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Hyperactive neutrophils infiltrate vital organs of tumor bearing host and contribute to gradual systemic deterioration with tumor progression

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Abstract

Various studies have addressed the role of neutrophils in cancer wherein the focus has been drawn on the elevated neutrophil count in blood or at tumor loci. However, cancer has a systemic impact which targets various organs thus challenging the overall physiology of the host. So, it is worthwhile to explore whether and how neutrophils contribute to systemic deterioration in cancer. To discern the systemic role of neutrophils, we monitored their number and function at different stages of tumor growth in Dalton's lymphoma mice model. Notably, we observed a gradual increase in neutrophil count in blood and their infiltration in vital organs of tumor bearing mice. In parallel, we observed damaged histoarchitecture with significant alterations in biochemical parameters that aggravated with tumor progression. We next examined systemic impact of neutrophil by assessing neutrophil elastase, myeloperoxidase, and matrix metalloproteinases (MMP-8 and MMP-9) wherein we found their upregulated expression and activity in tumor condition. Taken together, our results demonstrate high infiltration and hyperactivation of neutrophils which possibly account for gradual systemic deterioration during cancer progression. Our findings thus implicate neutrophils as a potential therapeutic target that may help to reduce the overall fatality rate of cancer.

Keywords: Neutrophils, systemic deterioration, hyperactivation, neutrophil elastase, matrix metalloproteinases, myeloperoxidase

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1. Introduction

Cancer initially grows as a local disease but eventually turns into a complex systemic disease which targets various organs in the tumor bearing host (Shinko, Diakos et al. 2017). The systemic effects comprise metastasis, inflammation, thrombosis, cachexia that gradually leads to functional impairment of organs. Indeed, these systemic effects cause the majority of cancer-related deaths, rather than the primary or metastatic tumors (Cedervall, Dimberg et al. 2015, Rutkowski, Svoronos et al. 2015). Importantly, the majority of these systemic manifestations appear to arise from chronic inflammation caused by the cancer cells and aberrant immune response of the host. Substantial evidence suggests that these states of immune dysregulation and excessive inflammation target different organ systems with potentially lethal or highly morbid conditions (Greten and Grivennikov 2019). Hence, the view of cancer as a systemic disease is now emerging into the research spotlight and the quest is to comprehend the underlying mechanism behind the evolution of a local disease to an intricate systemic ailment.

Chronic inflammation is one of the several hallmarks predisposing to cancer growth and progression (Schmidt and Weber 2006). The inflammatory immune component of a developing tumor may include a diverse leukocyte population such as, macrophages, eosinophils, mast cells, lymphocytes and neutrophils which are capable of producing an array of inflammatory mediators (Wahl and Kleinman 1998, Kuper, Adami et al. 2001). Neutrophils, which are the most important effector cells of innate immunity, have been shown to play a crucial role in tumor biology. They have recently become the subject of intense research with focus on the association between inflammation and cancer progression. Being the professional phagocytes, they are the first one to migrate at the site of infection, thus constitute the first line of defense. At the site of infection, once neutrophil function is over, their clearance is essential for resolution of inflammation to maintain tissue homeostasis. But, failure in the resolution machinery and prolonged neutrophil accumulation can damage the host tissue and reflect a state of chronic inflammation (Van Leeuwen, Gijbels et al. 2008, Mollinedo 2019). Of note, cancer patients show remarkable increase in peripheral blood neutrophil count and their infiltration in tumors. Based on this observation, neutrophil-to-lymphocyte ratio (NLR), an indicator of inflammation, has been adopted as a prognostic sign of poor survival in cancer patients. High neutrophil infiltration in patients with renal cell carcinoma, bronchi alveolar carcinoma, melanoma and colon cancer

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has been correlated with increased mortality (Gregory and Houghton 2011, Eruslanov, Bhojnagarwala et al. 2014). Activation of neutrophils, in response to various stimuli can extend their life span which can further influence tumor cells. In turn, the tumor-derived stimuli can induce phenotypic and functional changes in neutrophils which are known to support tumor growth, metastasis and immunosuppression (Zhang, Zhang et al. 2016).

Neutrophils are equipped with multiple mechanisms to eradicate the invading microbes such as respiratory burst, phagocytosis, neutrophils extracellular traps (NET) formation and degranulation (Yin and Heit 2018). In the process of degranulation, neutrophils release an array of proteins stored in their cytosolic granules into the extracellular spaces (Jasper, McIver et al. 2019). These granules play a major role in every step of neutrophil inflammatory response. They have potent anti-microbial properties and regulate inflammation via processing of chemokines, cytokines and various signaling molecules (Harbort, Soeiro-Pereira et al. 2015). These granules include antimicrobial proteins (lysozyme, lactoferrin), serine proteases (neutrophil elastase, cathepsin G), matrixmetalloproteinases (MMP-8, MMP-9) and myeloperoxidase (Borregaard and Cowland 1997). Effector functions of neutrophils largely depend upon the release of these granular cargoes. The controlled mobilization and release of these cargoes allows the transformation of neutrophils from inactive circulating cells to active effector cells of the innate immune system**.** However, under certain circumstances, unregulated release of these effector molecules can aggravate tissue damage and could be detrimental to the host (Pham 2008). In cancer setting, their excessive release can modulate tissue microenvironment (Coffelt, Wellenstein et al. 2016) and ultimately lead the way for tumor initiation, growth and metastasis (Rawat, Syeda et al. 2021). Their aberrant activation or persistence at inflammatory site is also associated with various inflammatory disorders ranging from chronic obstructive pulmonary disease (COPD) (Meijer, Rijkers et al. 2013), neutrophilic asthma (Chung 2016), rheumatoid arthritis (Apel, Zychlinsky et al. 2018) to the recent pandemic COVID-19 (Ackermann, Anders et al. 2021).

Substantial reports have focused on the role of neutrophils particularly within the tumor microenvironment or at the inflammation site; however, whether they impact systemic milieu or not is still a question. In the present study, we aimed to address this question for which we used a well-accepted Dalton's lymphoma (DL) mice model. DL is a murine non-Hodgkin's T-cell

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lymphoma which represents an excellent model to examine various parameters of cancer development, signaling mechanisms, and also for therapeutic drug screening (Prasad, Rosangkima et al. 2010, Kumari, Rawat et al. 2017). Here, we first examined the status of neutrophil count in peripheral blood and their infiltration in vital organs of tumor bearing mice at different stages of tumor growth. Further, we monitored the organ functions by evaluating the histo-architecture and biochemical enzymes level with disease progression. In addition, we monitored the status of neutrophil-derived granule cargoes including neutrophil elastase (NE), myeloperoxidase (MPO) and MMPs (MMP-8 and MMP-9) in order to evaluate neutrophil function in the systemic environment. Our results demonstrated the potential involvement of neutrophils in mediating systemic effects during tumor progression.

2. Materials and Methods

Leishman's stain, paraformaldehyde, methanol, glycerol, Coomassie brilliant blue (R-250), hematoxylin and eosin were obtained from SRL India. DAB (3,3'-diaminobenzidine), RNA Later, Revert-Aid first strand cDNA synthesis kit was purchased from Thermo Scientific. Poly-L-lysine, $H_2O_2$and citrate buffer were from Sigma-Aldrich. DHE (dihydroethidium) dye was from Invitrogen Molecular Probes, D11347.The elastase kit was purchased from Elabscience (Houston, Texas). Anti-Ly6G and goat anti-rat secondary were purchased from Abcam (Cambridge, USA). Anti-MPO, anti-elastase were purchased from cloud clone corp. Xylene was purchased from Fisher scientific, absolute alcohol was from Merck and RNeasy Micro Kit was from Qiagen. DPX, slides, cover slips and other reagents were of the highest analytical grade and were obtained from the common source**.**

2.1 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, where fresh and clean drinking water was supplied ad libitum with a standard pellet diet. Throughout the period of the experiment, animals were kept in 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.

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2.2 Tumor induction and maintenance: Dalton's lymphoma (DL) cells were obtained from Department of Biotechnology, Banaras Hindu University. The cells were maintained in the peritoneum of BALB/c mice by serial intraperitoneal transplantation as described earlier (Manjula Vinayak, 2015). 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.

2.3 Experimental Groups: BALB/c mice of either sex, each weighing 22-25g and aged 3-4 months were divided into 4 groups, with each group consisting of six mice. Group I served as control and in the rest three groups, DL was induced by injecting 1×10^6 cell/ml of tumor cell suspension i.p. The tumor was allowed to grow and mice were sacrificed through cervical dislocation on different time points i.e. on 7, 14 and 21 day post tumor transplantation.

2.4 Hematological and biochemical parameters: Animals were sacrificed at different time points of tumor growth. The blood samples were collected into collection tubes with and without EDTA (anti-coagulant). EDTA containing tubes were used for analysis of polymorphs, lymphocytes and total leukocyte count (TLC). The blood in non-EDTA tubes was allowed to stand for one hour at room temperature and then centrifuged at 1000g for 10 minutes. Serum was obtained which was used for the determination of various biochemical parameters like total protein, urea, albumin, ALT, AST and creatinine using commercially available kits (Erba diagnostic kits).

2.5 Histological Examination: Mice were sacrificed at different time points and organs such as lungs, liver, kidney, spleen, lymph node and peritoneum were collected and rinsed with PBS. Subsequently, tissues were fixed in neutral buffer formalin (NBF) and then embedded in paraffin for histopathological examination. The thin sections of 6µm were cut and transferred on clean slides. The slides were further processed and then stained with hematoxylin and eosin and observed under a light microscope using NIS Element software.

2.6 Immunohistochemistry: Samples were fixed in 4%PFA, embedded in paraffin and were cut into 5µm sections. Sections were deparaffinized in xylene twice, for 10 minutes each, rehydrated

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

2.7 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. Slides were deparaffinized in xylene twice, for 10 minutes each, rehydrated with graded ethanol, 100%, 95%, 80%, 70% and 50%, for 5 minutes each, and transferred to tap water. Slides were heated in sodium citrate buffer (pH 6.0) solution at 95°C for 20 minutes for antigen retrieval. The endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 for 20 min. Further, non-specific reactivity was blocked by incubating the slides with 5% normal goat serum for 1h. For NE expressions in blood and various tissues, 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).

2.8 Flow cytometry: 200µlofblood was diluted 1:1 with PBS. After RBC lysis, cells were incubated in 4%PFA for 20 minutes. Cells were washed two times with PBST and incubated with anti-Ly6G primary antibody (1:100) and kept on ice at 4°Cfor 1h. After washing with PBST

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(0.2% Tween-20), cells were incubated with Alexa fluor 488 goat anti-rat secondary antibody (1:200) for 30 minutes in dark at room temperature. Finally the cells were resuspended in 500µl PBS and acquired in BDAccuri C6 cytometer.

2.9 Preparation of tissue sections for O_2 detection: The exteriorized tissues were rinsed immediately in chilled PBS and snap frozen in liquid nitrogen. 20µm thick cryosections were obtained using cryostat and placed on clean poly L-lysine coated slides. DHE was suspended in DMSO at 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 analyzed under fluorescence microscope (Nikon). Minimum six slides per condition were analyzed for ROS quantification.

2.10 Detection of NE by ELISA: Blood samples were collected in microcentrifuge tubes under sterile conditions. Samples were allowed to clot and centrifuged at 1000g for 10 minutes. Serum was separated and stored in aliquots at -80°C. Ascitic fluid was also collected from tumor bearing mice and stored at -80°C. The tissues were removed and rinsed in sterile PBS, blotted on tissue (to remove excess buffer) and weighed. 600µl of RIPA lysis buffer containing protease inhibitor cocktail was added to 5mg tissue and homogenized using electric homogenizer. After homogenization, samples were kept on ice for 30 minutes before transfer into pre-labeled micro centrifuge tubes. Samples were centrifuged at 4°C, 10000g for 10 minutes. Supernatants from each sample were removed, aliquoted and stored at -80°Cfor further analysis. The levels of NE in serum, ascites and tissue specimen were detected using Elisa kit according to the manufacturer's protocol (Elabscience) and the absorbance was measured by a multi detection microplate reader (Biotek).

2.11 Gelatin Zymography: MMP-9 enzymatic activity in serum, ascitic fluid and tissue lysates was determined by SDS-PAGE gelatin zymography. Gelatinases present in the samples degrade the gelatin matrix in the gel thus leaving a clear band after staining the gel. Tissue samples were homogenized mechanically in RIPA lysis buffer containing protease inhibitor cocktail and then centrifuged at 4ºC to obtain clear supernatant. Protein concentrations in different samples were then estimated with Bradford assay. Briefly, serum, ascitic fluid and homogenized tissue samples normalized to an equal amount of protein i.e. 30µg were electrophoresed on 10% SDS-PAGE

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containing 1mg/ml gelatin in tris-glycine SDS running buffer. Positive control MMP-9 recombinant protein was also included. Gels were then incubated in 2% Triton X-100 solution at room temperature for 1h on shaker to remove the SDS and allow protein renaturation. Gels were subsequently incubated at 37°C for 48h in the developing buffer. Thereafter, gels were stained with 0.25% Coomassie brilliant Blue (R-250) for 30 minutes and destained with 30% methanol, 10% acetic acid solution. Gelatinase activity was detected as a white band against a blue background. Gel images were captured using Amersham imager 600 (GE Healthcare) and band densitometry was assessed using ImageJ software to obtain a semi-quantitative presentation of enzymatic activity.

2.12 RNA extraction and PCR analysis: Total RNA from various tissues 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, K1622). The cDNA was used to perform semi-quantitative PCR for NE, MPO, MMP-8, MMP-9 and GAPDH. 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
GAPDH	CACACCGACCTTCACCATTTT	AGACAGCCGCATCTTCTTGT

2.13 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 two groups was

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determined by two-tailed Student's t-test otherwise 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 Experimental scheme. The present study was carried out in DL mice model which is a murine non-Hodgkin's transplantable T cell lymphoma. It represents an excellent model to examine various parameters of cancer development, signaling mechanisms, and also for therapeutic drug screening (Prasad, Rosangkima et al. 2010, Kumari, Rawat et al. 2017). DL was induced by injecting 1×10^6 cell/ml of tumor cell suspension i.p. The tumor was allowed to grow and mice were sacrificed at different time points i.e. on day 7 which represents the early phase, day 14 which represents the mid phase and day 21 which represents the late phase of tumor growth. For the experiments, we collected blood samples and major tissues which include liver, lungs, spleen, peritoneum, kidney and lymph nodes.

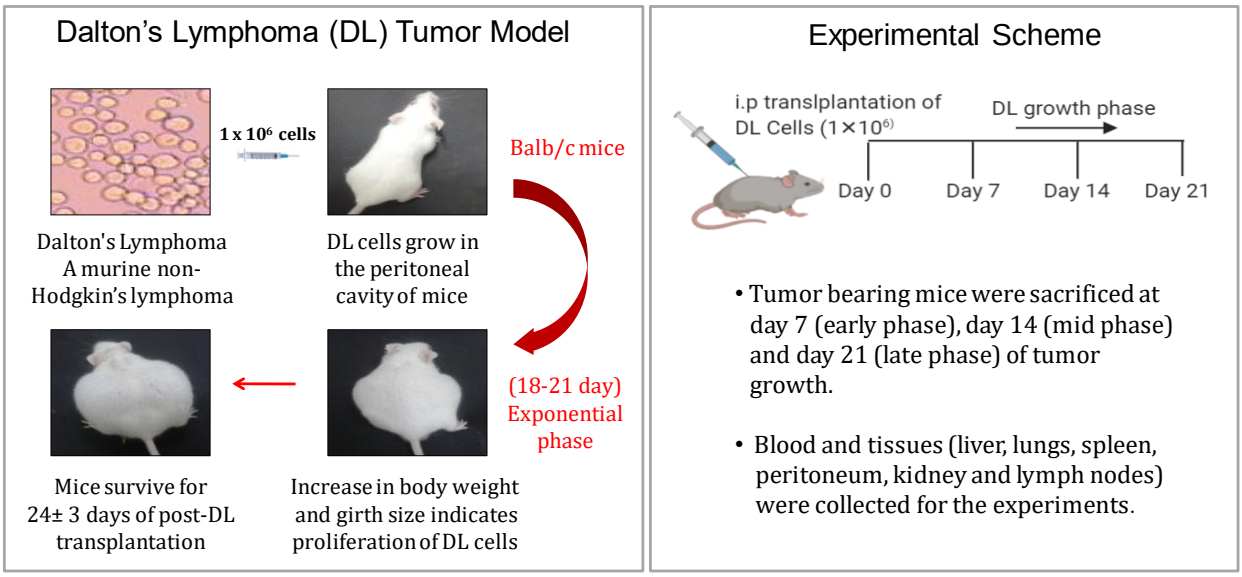

Figure 1 Schematic representation of tumor model and experimental design.

3.2 Neutrophil count increases in peripheral blood and vital organs of tumor bearing mice. To explore the involvement of neutrophils in mediating systemic effect with tumor progression, we first evaluated their presence in the peripheral blood of DL bearing mice (fig. 2A). DL cells (1×10^6) were intraperitoneally transplanted in healthy mice and the tumor was allowed to grow.

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Increase in body weight and girth size evaluated the tumor growth (fig. 2B). DL bearing mice generally have a survival of 24±3 days and we selected three time points i.e. day 7 (early phase), day 14 (mid phase), and day 21 (late phase) for the study. Blood samples were collected and processed for hematological analysis. We observed a gradual increase in the total leucocytes count (TLC) and polymorphs count with a parallel reduction in lymphocyte number with tumor progression (fig. 2C). Also, we confirmed the presence of neutrophils using anti-Ly6G antibody, neutrophil marker, wherein we observed increased $Ly6G^+$cells in peripheral blood of tumor bearing mice (fig. 2D). To further quantitate the elevated neutrophil count in blood, WBCs were isolated from peripheral blood at different time points, stained with anti-Ly6G and subjected to flow cytometry (fig. 2E). The percentage of $Ly6G^+$ cells in normal mice was around 34.6% which showed a gradual increase with tumor progression. On the 7th day of tumor growth, we observed 72.9% of $Ly6G^+$cells which increased to 77.4% on the 14th day of tumor growth. At the later phase of tumor growth i.e. on the 21^{st} day, the percentage of $Ly6G^+$cells further increased to 81.6%. As we observed a gradual increase in neutrophil count in peripheral blood we speculated that the presence of neutrophils in different tissues could be a more relevant determinant and marker of persistent inflammation than the circulating neutrophils. Therefore, we next examined the presence of neutrophils in vital organs of tumor bearing mice. The organs selected for the study were liver, lungs, spleen, peritoneum, kidney and lymph nodes. We performed immunohistochemistry wherein 6µm thick paraffin embedded tissue sections were processed and incubated with primary anti-Ly6G. Incubation with appropriate secondary antibody was followed by direct DAB staining and counterstained with hematoxylin. Interestingly, we found a systemic presence of neutrophils as the number of positive cells for Ly6G were significantly high ($p<0.05$) in all the examined organs of tumor bearing mice as compared to the control (fig. 2F). Collectively, our results showed a systemic increase in neutrophil infiltration in vital organs which suggest their crucial role in mediating systemic effects during tumor progression.

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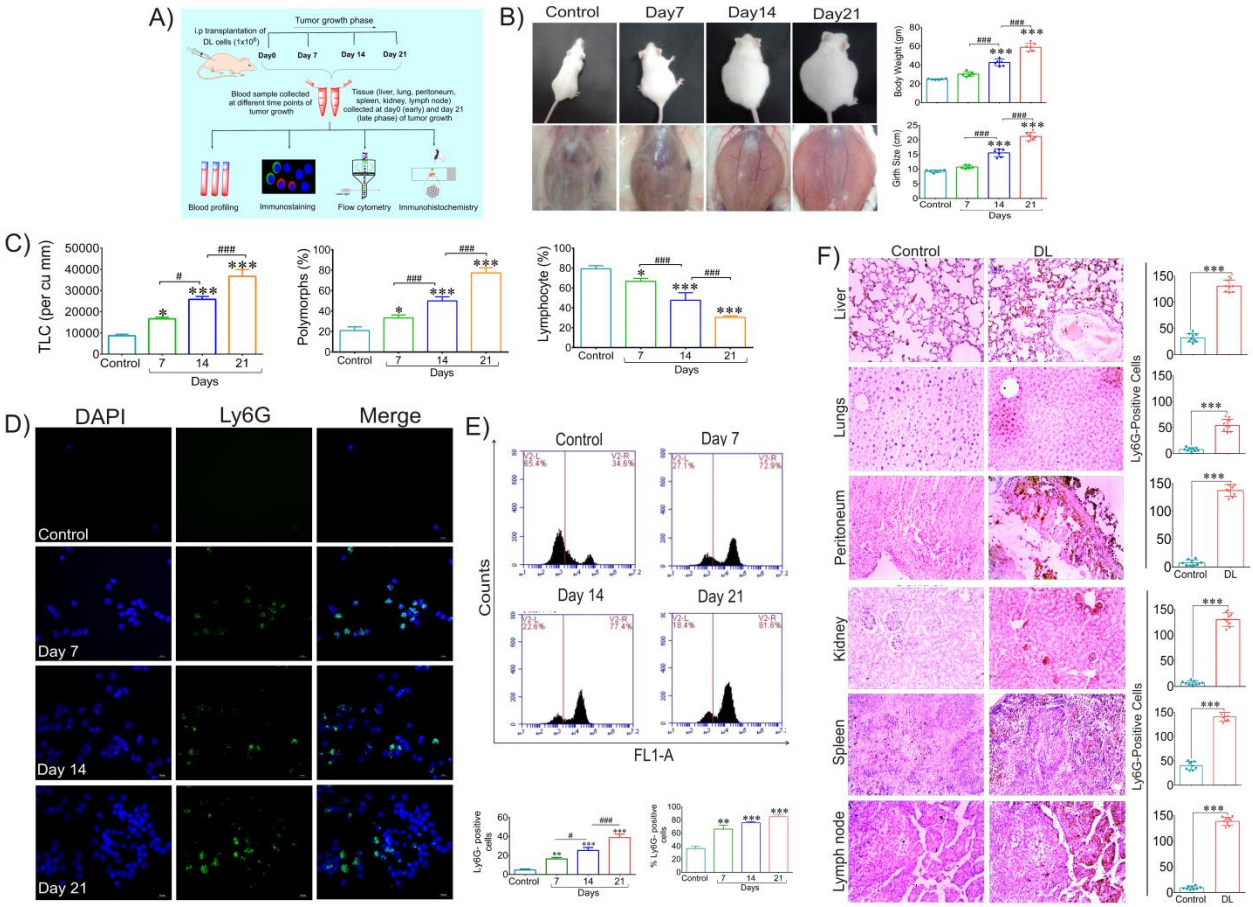

Figure 2 Neutrophil count increases in peripheral blood and vital organs with tumor progression. A) Schematic of the experimental design. B) Increase in body weight and girth size of DL bearing mice. C) Total leukocyte count, polymorphs and lymphocyte count determined in the peripheral blood of DL bearing mice with tumor progression. D) Immunofluorescence staining of blood smear at different time points with antibody directed against Ly6G (green), (magnification, × 600, scale bar, 10 μm). E) WBCs were separated from blood, stained with Ly6G antibody and subjected to flow cytometry at different time points of tumor growth. The bar graphs showing quantitative analysis of immunostaining data (Ly6G positive cells) performed using ImageJ software and flow cytometry data (percentage of Ly6G positive cells). 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 $p<0.05$(*), $p<0.02$(**) and $p<0.001$(***). F) Positive staining for neutrophils (Ly6G) was examined by immunohistochemistry in liver, lungs, peritoneum, kidney, spleen and lymph nodes wherein bar graphs showing $Ly6G^+$ cells counted in six different regions. Every image is the representative of six sections analyzed per condition. Data are shown from one of the three independent experiments with a similar pattern of results. The statistical significance between two groups was determined by two-tailed Student's t-test and results are expressed as mean ±SD and differences were considered significant where $p < 0.05$ (*), $p<0.02$(**) and $p<0.001$(***).

3.3 Systemic deterioration aggravates with tumor progression. Neutrophils are inflammatory cells and their aberrant accumulation is often associated with tissue injury (Castanheira and Kubes 2019). We observed increased infiltration of neutrophils in vital organs of tumor bearing mice; therefore, we next investigated the histoarchitecture of liver, lungs, spleen, peritoneum,

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kidney and lymph nodes. Histological studies are routinely performed to have a broad vision of disease and delineate its consequence on tissues, as the histological procedure preserves the original tissue architecture. Important characteristics of disease such as tissue morphology, nuclei structure and lymphocytic infiltrations can be deduced only from a histopathological image (Gurcan, Boucheron et al. 2009).We performed histological analysis at different time points i.e. on 7, 14 and 21st day of tumor growth to correlate it with gradually increased neutrophil number in the vital organs. Interestingly, it revealed a high cellular infiltration in all the examined organs (fig. 3B) which was further accompanied by a disturbed histoarchitecture. In the lungs of tumor-bearing mice, a high number of infiltrating polymorphic cells (black arrow) was correlated with congestion in blood vessels which indicate the inflammatory response. In contrast, the lung section of normal mice showed clear tissue architecture with no cellular infiltration or congestion. Similarly, the liver section of DL mice showed congestion in the portal vein (b) and disturbed hepatocytes morphology (d). Dilations of blood sinusoids (e) were also observed with an increased number of kupffer cells (c) as well as cellular infiltrations (a). No such alterations were observed in the control sections. In the peritoneum which is the site of tumor growth, we observed an increase in the level of peritonitis i.e. the inflammation of peritoneum due to increased cellular infiltration (black arrow). Such cellular infiltrates were strikingly less abundant in the peritoneum sections of control mice. We also examined the kidney section of normal mice wherein we found that the glomerulus was surrounded by the intact basement membrane and Bowman's capsule, with no cellular infiltration while in DL bearing mice thickening of basement membrane, disruption of glomerulus architecture (thin arrow) and increase in the number of mesangial cells (thick arrow) was observed. Looking at the spleen section, control mice showed a well distributed red and white pulp region with clear marginal zone and normal histoarchitecture. But with tumor progression, we observed a high rate of infiltrating cells (black arrow) and the margin between red pulp and white pulp started to disappear. Splenomegaly i.e. enlargement of the spleen is a common characteristic of spleen damage and spleen hyperfunction (McKenzie, Colonne et al. 2018), which was observed in the spleen sections of DL bearing mice. Lymph node sections obtained from DL bearing mice showed reduced size and number of germinal follicles (G) with depletion of cortical lymphocytes. Also, gradually increased leukocyte infiltration (black arrow) with tumor progression resulted in lymphadenitis i.e. inflammation of lymph nodes. No such changes were

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observed in the control sections. Further, we monitored the biochemical enzyme levels to investigate the function of the vital organs in the tumor bearing mice. In healthy conditions, the enzymes are present in the normal range in serum however in the state of inflammation or tissue damage, their levels increase (Wang, Feng et al. 2006). Serum was collected on 7, 14 and 21st day of tumor growth and analyzed for AST, ALT, urea, albumin, creatinine and total protein levels. With tumor progression, we observed a significant rise in the serum ALT and AST levels that suggest liver damage. Albumin, creatinine and total protein levels were also elevated and showed a gradual increase ($p<0.05$) with tumor growth. Similarly, we found a significant difference ($p<0.05$) in urea levels on the later stage of tumor progression (fig. 3B). These altered enzyme levels and the damaged histoarchitecture thus suggest systemic organ dysfunction in tumor bearing mice and point towards the crucial involvement of neutrophils.

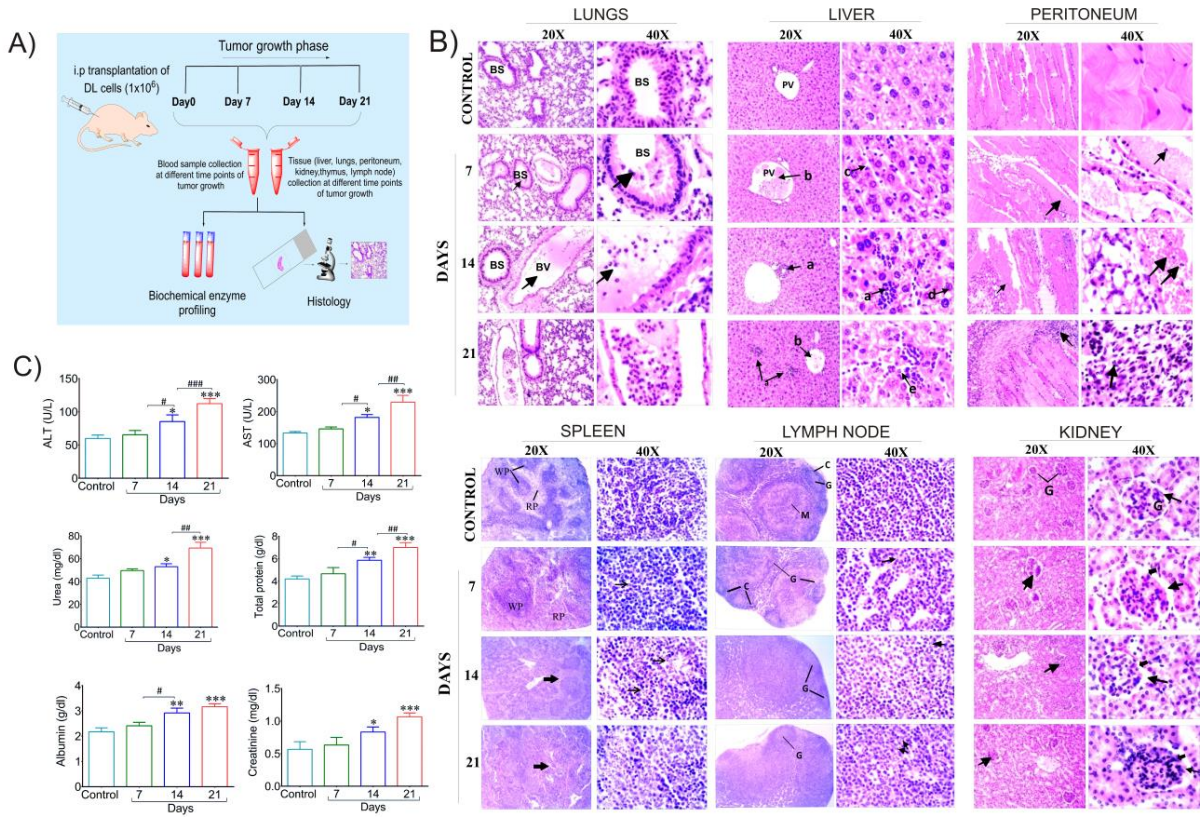

Figure 3 Systemic deterioration aggravates with tumor progression. A) Schematic of the experimental design. B) Lung, liver, peritoneum, kidney, spleen and lymph nodes were harvested on 7, 14 and 21st day post tumor transplantation. 6µm thick tissue sections were prepared and processed for hematoxylin and eosin staining**.** Lungs: BV-blood vessel, BS-bronchiole, Liver: PV- portal vein, a-cellular infiltrations, b-congestion in portal vein, c-increase in number of kupffer cells, d-disturbance of hepatocytes, e-dilations in blood sinusoids, Peritoneum: arrow indicates infiltrating cells, Spleen: WP- white pulp, RP- red pulp, Lymph node: G-germinal follicles, M- medulla**, C-**cortex, Kidney: thin arrow indicates infiltrating cells, thick arrow- mesangial cells, (magnification, × 100 and × 400

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is applicable to all panels. Every image is the representative of six sections analyzed per condition. C) Assessment of ALT (alanine transaminase), AST (aspartate transaminase), urea, total protein, albumin and creatinine in serum of tumor bearing mice at different time points of tumor growth. 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 $p<0.05$(*), $p<0.02$(**) and $p<0.001$(***).

3.4 Peripheral blood neutrophils attain a hyperactive state with tumor progression. We were further interested in knowing the change in the activation state of neutrophils and whether it correlates with the histological changes observed during tumor progression. Interestingly, it has been revealed that inflammation is an early and sensitive event which represents the hyperactive state of neutrophils in peripheral blood (Ling, Chapple et al. 2015). These neutrophils release toxic mediators packed in their distinct granule subsets and thus contribute to tissue injury (Moraes, Zurawska et al. 2006). To assess neutrophil function, we first examined the expression of NE and MPO in peripheral blood neutrophils. 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 (Suzuki, Okada et al. 2019). Similarly, MPO is a heme peroxidase enzyme abundantly expressed in neutrophils. It catalyzes the reaction between hydrogen peroxide and chloride to generate hypochlorous acid which further generates reactive oxygen intermediates for pathogen removal. However, excess generation of MPO-derived oxidants has been linked to tissue destruction and chronic inflammation (Aratani 2018). We observed a gradual increase in expression of NE (fig. 4B) and MPO (fig. 4C) in peripheral blood neutrophils which were accompanied with characteristic changes in the morphology of these cells with tumor progression. Interestingly, neutrophils from control mice exhibited a smaller size and spherical morphology while neutrophils of tumor bearing mice showed irregular shapes. Neutrophils ($Ly6G^+$) became more hypersegmented with an increase in NE and MPO expression suggesting that neutrophils attain a hyperactive state with tumor progression (fig. 4D).

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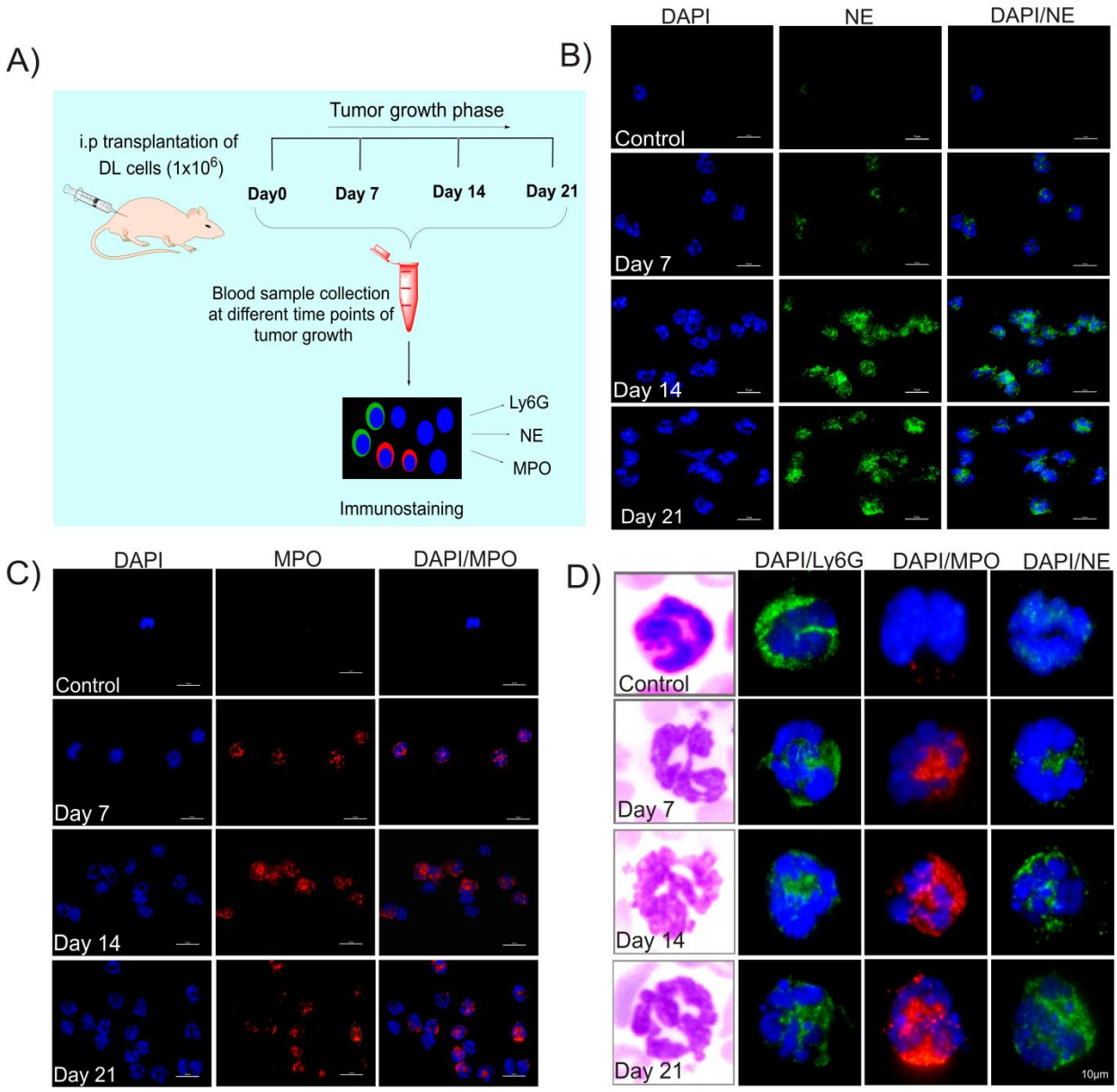

Figure 4 Peripheral blood neutrophils attain a hyperactive state with tumor progression. A) Schematic of the experimental design. Peripheral blood was collected from tumor bearing mice at 7, 14 and 21st day post tumor transplantation and immunofluorescent staining was performed using B) anti-elastase and C) anti-MPO as described in materials and methods. D) Neutrophils were stained with Leishman's dye, anti-Ly6G, anti-elastase and anti-MPO and examined by fluorescence microscopy (magnification, × 1000, scale bar, 10 μm).

3.5 Neutrophil infiltration contributes to high NE release in tissues. We first examined NE levels in serum and ascites (obtained from peritoneum cavity) by ELISA. Serum and ascites were collected similarly from tumor bearing mice on 7, 14 and 21st day post tumor transplantation. The results showed a significant ($p<0.05$) increase in NE levels in serum (fig. 5B) and ascites (fig. 5C) with tumor progression. The level of NE was high by 19-fold in serum whereas it was

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12-fold elevated in the ascites by the 21st day of tumor growth. We observed contrast difference between the control and 21st day DL, which represents the late phase of tumor growth, so we performed all the studies in tissues at the 21st day of tumor growth. We examined NE levels in liver, lungs, spleen and peritoneum harvested from tumor bearing mice. Interestingly, the level of NE was also markedly higher in the vital organs of tumor bearing mice as compared to the control group (fig. 5C). In DL mice, NE level increased by 4-fold in the liver whereas it was 6-fold higher in the peritoneum (site of tumor growth) as compared to the control. Similarly, in spleen and lungs of tumor-bearing mice, the rise in NE level was 10-and 3-fold, respectively, when compared to the control. We also looked into the mRNA expression profile of NE by semi-quantitative PCR in various organs of tumor bearing mice at 21st day of tumor growth. As compared to the control, we observed a high expression of NE transcript in all the examined organs (fig. 5D). The results were in line with the immunostaining data which also showed high expression of NE in the liver, peritoneum, lungs and spleen (fig. 5E) thus corroborating the ELISA results.

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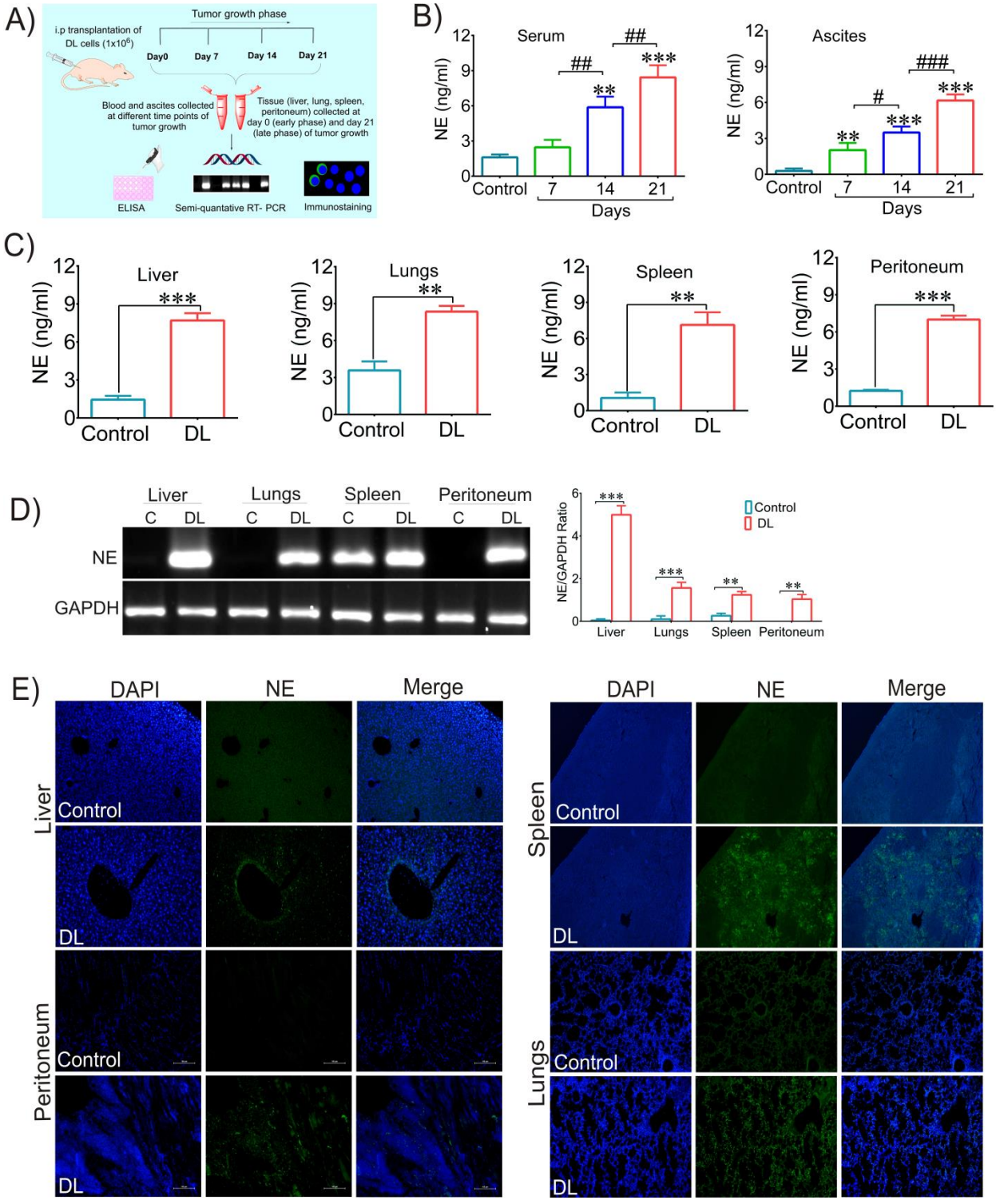

Figure 5 Neutrophil infiltration contributes to high NE release in tissues. A) Schematic of the experimental design. B) Serum and ascites (peritoneal fluid) was harvested from tumor bearing mice at 7, 14 and 21st day post tumor transplantation and assayed for NE by ELISA. C) Liver, lungs, spleen and peritoneum were harvested at day 21 of tumor growth. Tissues were homogenized and supernatant was collected for estimation of NE by ELISA. D) Representative mRNA expression profile of NE as demonstrated by band intensities of PCR amplicons. GAPDH was used as an internal control and bar graphs showing densitometric analysis of mRNA expression level compared

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to GAPDH. E) Immunofluorescent staining using anti-elastase was performed in liver, lungs, spleen and peritoneum as described in materials and methods (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 or two-tailed Student's t-test for two groups where $p<0.05$(*), $p<0.02$(**) and $p<0.001$(***).

3.6 Neutrophil infiltration enhances MPO and ROS production in tumor bearing mice. Excess generation of MPO-derived oxidants has been linked to tissue destruction and chronic inflammation(Aratani 2018). Having demonstrated an increase in MPO expression in blood, we next examined MPO expression in the vital organs of tumor bearing mice. We looked for MPO gene expression at transcriptional and translational level in liver, peritoneum, spleen, and lungs. Tissues were harvested on 21st day post tumor transplantation and processed for semi-quantitative PCR and immunostaining. RT-PCR results (fig. 6B) showed high mRNA expression of MPO in all the vital organs of tumor bearing mice. Similarly, high expression of MPO was observed at protein level (fig. 6C) in tissue sections of tumor bearing mice as compared to the control. MPO catalyzes the formation of reactive oxygen intermediates; therefore, we next looked into the reactive oxygen species (ROS) level in the liver, peritoneum, spleen and lungs. We observed high fluorescent intensity in the tissue sections of tumor-bearing mice as compared to the control (fig. 6D). These results indicate that high infiltration of activated neutrophils in tissues resulted in high MPO release which enhanced ROS production that might be contributing to systemic tissue damage in tumor-bearing mice.

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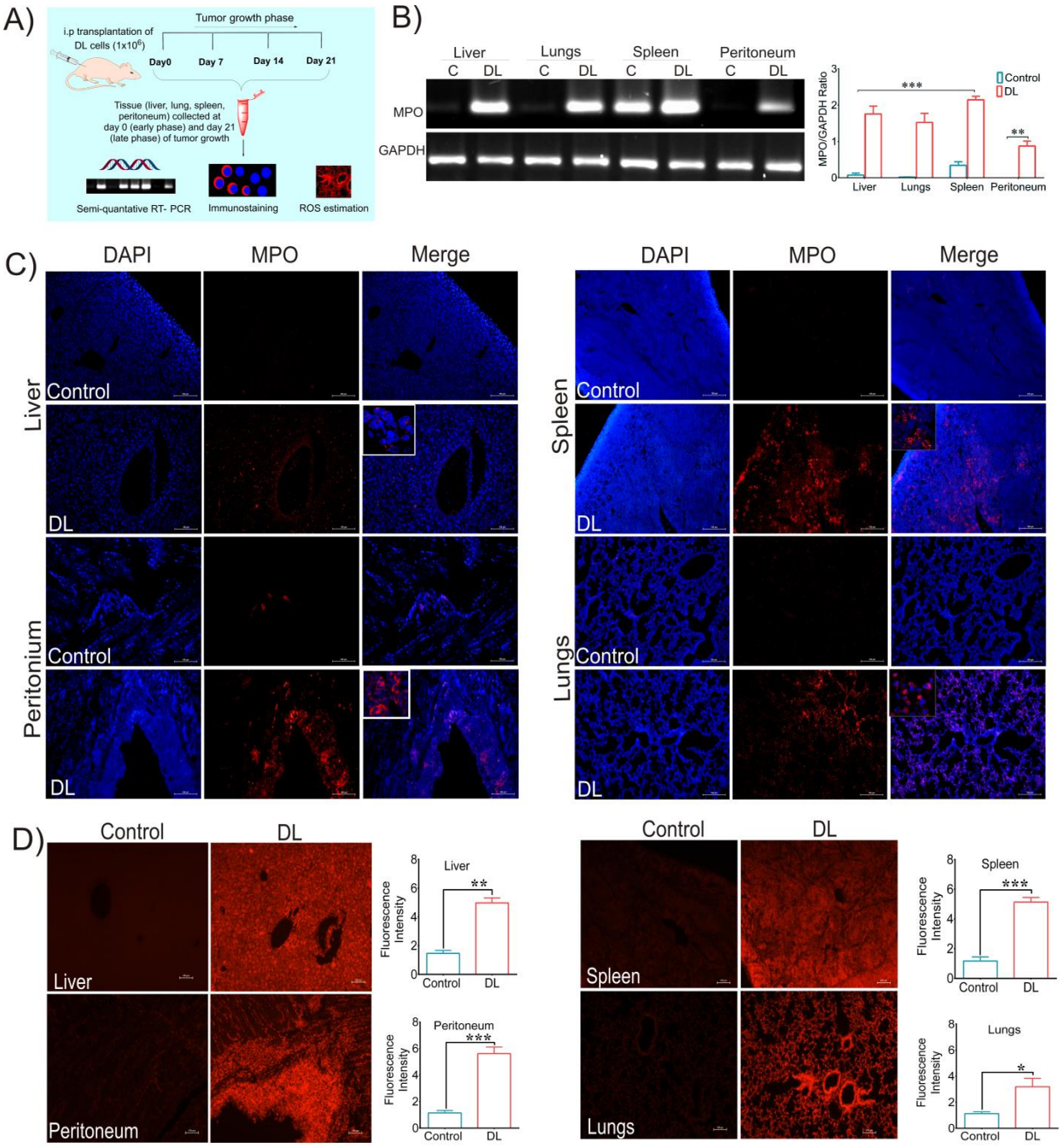

Figure 6 Neutrophil infiltration enhances MPO and ROS production in tumor bearing mice. A) Schematic of the experimental design. B) Representative mRNA expression profile of MPO as demonstrated by band intensities of PCR amplicons. GAPDH was used as an internal control and bar graphs showing densitometric analysis of mRNA expression level compared to GAPDH. C) Immunofluorescent staining using anti-MPO was performed in liver, lungs, spleen and peritoneum as described in materials and methods. D) Cryosections of liver, peritoneum, spleen and lungs were prepared and processed for DHE staining as mentioned in materials and methods. Histogram shows DHE fluorescence intensity in vital organs of mice with and without tumor (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 or two-tailed Student's t-test for two groups where $p<0.05$(*), $p<0.02$(**) and $p<0.001$(***).

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3.7 Enzymatic activity of MMPs increases in tumor bearing mice: MMPs play a critical role in tumor progression by facilitating tumor cell invasion and show broad catalytic activity against components of ECM (Roy, Yang et al. 2009). Neutrophils are known to be the major source of MMPs (specifically, MMP-8 and -9) (Fligiel, Standiford et al. 2006, Rawat, Syeda et al. 2021). Therefore, we further looked for the mRNA expression of MMP-8 and MMP-9 in tissues of tumor bearing mice (fig. 7B). RT-PCR results showed high expression of MMP-8 and MMP-9 in liver, peritoneum, spleen and lungs of tumor bearing mice as compared to the control. Further, we looked for the enzymatic activity of MMP-9 in serum, ascitic fluid and tissue extracts of tumor bearing mice by SDS-PAGE gelatin zymography. Gelatin is used as a substrate and gelatinase (MMP-9) present in the sample degrades the gelatin matrix leaving a clear band which is visualized upon staining the gel. Zymography results (fig. 7C) showed either complete absence or low gelatinase activity in the control groups. Gelatinase activity was dramatically elevated serum, ascitic fluid and in vital organs which include liver, lungs, peritoneum and spleen of tumor-bearing mice.

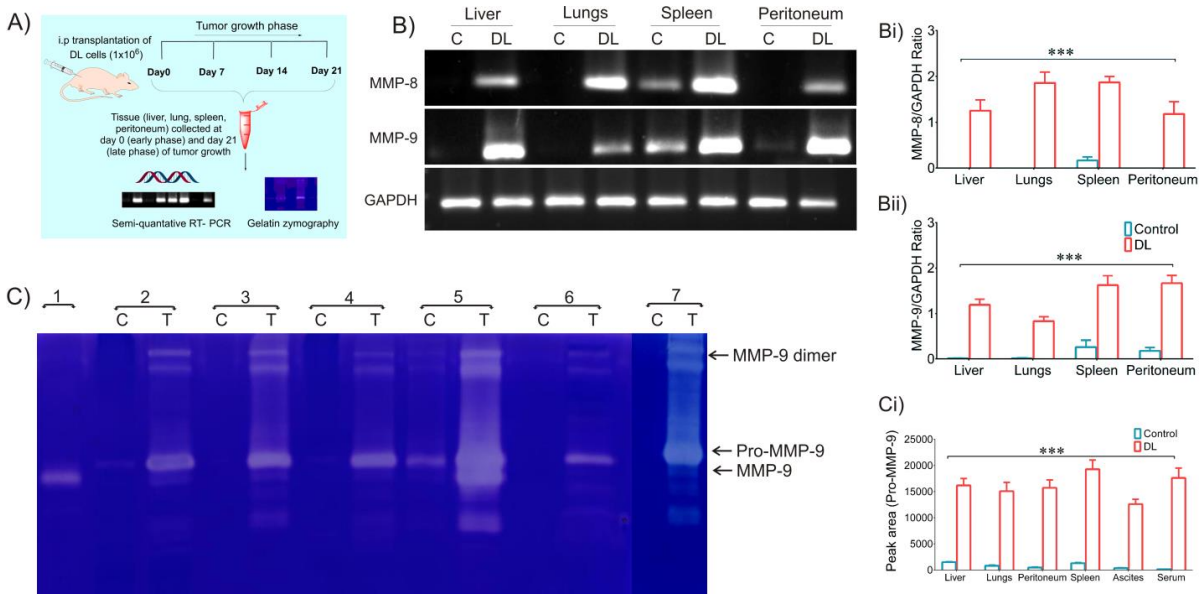

Figure 7 Enzymatic activity of MMPs increases in tumor bearing mice. A) Schematic of the experimental design. B) Representative mRNA expression profile of MMP-8 and MMP-9 as demonstrated by band intensities of PCR amplicons. GAPDH was used as an internal control and bar graphs showing densitometric analysis of mRNA expression level compared to GAPDH. C) Activity of MMP-9 in 2 (liver), 3 (lungs), 4 (peritoneum), 5 (spleen), 6 (ascitic fluid) and 7 (serum) was assessed by gelatin zymography. Lane 1 denotes MMP-9 positive control. The tissue samples were homogenized and the supernatant of proteins were resolved on SDS-PAGE gel containing 1 mg/ml gelatin. Gels were developed as mentioned in the material and methods section to show clear bands of gelatinolytic activity. Bar graph shows band intensities measured using ImageJ software. Lanes marked as C represents the control group whereas lanes marked as T denote the tumor group. The results represent three

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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 $p<0.05$(*), $p<0.02$(**) and $p<0.001$(***).

4. Discussion

Our understanding of the role of neutrophils in cancer has substantially evolved. Neutrophils have recently been shown to play a major role in the various phases of cancer initiation to its metastatic progression (Piccard, Muschel et al. 2012). Accumulating evidences suggest that neutrophil infiltration within tumor microenvironment is associated with poor clinical outcomes in many cancer types such as renal cell carcinoma, human hepatocellular carcinoma, bronchoalveolar carcinomas as well as head and neck squamous cell carcinomas. Moreover, neutrophil infiltration also correlates with tumor grade in human gliomas and pancreatic tumors (Fridlender and Albelda 2012). Importantly, all these reports have focused on the presence of neutrophils within the tumor microenvironment. However, the host response to malignant tumors comprises not only local changes at tumor loci, but also shows systemic effects. Cancer becomes life-threatening when it starts affecting the functioning of major organs of the host. In fact, majority of cancer-related deaths are due to systemic deterioration which ultimately leads to organs failure. In view of this, we investigated the role of neutrophils in mediating systemic deterioration associated with tumor progression. In the present study we found that neutrophils are the major cell population accumulated in the peripheral blood and vital organs of tumor-bearing mice and their number increases significantly from early to the late stages of tumor growth. Also, with an increase in systemic infiltration of neutrophils, we observed their hyper-activation which concomitantly resulted in gradual tissue damage.

Cancer is an inflammatory disease wherein neutrophilia is considered to be the most frequent alteration detected in the patients (Howard, Kanetsky et al. 2019). It has been significantly correlated with advanced disease and found to be an independent prognostic factor, associated with reduced survival in human metastatic melanoma, pancreatic carcinoma, and renal carcinoma. Also, reports have suggested the significance of NLR as a simple measure of systemic inflammation and stress in critically ill cancer patients (Martins Tavares-Murta and Murta 2008). In fact, neutrophilia is the most common SARS-CoV2 infection-induced alteration detected in the blood of COVID-19 patients (QinC 2020).Our results demonstrated an increase in neutrophil count in the peripheral blood of tumor bearing mice which was accompanied by a

reduction in lymphocyte number (fig. 2C). Interestingly, we also observed a high infiltration of neutrophils in the vital organs (liver, lungs, spleen, peritoneum, kidney and lymph nodes) of tumor bearing mice (fig. 2F). This finding undoubtedly points towards a systemic role of neutrophils during cancer progression. Previous studies have also associated aberrant accumulation of neutrophils at tumor sites with tissue injury and poor outcomes in cancer patients (Mayadas, Tsokos et al. 2009). In acute respiratory distress syndrome (ARDS) and sepsis, intensity of neutrophil infiltration in lungs has been correlated with organ failure (Adams, Hauser et al. 2001). Likewise, we found a correlation between neutrophil infiltration and histopathological aberration in all the examined organs of tumor bearing mice with disease progression (fig. 3B). Further, altered biochemical enzyme levels in serum confirmed the tissue injury (fig. 3C). The serum biochemical tests are mostly used in diagnosis of liver, kidney, cardiovascular and many other diseases as well as monitoring the response to inflammation and toxin exposure (Wang, Feng et al. 2006).The marker enzymes of the liver leak into the blood when there is any damage to the liver and their levels increase in the serum (Zimmerman 1999).We observed elevated serum levels of AST and ALT in tumor bearing mice which indicate liver damage (fig. 3C).Kind, P. R., et al also reported an increased liver transaminases in tumor-bearing animals (Kind, Gordon et al. 1985). Urea is the main excretory product of protein metabolism and its high level in blood may reflect an imbalance between urea formation by protein catabolism and urea excretion by the kidney. We observed an elevation in serum urea levels of tumor bearing mice that reflects altered kidney functioning. The high level of urea in blood can also be attributed to the inflammatory condition which is known to cause the decline in renal functioning even in persons without renal disease (Harirforoosh and Jamali 2008). In addition, we also observed an increase in albumin and total protein level with tumor progression. Neutrophil-mediated organ dysfunction has been implicated as playing a causative role in the high rates of morbidity and mortality in SIRS (systemic inflammatory response syndrome) patients (Gando, Kameue et al. 2002). In fact, neutrophils have gained major attention in the ongoing pandemic COVID-19 and are reported to play a crucial role in the development of sepsis, cytokine storm, and multi-organ failure (Arcanjo, Logullo et al. 2020, Tomar, Anders et al. 2020).Increased levels of AST, ALT, albumin, creatinine and urea have also been associated with disease severity and worse prognosis in COVID-19 patients (Lippi and Plebani 2020).

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Upon infiltration in the inflamed tissue, neutrophils are activated by a multitude of inflammatory mediators, which triggers the release of its key effector molecules which are encapsulated within their distinct granules (Mantovani, Cassatella et al. 2011). Various reports have discussed the involvement of these effectors mediators in contributing to tissue damage (Wilgus, Roy et al. 2013). NE, a key cargo stored in the primary granules of neutrophils, can hydrolyze a variety of substrates, including elastin and other ECM component (Korkmaz, Moreau et al. 2008). In addition to its role in host defense, evidence suggests an important contribution of NE in various chronic inflammatory diseases, including asthma, respiratory syncytial virus (RSV) (Yasui, Baba et al. 2005) and cancer (Gaida, Steffen et al. 2012). Up regulated activity and expression of NE has been observed in various cancer types and its concentration is often correlated with cancer grade, stage and survival of patients. In our study, we observed an increase in NE in peripheral blood neutrophils (fig. 4) and in the vital organs of tumor bearing mice at both transcriptional and translational level (fig. 5). NE was also found to be elevated in human ARDS samples, and its inhibition further resulted in reduced epithelial injury in animal models (Sun and Yang 2004, Fujino, Kubo et al. 2012). Also, Baines et al. correlated high gene expression of neutrophil elastase with over-activation of neutrophils in neutrophilic asthma (Baines, Simpson et al. 2011). Similarly, our results reflect the hyper-activation of neutrophils which might have contributed to tissue damage (fig. 4). Like NE, MPO is another key cargo of primary granules in neutrophils which has been found to enhance the inflammatory response in various inflammatory diseases (Loria, Dato et al. 2008). MPO, being an indicator of neutrophils presence in tissues, has been widely used as an inflammatory marker of both acute and chronic conditions (Faith, Sukumaran et al. 2008). Also, MPO changes have been associated with the severity of many diseases and its level reflects the existence of a systemic inflammation, rather than a local inflammatory condition (Schierwagen, Bylund-Fellenius et al. 1990). Similarly, we observed a high expression of MPO in peripheral blood neutrophils (fig. 4) and in the vital organs of tumor bearing mice (fig. 6B) reflecting a systemic inflammatory environment. Moreover, several types of tissue injuries and the pathogenesis of various chronic diseases such as atherosclerosis, renal disease, lung injury, multiple sclerosis and cancer have been reported to be directly or indirectly linked with MPO-derived oxidants (Davies 2020). MPO has been associated with enhancing inflammatory response in COPD patients via producing high reactive oxygen intermediates (Zhu, Ge et al. 2014). MPO catalyzes the formation of reactive oxygen intermediates which play a

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significant role in disease pathogenesis (Yang, Preston et al. 2001). High amounts of oxygen radicals are found in the bronchoalveolar lavage (BAL) fluid of asthmatic patients as compared to healthy controls (Monteseirin 2009). Similarly in RSV bronchiolitis patients, abundant release of ROS damages the host cellular structures and contributes to lung injury (Bataki, Evans et al. 2005). Our results also resonate with these studies showing an increase in ROS levels in liver, lungs, spleen and peritoneum of tumor bearing mice (fig. 6C) implicating a crucial role of MPO and ROS in mediating tissue damage.

Neutrophils are known to be the major source of MMPs (specifically, MMP-8 and -9). MMP-8 also known as neutrophil collagenase is highly expressed in neutrophils and stored as proenzyme in specific granules. Activated neutrophils quickly release MMP-8 to ensure its availability at the site of infection or inflammation. Various studies have reported the upregulation of MMP-8 in a wide range of inflammatory disorders including cancer (Van Lint and Libert 2006). It has been reported to be upregulated in the tissues of individuals with periodontitis and considered to represent an appropriate therapeutic target for the prevention of periodontal disease progression (Liu, Hynes et al. 2006). High expression of MMP-8 has been observed in tissue samples of pancreatic adenocarcinoma (Jones, Humphreys et al. 2004) and uterine cancer (Ueno, Yamashita et al. 1999) patients as compared with normal tissues. Likewise, we observed an upregulation of MMP-8 expression in liver, lungs, spleen and peritoneum of tumor bearing mice as compared to the control (fig. 7B). Sirnio and group showed high serum MMP-8 levels in colorectal cancer patients and suggested a physiological link between MMP-8 and systemic inflammation (Sirniö, Tuomisto et al. 2018). Similarly, MMP-9 is one of the most important contributors of tumor progression and a key player in ECM degradation (Ardi, Kupriyanova et al. 2007). In a murine model of pancreatic ductal adenocarcinoma, neutrophils were found to be the major cell group producing MMP-9 and strikingly these neutrophils were predominantly present at the invasive fronts of metastatic tumours. Interestingly in infectious inflammatory conditions neutrophil entry into tissues relies on the production of MMP-9 by neutrophils. Neutrophil-derived MMP-9 is also known to cause airway remodeling in asthma (Ventura, Vega et al. 2014). High levels of MMP-9 in the sputum and BAL fluid of asthma patients has also been correlated with the extent of cellular infiltration and disease severity (Ventura, Vega et al. 2014). Similarly, high expression of MMP-9 at the gene and protein levels has been found in the bronchial walls of

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asthma patients (Hoshino, Nakamura et al. 1998). BAL fluid from COPD and emphysematous patients also showed upregulation of neutrophil-derived MMP-9, which caused parenchymal destruction and disease extremity (Finlay, Russell et al. 1997, Vlahos, Wark et al. 2012). We showed that MMP-9 expression and activity was dramatically elevated in the liver, lungs, peritoneum and spleen of tumor bearing mice which was further corroborated by its high gelatinase activity in serum and ascitic fluid obtained from tumor bearing mice (fig. 7C).The circulating leucocytes were considered to be one of the major sources of elevated MMP-9 in the serum of patients suffering from Kawasaki disease (KD), a multi-systemic type of vasculitis (Takeshita, Tokutomi et al. 2001).Taken together, our findings strongly support the hypothesis that neutrophils might be majorly responsible for the systemic manifestations observed in cancer patients with disease progression.

5. Conclusion

Various studies have well established the role of neutrophils in influencing the tumor microenvironment. However, we hypothesized that neutrophils also play an important role in modulating the systemic environment during cancer development. We observed elevated count and hyperactivation of neutrophils in the peripheral blood and vital organs of tumor bearing mice. In addition, we also found damaged histoarchitecture and altered biochemical enzyme levels suggesting organ dysfunction. We convincingly showed that high neutrophil infiltration was accompanied with the excessive release of its key effector molecules (NE, MPO, MMP-8 and MMP-9). This excessive release of effector molecules represents the hyperactive response of neutrophils, which might be leading to the tissue damage and subsequent organ dysfunction. Systemic effects cause the majority of cancer-related deaths therefore; taming neutrophils could be a better approach to prevent systemic deterioration in cancer patients thereby leading to increased survival. Our present study demonstrates that significant systemic impact makes the neutrophils as a potential target and further expands the horizon of neutrophil-centered approach for cancer therapeutics.

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Declaration

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. Approval number: DU/ZOOL/IAEC/EXT-R/2018/07.

Consent to participate Not applicable

Consent to publication Not applicable

Conflict of interest The authors declare no competing interests

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