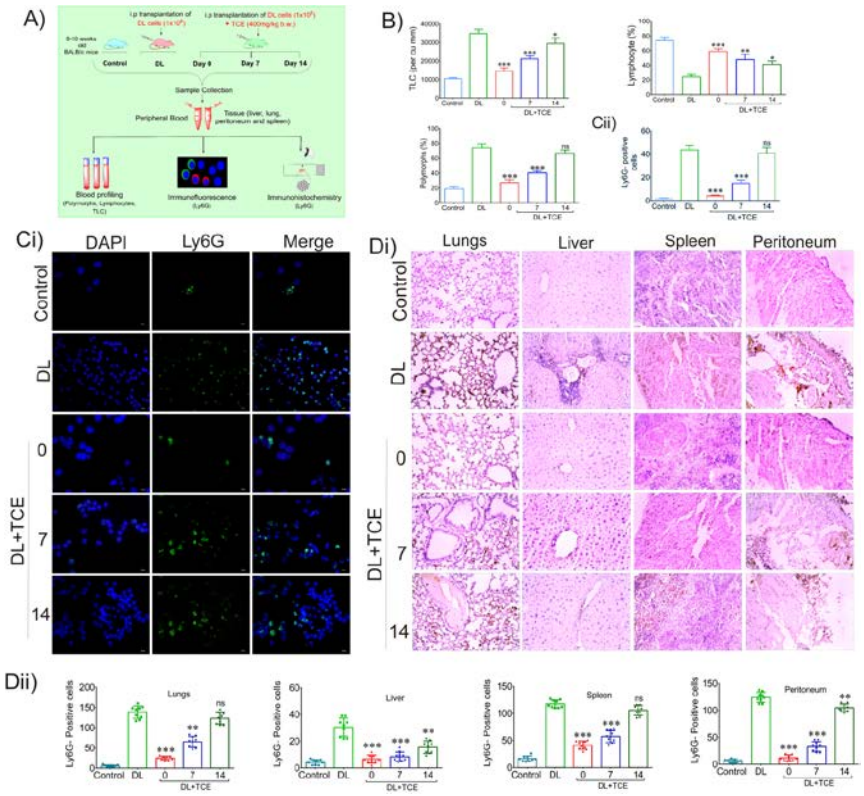

Figure 1. TCE regulates neutrophil count in peripheral blood and their infiltration in vital organs. A) Schematic of the experimental design. B) Total leukocyte count, lymphocyte, and polymorph count determined in the peripheral blood of tumor bearing mice post TCE treatment. Ci) Immunofluorescence images of blood smear at different time points with antibody directed against Ly6G (green), (magnification, × 600, scale bar, 10 µm). Cii) bar graph showing quantitative analysis of immunostaining data (Ly6G positive cells) performed using ImageJ software. D) Positive staining for neutrophils (Ly6G) examined by immunohistochemistry in liver, lungs, spleen and peritoneum. Dii) bar graphs showing number of $Ly6G^+$ cells counted in six different regions. Every image is the representative of nine sections analyzed per condition. Data are shown from one of the three independent experiments with a similar pattern of results. Data are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant. .

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3.2. TCE regulates systemic NE in tumor conditions

After exploring the effect of TCE on systemic neutrophil infiltration, we were interested in exploring systemic neutrophil functions. Neutrophils release toxic mediators packed in their distinct granule subsets which contribute to tissue injury [2]. NE is a key effector molecule encapsulated in the primary granules of neutrophils which hydrolyzes a variety of extracellular matrix (ECM) components, thus playing an important role in tissue destruction, tumor invasion and metastasis [27]. To assess the therapeutic efficacy of TCE on neutrophil function, we first examined its effect on NE expression in peripheral blood neutrophils. We observed high NE expression in tumor condition which was reduced to a significant level and comparable to the control group upon TCE treatment in early and mid stage of tumor growth (fig. 2C). TCE treatment in the advanced stage of tumor growth also showed reduced NE expression as compared to the tumor bearing group. Then we assayed NE level in serum, ascitic fluid and tissue lysates of liver, lungs, spleen and peritoneum by ELISA wherein we observed its high level in tumor condition. Interestingly, TCE treatment significantly reduced NE levels in serum, ascitic fluid (peritoneal fluid) and tissue lysates of all the examined organs in early and mid stage of tumor growth. However, the effect was not significant in the advanced stage (fig.2B). Further, to corroborate these results, we investigated the expression of NE at transcriptional level in liver, lungs, spleen and peritoneum. Similarly, we observed high mRNA expression of NE in tumor condition in all the examined organs which was significantly reduced upon TCE administration in early and mid stage treatment groups. Importantly, we observed significant reduction in NE expression in spleen and peritoneum tissues with late stage treatment, however no such effect was observed in liver and lung tissues (fig.2D).

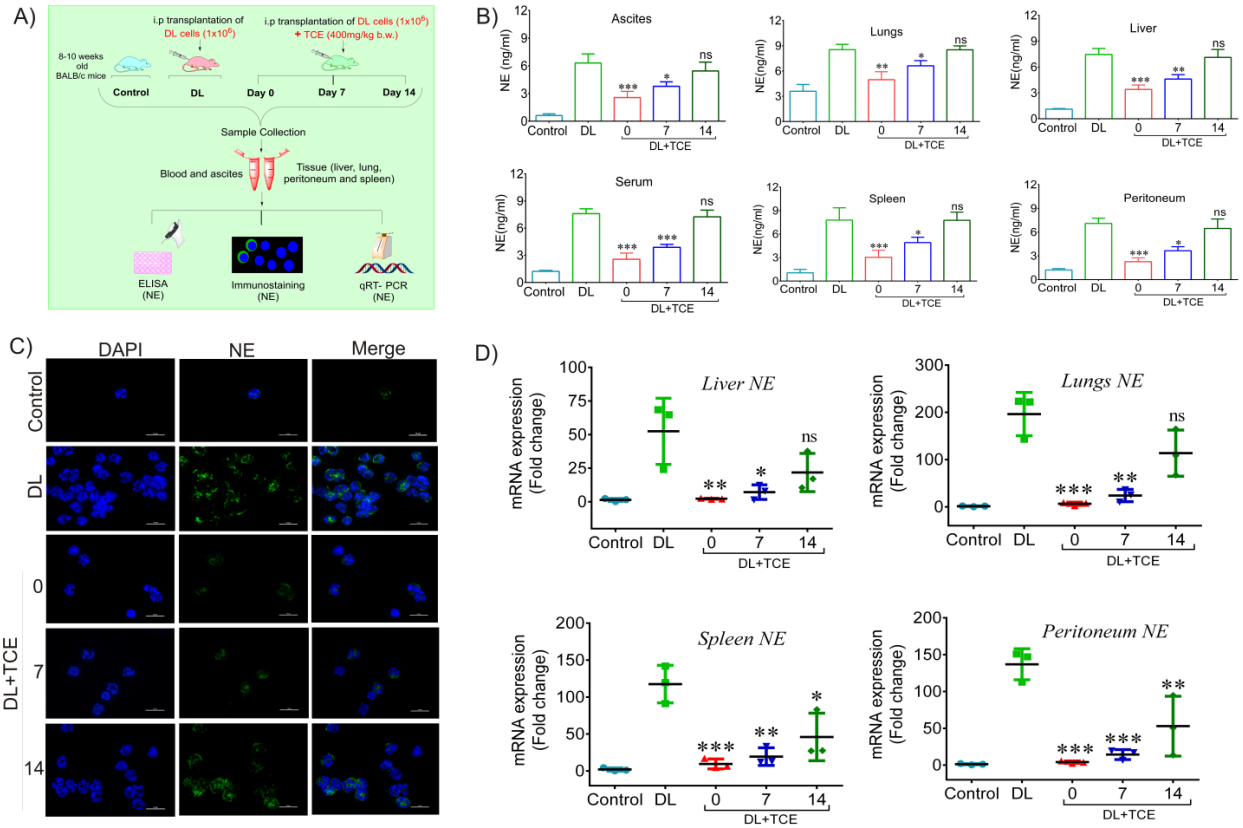

Figure 2: TCE regulates systemic NE in tumor conditions. A) Schematic of the experimental design. B) Serum and ascites fluid was harvested from tumor bearing mice post TCE treatment and assayed for NE by ELISA. Similarly tissues including liver, lungs,

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spleen and peritoneum were harvested at different time points. Tissues were homogenized and supernatant was collected for estimation of NE by ELISA. C) Immunofluorescent images of peripheral blood cells stained with anti-elastase were performed as described in materials and methods (magnification, × 100, scale bar, 100 μm). D) Representative mRNA expression of NE assessed by quantitative RT-PCR in liver, lungs, spleen and peritoneum post TCE treatment. β-actin was used as an internal control. The results represent three independent experiments and are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant.

3.3. TCE regulates systemic MPO and ROS production in tumor bearing mice

MPO is another key cargo present in neutrophil granules which has been found to enhance the inflammatory response and cause tissue destruction in various inflammatory diseases [28]. Previously, we observed increased expression of MPO in peripheral blood and vital organs with tumor progression that was correlated with systemic deterioration. Therefore, we further extended our study to assess the effect of TCE on MPO expression in blood and tissues of tumor bearing hosts. Our study revealed that in peripheral blood high expression of MPO in tumor conditions showed a significant reduction with TCE treatment at early and mid stage of tumor growth. However, no such significant difference was observed in the group receiving treatment in the advanced stage of tumor growth (fig.3C). Next, we investigated the effect of TCE treatment on the expression profile of MPO in liver, lungs, spleen and peritoneum. We observed high expression of MPO in all the examined organs which reduced significantly with TCE treatment at the early and mid stage of tumor growth. In contrast, with TCE treatment at the advanced stage, MPO expression was significantly reduced only in spleen tissue while its expression was unaffected in liver; lungs and peritoneum (fig.3B). MPO is a key enzyme that catalyzes the formation of reactive oxygen intermediates [29]; therefore, we next looked into the effect of TCE on reactive oxygen species (ROS) production in the liver, lungs, spleen and peritoneum. For ROS detection we used DHE, a cell permeable dye which shows specificity for superoxides [30], wherein the intensity of DHE staining in tissues is proportional to the ROS levels. Tissues obtained from the tumor bearing mice exhibited high fluorescent intensity as compared to the control tissues. Interestingly, we observed a significant reduction in ROS intensity in vital tissues excised from early and mid stage treatment groups. But, no significant effect was observed in the advanced stage treatment group (fig.3D).

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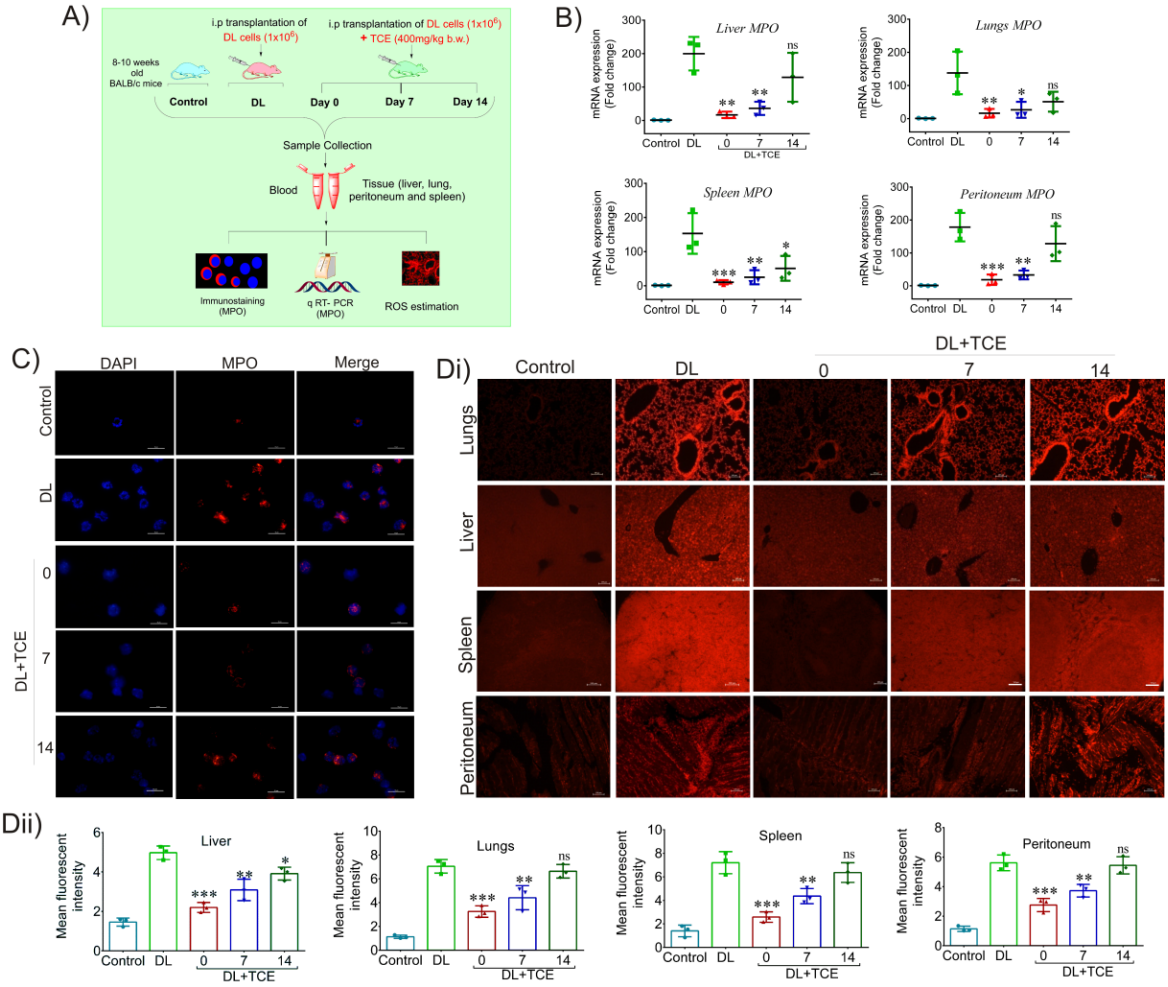

Figure 3: TCE regulates systemic MPO and ROS production in tumor bearing mice. A) Schematic of the experimental design. B) Representative mRNA expression of MPO by quantitative RT-PCR in liver, lungs, spleen and peritoneum post TCE treatment at different time points. β-actin was used as an internal control. C) Immunofluorescent images of peripheral blood cells stained with anti-MPO as described in materials and methods. Di) Cryosections of lungs, liver, spleen and peritoneum were prepared and processed for ROS detection using DHE staining as mentioned in materials and methods. Dii) Histogram shows DHE fluorescence intensity in vital organs of mice in various groups, (magnification, × 100, scale bar, 100 μm). The results represent three independent experiments and are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant.

3.4. TCE regulates MMPs expression at different stages of tumor growth

In addition to NE and MPO, neutrophils are known to be the major source of MMPs (specifically, MMP-8 and -9). Importantly These MMPs play a critical role in tumor progression by facilitating tumor cell invasion and show broad catalytic activity against components of ECM [31, 32]. Previously, we also observed high systemic expression of MMP-8 and -9 in tumor conditions. Therefore, we further looked for the regulatory effect of TCE on MMP-8 and MMP-9 expression in liver, lungs, spleen and peritoneum tissue of tumor bearing mice. We found that TCE treatment at early and mid stage of tumor growth showed a significant reduction in MMP-8 expression in all the examined organs, however, no significant reduction was observed in the late treatment group (Fig. 4B). Similarly, TCE significantly reduced the elevated MMP-9 levels in early and mid stage treatment

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groups. In contrast, the late treatment group showed significant reduction only in lungs and spleen tissues whereas liver and peritoneum tissues did not show any significant effect of TCE treatment (Fig. 4C).

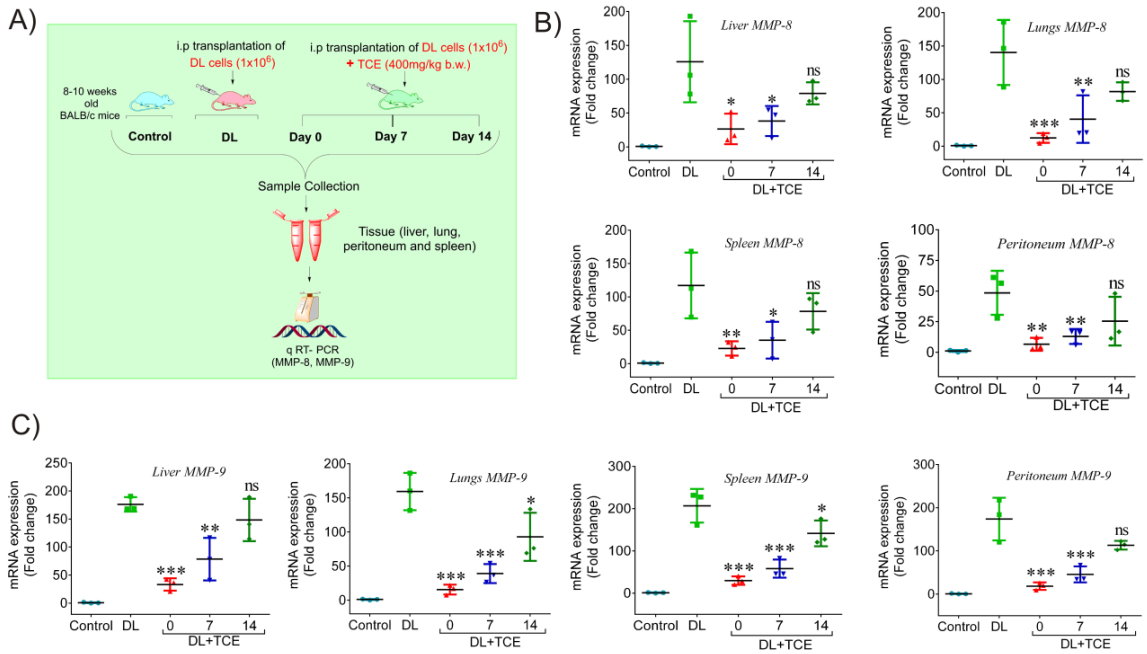

Figure 4: TCE regulates MMPs expression at different stages of tumor growth. A) Schematic of the experimental design. B) Representative mRNA expression profile of MMP-8 and MMP-9 by quantitative RT-PCR in liver, lungs, spleen and peritoneum post TCE treatment at different stages of tumor growth. β-actin was used as an internal control. The results represent three independent experiments and are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant.

3.5. TCE regulates elevated CSTG expression in tumor bearing mice

Cathepsins represent an extensive family of proteases which play crucial roles in many physiological as well as pathological conditions. Particularly, CTSG is a serine cathepsin which is stored in the azurophilic granules of neutrophils together with NE [33]. Being a degradative enzyme, CTSG can kill the ingested pathogen and perform extracellular functions such as degradation of ECM and hydrolysis of host plasma proteins. Besides its anti-microbial role, CTSG has been identified to play a very important role in inflammation, tumor growth, and progression [34, 35]. Like other granular cargoes, we observed an elevation in CTSG levels in tumor bearing mice. Further, we observed that TCE treatment at early and mid stages of tumor growth significantly reduced CTSG expression, while the expression was not affected by TCE treatment in the advanced stage of tumor growth (Fig. 5B).

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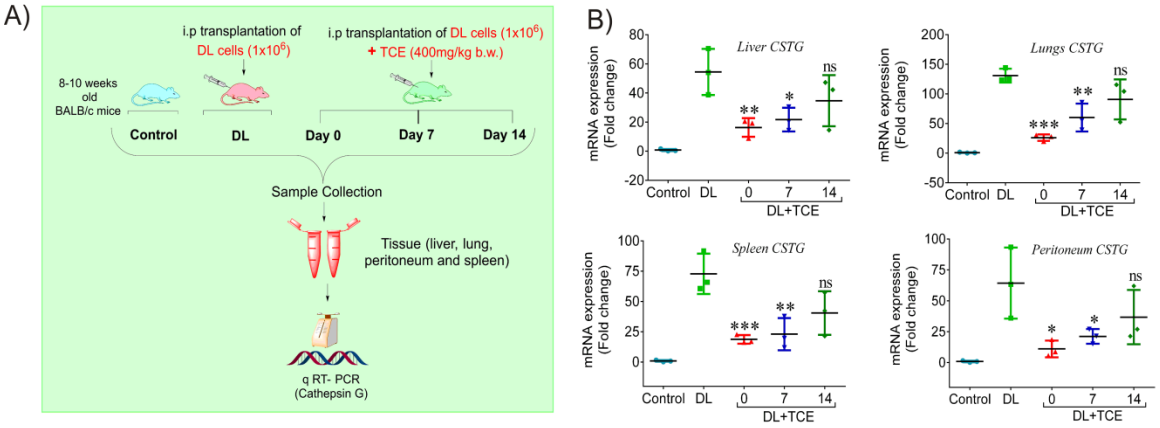

Figure 5: TCE regulates elevated CSTG expression in tumor bearing mice. A) Schematic of the experimental design. B) Representative mRNA expression of CSTG by quantitative RT-PCR in liver, lungs, spleen and peritoneum post TCE treatment at different stages of tumor growth. β-actin was used as an internal control. The results represent three independent experiments and are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant.

3.6. TCE restores tissue histoarchitecture and serum enzyme levels in tumor bearing mice

Earlier we earlier showed that with tumor progression, there was a gradual tissue destruction which was accompanied with altered serum enzyme levels [13]. This systemic deterioration was found to be correlated with increased neutrophil infiltration and their hyperactive function. Therefore, after investigating the effect of TCE in regulating neutrophil count and hyperactivation systemically, we were interested to explore the effect of TCE on histoarchitecture and function of the examined organs. Liver section of tumor mice showed disturbed histo-architecture with dilated sinusoids, congestion in portal vein and increased cellular infiltrations. Interestingly, no such histopathological alterations were observed in the group receiving TCE treatment in the early stage (day 0) of tumor growth and tissue appearance was similar to the control section. Also, TCE treatment in the mid stage (day 7) of tumor growth reduced the histopathological damage and showed few cellular infiltrations. However, TCE treatment in the advanced stage of tumor growth also showed infiltrations and sinusoidal dilation but comparatively less as compared to the tumor bearing group (Fig. 6B). Similarly, lung sections from tumor bearing mice showed high cellular infiltrations which upon TCE treatment in the early stage preserved the tissue architecture and was comparable to the control. Moreover, TCE treatment in mid and advanced stages of tumor growth showed protective effects with reduced level of cellular infiltration (fig.6B). Spleen section of the tumor bearing group showed sinusoidal dilation with diffused margin between red and white pulp region as well as cellular infiltrations. In contrast, mice receiving early treatment showed normal histoarchitecture with well distributed white and red pulp showing a clear marginal zone similar to the control section. Also, TCE treatment in mid stage of tumor progression preserved the red and white pulp region with few cellular infiltrations. However, the late treatment group showed few sinusoidal dilations and cellular infiltrations (fig.6B). Similarly, peritoneum section from tumor bearing mice showed peritonitis i.e. inflammation of the peritoneum due to increased leukocyte infiltrations. Mice receiving TCE treatment in the early stage restored normal histological appearance of peritoneum as compared to the control. However, TCE treatment in the mid and advanced stage of tumor progression showed reduced cellular infiltration as compared to tumor bearing group (fig.6B). Further, we investigated the effect of TCE on the functional

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aspect of these vital organs by monitoring the level of serum enzyme levels In healthy conditions, the enzymes are present in the normal range in serum, however, during inflammation or tissue damage, their levels increase significantly which represent organ dysfunction. Fig. 6C shows the biochemical profile of mice receiving TCE treatment at different time points. Increase in aspartate aminotransferase (AST) level was observed in the tumor bearing group which showed a significant ($p<0.05$) reduction compared to the tumor group with early, mid and advanced stages of TCE treatment. Similarly, the tumor bearing group showed high alanine transaminase (ALT) levels which reduced significantly ($p<0.05$) in early, mid and advanced stages of tumor growth. Additionally, total protein content was high in case of tumor bearing groups which significantly reduced ($p<0.05$) to normal level in the early and mid stage treatment groups. However, no significant difference was observed in the total protein level in the advanced stage treatment group. We also assessed the serum creatinine level and found it to be high in the tumor bearing group as compared to the control. TCE treatment in the early and mid stage of tumor growth maintained significant ($p<0.05$) creatinine level. Furthermore, we observed high urea and albumin levels in tumor condition which showed a significant reduction upon TCE treatment in early and mid stages of tumor growth. However, the effect was not significant in the advanced stage treatment group. These results demonstrated that TCE treatment protected tissue injury and maintained the normal functioning of organs in the tumor bearing host.

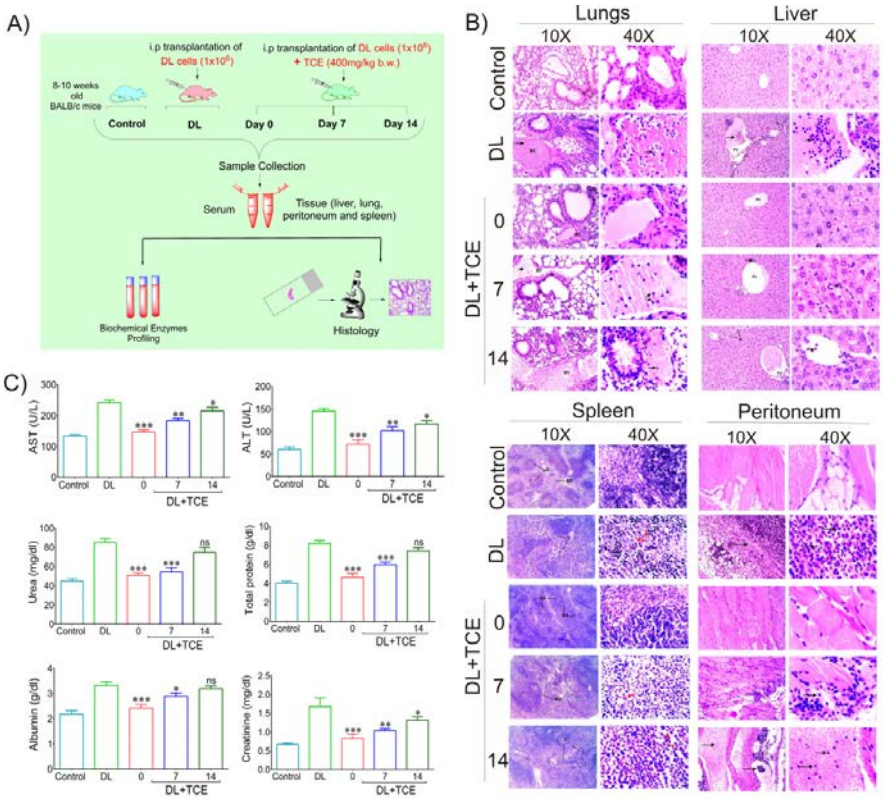

Figure 6: TCE restores tissue histoarchitecture and serum enzyme levels in tumor bearing mice. A) Schematic of the experimental design. B) Lung, liver, spleen and peritoneum were harvested at different time points post TCE treatment. 6μm thick tissue sections were prepared and processed for hematoxylin and eosin staining. Lungs: BV-blood vessel, black arrow- cellular infiltrations, Liver: PV- portal vein, a- dilations in blood sinusoids, black arrow- cellular infiltrations, Peritoneum: arrow indicates infiltrating cells, Spleen: WP- white pulp, RP- red pulp, red arrow indicates infiltrating cells, s- diffused margin between red and white pulp (magnification, × 100 and × 400 is applicable to all panels). Every image is the representative of six sections analyzed per condition. C) Assessment of AST (aspartate

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transaminase), ALT (alanine transaminase), urea, total protein, albumin and creatinine in serum of tumor bearing mice post TCE treatment in different groups. The results represent three independent experiments and are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant.

3.7. TCE limits tumor growth and increases the survival of tumor bearing mice.

Considering the significant effects of TCE treatment in limiting neutrophil-induced systemic damage in tumor bearing mice, we further examined the effect of TCE on other parameters such as body weight, girth size and anatomy of lymphoid organs. DL mice usually show an increase in body weight and girth size due to over secretion of ascites in the peritoneum and thus contribute to tumor burden. Considering the aforementioned results, we further examined the effect of TCE on the physiological parameters such as body weight, girth size and anatomy of lymphoid organs. Mice bearing Dalton's lymphoma (DL) show an increase in body weight and girth size due to over secretion of ascites which contribute to tumor burden. Interestingly, we observed a significant reduction in body weight and girth size in the groups receiving TCE treatment in early and mid stages of tumor growth (Fig. 7B). Additionally, DL mice showed enlargement of spleen i.e. splenomegaly, lymph nodes and reduction in thymus with tumor progression. TCE treatment in early, mid and advanced stages of tumor growth showed a significantly restored the anatomy of lymph nodes and thymus as compared to the tumor bearing group. However, splenomegaly was restored in early and mid stages of TCE treatment while the effect was insignificant in the advanced stage of tumor growth (Fig. 7C). Further, survival of mice was also monitored in all the experimental groups. We observed a significant increase in life span of TCE treated mice as compared to the untreated tumor controls. Interestingly, in the case of the early treatment group, mice survived for more than 70 days in contrast to untreated tumor controls, which survived only up to 25-28 days. TCE treatment in mid stage also increased the life span, up to 50 days, while TCE treatment in advanced stage increased the survival up to 40 days of tumor bearing mice (Fig. 7D). These results demonstrated that TCE exerted its therapeutic effect via mitigating neutrophil-induced systemic deterioration which would have elevated the survival of tumor bearing hosts.

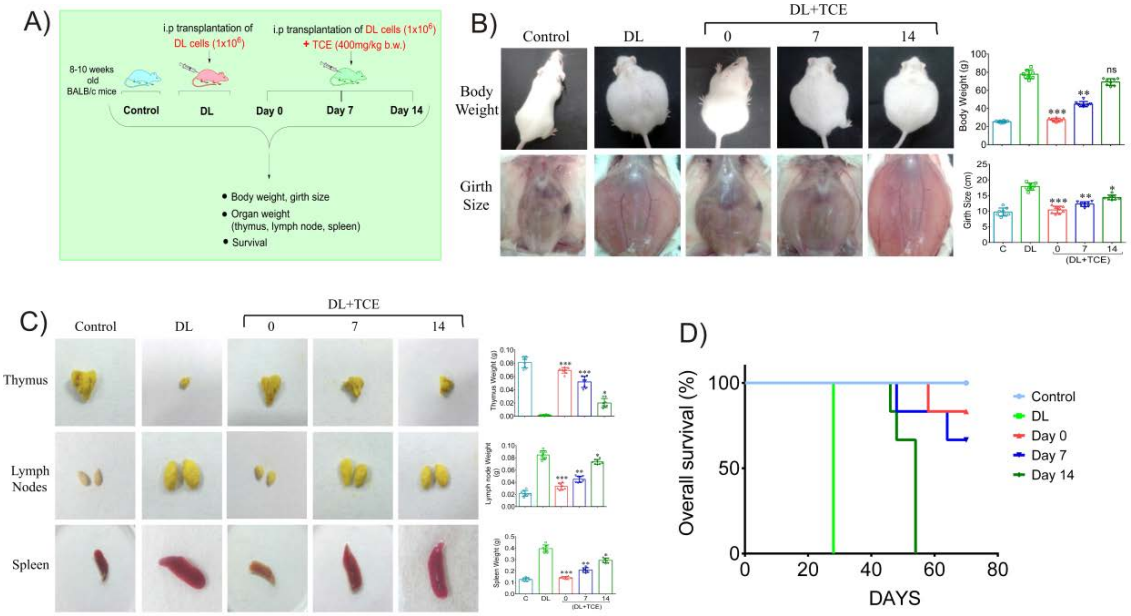

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Figure 7: TCE limits tumor growth and increases the survival of tumor bearing mice. A) Schematic of the experimental design. B) Reduction in body weight and girth size post TCE treatment. C) Restoration of lymphoid organs including thymus, lymph nodes and spleen post TCE treatment at different stages of tumor growth. D) Kaplan-Meier survival curve showing the effect of TCE on survival of tumor bearing mice post TCE treatment. The results represent three independent experiments and are expressed as the mean ±SD; statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by a tukey post hoc test where $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***), ns; non-significant.

4. Discussion

Immunomodulation by various phytoconstituents is gaining importance in the management of chronic inflammatory diseases, autoimmune diseases and neoplastic disorders due to their potent therapeutic properties with minimal side-effects [36]. In Ayurveda, *Tinospora cordifolia* (TC) is considered as one of the most divine herbs for a wide range of medicinal properties [37]. The active components of TC display promising cures for various disorders. In recent decades, research has been carried out to demonstrate the immunomodulatory potential of TC extensively on macrophages [38, 39]. However, the effect of TC on neutrophil function has not been revealed so far. Neutrophils are emerging as a crucial player in various inflammatory diseases. Recently we have shown that neutrophil infiltration and its hyperactive function with tumor progression is associated with systemic deterioration. Therefore, in the present study, we aimed to investigate the effect of TC extract (TCE) on regulation of neutrophil infiltration and hyperactivation to ameliorate cancer-induced systemic deterioration. We found that TCE treatment significantly reduced neutrophil number and function in peripheral blood as well as in vital organs of tumor bearing hosts. In parallel, with TCE treatment, we observed restoration of histoarchitecture of all the examined organs and serum enzyme levels in the tumor bearing host. Further, TCE treatment restored the body parameters as well as anatomy of lymphoid organs which were found to be affected in tumor condition. Finally, TCE treatment also increased the life span of tumor bearing mice.

Cancer is an inflammatory disease wherein neutrophils have emerged as a crucial player. Importantly, cancer patients show remarkable increase in peripheral blood neutrophil count and their infiltration in tumors [40]. Based on this NLR has been adopted as a significant measure of systemic inflammation in cancer patients [41]. In our previous study, we also observed an increase in neutrophil count in peripheral blood during tumor condition [13]. To investigate the potent immunomodulatory role of TC, we first examined its effect on neutrophil count in peripheral blood. For this, we administered TCE treatment at the early (0 day), mid (7 day) and late (14 day) stage of tumor growth. Interestingly, TCE administration at early and mid stage of tumor growth showed a significant reduction in neutrophil count in peripheral blood while reduction was insignificant in the advanced stage. Additionally, TCE treatment restored the altered levels of TLC, lymphocytes and polymorphs in tumor bearing hosts. In line with our results, previous studies have also reported a beneficial effect of TCE on blood parameters [42] however, there are no reports evaluating its effect on neutrophil count. Various reports have associated aberrant accumulation of neutrophils at tumor sites with tissue injury and poor outcomes in cancer patients [43]. Importantly, we recently reported significant findings that neutrophils also infiltrate vital organs of the tumor bearing host and their count increases significantly from early to late stages of tumor growth [13]. Therefore, we further tested the efficacy of TCE in regulation of systemic neutrophil infiltration. Interestingly, TCE treatment at early and mid stage of tumor growth showed a significant reduction in neutrophil infiltration in liver, lungs, spleen and peritoneum. A recent study also showed that TCE treatment reduced infiltration of pro-inflammatory immune cells such as granulocytes and macrophages into the swim bladder of humanized zebrafish model of SARS-CoV-2 spike-protein induced disease phenotype [44]. Based on the reduction in

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systemic neutrophil infiltration, it can be speculated that TCE exerts its ameliorating effects via regulating the neutrophil functions. To decipher this, we further looked into the effect of TCE on the status of neutrophil-derived granular cargoes in the vital organs.

The dynamic functions of neutrophils are dedicated to the different cytoplasmic granules present in the mature neutrophil which house an array of key effector molecules [4]. Upon reaching the site of infection, neutrophils are activated by a multitude of inflammatory mediators, which triggers the release of its key effector molecules. Various reports have shown the involvement of these granular cargoes in contributing to tissue damage [3]. NE, a key cargo stored in the primary granules of neutrophils, can hydrolyze a variety of ECM components [27]. In addition to its role in host defense, evidence suggests an important contribution of NE in various chronic inflammatory diseases, including cancer [45]. Recently, we also suggested a crucial role of NE in mediating systemic tissue damage via upregulated activity of NE in serum and vital organs of tumor bearing host [13]. In the present study, we found that TCE treatment significantly reduced high NE expression in peripheral blood neutrophils. In addition, TCE also reduced high NE activity in serum and ascites along with vital organs including liver, lungs, spleen and peritoneum. Other investigators have also shown the protective effects of TCE in animal models of osteoporosis and arthritis wherein TCE limits the production of inflammatory immune mediators [46, 47]. Like NE, MPO is another key cargo stored in the primary granules of neutrophils and its excessive release can further amplify inflammatory response [28]. MPO catalyzes the formation of ROS which is also known to play a key role in the severity of various diseases [48]. Under stress or disease conditions, production of ROS increases as compared to the enzymatic antioxidants. This imbalance causes cellular damage and results in severe health issues [49, 50]. We found that TCE treatment was able to reduce high expression of MPO in liver, lungs, spleen and peritoneum tissue. Impressively, it also showed a significant reduction in ROS levels in all the examined organs in early and mid stage of treatment. From these results, we apprehend that possibly reduction in MPO expression further resulted in a decrease in the oxidative stress in the vital organs which subsequently might be responsible for a reduction in tissue injury. Tiwari et al. also reported that TCE treatment reduced the high MPO activity with subsequent reduction in oxidative stress which ameliorated tissue injury in murine models of asthma [51]. Arunachalam et al. have recently reviewed an important role of *Tinospora cordifolia* in oxidative stress-related diseases [52]. These results suggest that the strong antioxidant activity of TCE might be attributed to the regulation of neutrophil derived MPO and ROS. Neutrophils are known to be the major source of MMP-8 and MMP-9 [53]. MMP-8, or neutrophil collagenase, is involved in degradation of all structural components of the extracellular matrix and plays a crucial role in a range of inflammatory disorders including cancer [31, 54]. Similarly, MMP-9, known as gelatinase B, is also one of the key players in ECM degradation and contributes to cancer metastasis via promoting angiogenesis [55]. We have earlier shown an upregulated expression of MMP-8 and MMP-9 in vital organs of tumor bearing mice with tumor progression and suggested their crucial role in tissue injury [13]. Here, TCE treatment at early and mid stages of tumor growth showed a significant reduction in MMP-8 and MMP-9 in all the examined organs. A previous study has also shown a marked reduction in MMP-9 activity upon TCE treatment in autoimmune arthritis [47] thus corroborates our results. Neutrophils are also equipped with CTSG, a serine cathepsin which is stored in their azurophilic granules together with NE [27]. Similar to MMP-8 and MMP-9, CSTG is also a degradative enzyme, which can perform extracellular functions such as degradation of ECM and hydrolysis of host plasma proteins [56]. Besides its potent anti-microbial role, CTSG has been identified to play a very important role in inflammation, tumor growth, and progression [57]. We observed high expression of CSTG in liver, lungs, spleen and peritoneum of tumor bearing mice. Interestingly, we observed a significant reduction in CSTG expression with TCE treatment at both early and mid stage of TCE treatment. However, we did not observe any significant effect of TCE treatment in the advanced stage of tumor growth.

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These results suggest that TCE can actively regulate the expression of effector mediators of neutrophils which could ameliorate neutrophil-induced systemic damage in cancer conditions. To investigate this, we extended our study to examine the effect of TCE on the histoarchitecture of vital organs wherein tissue morphology, nuclei structure and cellular infiltrations can be deduced [58]. Interestingly, upon TCE treatment, the vital organs including liver, lungs, spleen and peritoneum revealed a normal histoarchitecture with few or no cellular infiltrations at early and mid stages of tumor growth. However, moderate effects were observed at the advanced stage of tumor growth. We further monitored the effect of TCE on serum enzyme levels such as AST, ALT, urea, albumin, creatinine and total protein levels. The normal range of these enzymes in serum depict the normal tissue function however any alteration in their levels suggest tissue injury, inflammatory state and organ dysfunction [59]. We earlier reported gradual alterations in serum enzyme levels with tumor progression. In the present study, TCE treatment at early and mid stage of tumor growth showed a significant restoration of serum enzyme levels. Similarly, Sachdeva et al. showed a hepatoprotective, nephroprotective, and immunomodulatory function of TCE wherein it was found to reduce the level of AST, ALT, urea, creatinine and other electrolytes in serum [60]. Various other reports have also suggested the effect of *Tinospora cordifolia* in ameliorating histopathological and biochemical changes [24, 61, 62]. These findings corroborate our results and further suggest that TCE could be used as a strong candidate in ameliorating systemic deterioration.

Considering the regulatory role of TCE on neutrophil number and function systemically which further restored the organ structure and function, we next examined the effect of TCE on tumor burden and survival of the DL bearing mice. In DL mice, tumor burden is governed by gradual increase in body weight and girth size with tumor progression. It accounts for the exponential growth rate of DL cells with concomitant release of tumor factors contributing to increased volume of ascites. Interestingly, TCE treatment at early and mid stage of tumor growth reduced the tumor burden as evident by a significant reduction in body weight and girth size of the tumor bearing mice. In a study by Thippeswamy and Salimath, TCE inhibited cell proliferation and decreased ascites secretion in *Ehrlich ascites* tumor (EAT) mice [63]. Anti-tumor effects of TCE are very well reported in various cancer cell lines as well as in mice models [64-66]. DL bearing mice also show an alteration in lymphoid organs such as reduction in thymus, enlargement of spleen and lymph nodes [67]. TCE treatment revealed a protective effect by restoring the size of these organs. Singh et al. has also reported the restoration of thymic homeostasis upon TCE administration [68] and thus corroborates our study. Furthermore, TCE treatment at early and mid stage of tumor growth increased the survival of tumor bearing mice However, the group receiving TCE in the advanced stage of tumor growth did not display a significant reduction in tumor load but surprisingly we observed a remarkable increase in survival period. Considering these results, we can suggest that regulation of neutrophil number and its hyperactivation by TCE treatment could be attributed to amelioration of systemic deterioration compared to the tumor bearing counterparts which further increased the survival of tumor bearing hosts. To our knowledge, this is the first *in vivo* report which demonstrates the potential role of *Tinospora cordifolia* in ameliorating cancer-induced systemic damage via regulation of neutrophils. However more investigations are needed to precisely understand the regulatory mechanisms of TCE on neutrophils counts and their functions. The present study has some limitations. The protective effect of TCE was evaluated at a single dose i.e. 400mg/kg b.wt. At this dose, we observed significant results in early and mid stages of tumor growth. However no such effects were seen in the group receiving treatment in the advanced stage of tumor growth though it showed a remarkable increase in survival as compared to the tumor bearing counterparts. Therefore, a higher dose could be tested to investigate the effect of TCE at an advanced stage of tumor growth. Nonetheless it opens

the avenue for further research including mechanistic studies wherein neutrophils can be targeted with potential nutraceuticals.

5. Conclusion

Most of the cancer-related deaths are due to systemic deterioration which ultimately causes organ failure. In view of this, regulation of systemic damage can be a better therapeutic approach in cancer management. Recently, we have shown that neutrophils are important contributors in mediating systemic effects of cancer. Therefore, in the present study we explored the effect of potent immunomodulator, *Tinospora cordifolia* in regulating neutrophils to ameliorate systemic deterioration in cancer conditions. The novel observations of the present study convincingly show that TCE administration at early and mid stage of tumor growth regulates neutrophil infiltration and hyperactivation which ameliorated cancer-induced systemic damage and further substantiates the anti-tumor actions of TCE. However, detailed mechanistic insights will present a comprehensive outlook for its applicability as a potent anti-cancer drug to ameliorate systemic damage as well as to improve the quality of life in cancer patients.

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Author's contributions: Kavita Rawat and Anju Shrivastava conceptualized, designed, and analyzed the experiments. Kavita Rawat performed the experiments and wrote the original draft. Kavita Rawat, Saima Syeda and Anju Shrivastava reviewed and edited the manuscript. All authors read the manuscript carefully and approved the final manuscript.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable

Ethics approval The study was performed in accordance with the guidance for the care and use of laboratory animals with approval of the University of Delhi and Committee for the Purpose of Control and Suppression of Experiments on Animals (CPCSEA), India.

Consent to participate Not applicable

Consent to publication Not applicable

Conflicts of Interest The authors declare no competing interests

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