Tumour Extracellular Vesicles Induce Lymph Node Inflammatory Pre-Metastatic Niche Formation

Thesis Type: Traditional thesis

Xin SU

Department of Experimental Surgery

McGill University, Montreal

Supervisors: Dr. Lorenzo E. Ferri

Dr. Jonathan Cools-Lartigue

September 2022 (initial submission)

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy (PhD) in Experimental Surgery

Table of Contents

English Abstract

Lymph nodes (LNs) are frequently the first sites of metastasis in most solid tumours. Currently, the only relevant prognostic feature following LN assessment is the presence or absence of overt metastasis. There is emerging evidence suggesting that metastasis to distant tissues, including LNs, is facilitated by the creation of a pre-metastatic niche potentially mediated through the action of tumour-derived extracellular vesicles (EVs). Thus, environmental changes associated with LN metastasis may be detectable and modifiable based on the local milieu. Neutrophils play an important role within the tumour microenvironment, and our group have previously demonstrated that these granulocytes can sequester and promote metastasis through the formation of neutrophil extracellular traps (NETs). Furthermore, lymphatic neutrophil accumulation has been associated with adverse oncologic outcomes in many tumour types. Neither the mechanism of neutrophil accumulation within LNs of cancer patients nor its functional significance have been described to date. Given that EV can play a central role in pre-metastatic niche formation, we hypothesized that primary tumour derived EV facilitate neutrophil ingress and NETs deposition, thereby increasing cancer cell entrapment and progression within LNs. To establish a role for NETs in LN metastasis, we first confirmed that LN NETs are associated with reduced patient survival and that the formation of NETs precedes LN metastasis in vivo. Next, we demonstrated that neutrophil depletion or inhibition of NETs abolishes the development of LN metastases in experimental animal models. Furthermore, we discovered that EVs are essential to the formation of LN NETs. Lymphatic endothelial cells (LEC) secrete CXCL8/2 in response to the uptake of tumour-derived EVs inducing neutrophil

infiltration, the formation of NETs, and the promotion of LN metastasis. Finally, we compared proteomics of plasma EV from nodal positive and negative patients, the analysis demonstrated distinct, inflammation related profiles. Our findings are the first reveal the role of EV induced LN NETs in the development of LN metastasis and provide potential therapeutic vulnerabilities to enable the better management of cancer patients.

French Abstract

Les ganglions lymphatiques (GLs) sont souvent les premiers sites de métastase dans la plupart des tumeurs solides. Actuellement, la seule caractéristique pronostique pertinente après l'évaluation des GLs est la présence ou l'absence de métastases détectables. De nouvelles preuves suggèrent que les métastases allant vers des tissus distants, y compris les GLs, sont facilitées par la création d'une niche pré-métastatique, potentiellement médiée par l'action des vésicules extracellulaires (VEs) dérivées de la tumeur. Ainsi, les changements environnementaux associés aux métastases des GLs peuvent être détectés et modifiés en fonction du milieu local. Les neutrophiles jouent un rôle important dans le microenvironnement tumoral, et notre groupe a précédemment démontré que ce type de granulocytes peuvent séquestrer et favoriser les métastases par la formation de pièges extracellulaires à neutrophiles (NETs). De plus, l'accumulation lymphatique de neutrophiles a été associée à des résultats oncologiques défavorables dans un certain nombre de types de tumeurs. À ce jour, ni le mécanisme d'accumulation des neutrophiles dans les GLs des patients cancéreux, ni sa signification fonctionnelle n'ont été décrits. Étant donné que les VEs peuvent jouer un rôle central dans la formation de la niche pré-métastatique, nous avons émis l'hypothèse que les VEs dérivées des tumeurs primaires facilitent l'entrée des neutrophiles et le dépôt des NETs, augmentant ainsi le piégeage des cellules cancéreuses et la progression dans les GLs. Pour établir le rôle des NETs dans les métastases des GLs, nous avons d'abord confirmé que les NETs des GLs sont associées à une réduction de la survie des patients et que la formation de NETs précède les métastases des GLs in vivo. Ensuite, nous avons démontré que la réduction des neutrophiles ou l'inhibition des

NETs abolit le développement des métastases des GLs dans des modèles animaux expérimentaux. De plus, nous avons découvert que les VEs sont essentiels à la formation des NETs dans les GLs. Les cellules endothéliales lymphatiques (CEL) sécrètent CXCL8/2 en réponse à l'absorption des VEs dérivées de la tumeur, induisant une infiltration de neutrophiles, la formation de NETs et la promotion de métastases des GLs. Enfin, nous avons comparé la protéomique des VE plasmatiques des patients positifs et négatifs au niveau des ganglions. L'analyse a mis en évidence des profils distincts, liés à l'inflammation. Nos résultats sont les premiers à révéler le rôle des NETs des GLs induits par les VEs dans le développement des métastases des GLs et fournissent des vulnérabilités thérapeutiques potentielles pour permettre une meilleure gestion des patients atteints de cancer.

Acknowledgment

This thesis and the work behind it represent the joint efforts of many talented and hardworking researchers and scientists, within or outside of our group, without whom this work would never be able to come into reality.

First, I would like to thank both of my supervisors, Drs. Lorenzo Ferri and Jonathan Cools-Lartigue. Both of them are very brilliant surgeon scientists who have been continuously supporting me academically and financially. They are attentive and helpful, constantly communicating with me regarding the project progression, my academic development and career plan. They are patient and understanding. They respect my opinion and engagement in the project. The PhD training experience with them has greatly expanded my horizon and reinforced my interest and dedication in cancer research.

I am also very grateful to other members within our group. I express my thankfulness to our lab manager Betty Giannias and the animal health technician France Bourdeau for their constant help and sharing of their research experience. Other principal investigators in our groups, including Drs. Swneke Bailey and Jonathan Spicer and former member Dr. Veena Sangwan have been giving me generous guidance, especially on project design and manuscript writing. I would also like to thank other graduate students of Dr. Jonathan Cools-Lartigue including Ariane Brassard, Iqraa Dhoparee-Doomah, Sabrina Leo and Sabrina (Lixuan) Feng. Initially I mentored and helped them for their projects. As fast learners, they quickly gained acquisition of the scientific knowledge and techniques and in turn helped me a lot for my project.

In addition, the positive energy and working environment have been encouraging and supporting for me along this journey of PhD.

As I am the first student in this group to study extracellular vesicles—a vital yet super complicated biological system, the successful fulfilment of this work cannot be separated from the help and efforts of experts in this field, including Dr. Julia Burnier. She has been always remaining accessible to me, kindly helping me with any research question and patiently teaching me writing skills. Her lab members also helped me greatly, especially Thupten Tsering, an experienced technician, and Alexandra Bartolomucci, a young, ambitious, and hardworking PhD student who is also my best friend. I would like to thank Dr. Janusk Rak and his lab for the help in identification and validation of extracellular vesicles. I am also thankful to molecular imaging, immunophenotyping, clinical proteomics, biobank and histopathology platforms at the Research Institute of McGill University Health Centre for their technical support.

Last but not least, I would like to express my greatest thankfulness to my parents. They have been supporting, encouraging, and understanding. They saw my potential and enthusiasm in research and becoming a scientist, thus sparing all their efforts to support me. The path is long and winding, sometimes I got lost and discouraged, but never had I doubted that my parents would be there for me. My gratefulness for them is beyond words.

Contribution to original knowledge

This thesis focuses on the regulation of neutrophil extracellular traps (NETs) and extracellular vesicles (EVs) on promoting lymph node (LN) metastasis. LNs are often the first site of metastasis in solid tumours. The status of LN metastases is a potent outcome predictor and is crucial for clinical intervention. Most recent research works have been highlighting the role of detecting LN metastasis (whether there is tumour infiltration or not) and how to improve the test accuracy. However, clinical assessment of immune compartment during LN metastasis development has been long overlooked. Moreover, the biological mechanism underlying LN environmental changes remains largely unknown, which led to this PhD research work focusing on neutrophils, the most abundant human leukocytes and LN resident cells, and their activation bioproduct NETs. Furthermore, I elucidated the detailed regulation of tumour derived EVs on NETs formation, through the establishment of premetastatic niche implicated in LN metastasis, which was entirely unknown.

The significant contribution to original knowledge includes:

1. **LN NETs deposition precedes overt metastases and predicts patient survival**. Using a tissue microarray (TMA) of 175 gastroesophageal adenocarcinoma (GEA) patient samples, we confirmed that the level of LN NETs correlates with LN disease progression. Notably, even tumour free LNs, from nodal positive patients, have higher level of NETs indicating that LN NETs formation occurs in a pre-metastatic manner. We also validated

this hypothesis in animal models. In addition, high levels of LN NETs are associated with reduced overall survival in GEA patients. Taken together, this work was the first to highlight the prognostic value of LN NETs. Moreover, this finding is complementary to the current LN staging system, and it suggests the utility of assessing the immune compartment in pathological samples.

2. **Neutrophil depletion and all kinds of inhibition of NETs dramatically abolish LN metastasis**. Through extensive animal experiments, we confirmed that LN NETs formation contributes to LN metastasis development. This phenotype is universal, not subject to cancer type, but related to the malignancy of the neoplasm. We demonstrate the potential of targeting NETs as a novel anti-cancer treatment as several NETs based clinical trials are ongoing.

3. **LN NETs formation is EV dependent and mediated by LECs secreting CXCL8/2**. Using EV education and knockdown experiments, we demonstrated the essential role of EVs in the formation of LN NET. Using in vivo and in vitro experiments, together with data from human samples, we demonstrate that LECs secrete CXCL8/2 upon EV uptake, which leads to neutrophil recruitment and the formation of LN NETs. The identification of involvement of EVs and CXCL8/2 in the development of LN metastases is important as both are targetable markers. Moreover, our work focused on the synergy between tumour-derived cytokines and EVs and investigated the plasticity of neutrophil function in the milieu of cancer from a cancer secretome mindset.

4. **Clinical proteomics revealed distinct EV population in patient plasma.** Using 10 gastroesophageal cancer patient plasma samples, we isolated and then analysed the EVs using mass spectrometer and bioinformatic approaches. We found that EVs from patient with or without LN metastasis have very different profile of protein expression. The cluster of these proteins revealed distinct immune pathway and physiological changes corresponding to patient status. Furthermore, patients with LN metastasis have higher level of onco-proteins in their plasma EVs.

Our group was the first to describe the role of NETs in facilitating distant cancer metastasis (liver) under conditions of systemic inflammation. In these years of my PhD research work, we demonstrated and described the role that NETs and EVs play in facilitating lymph node metastasis, an earlier and more treatable stage in cancer progression. To the best of our knowledge, this represents the first study in this context. The progress of this work has been presented at many major academic conferences, notably including American Association of Cancer Research (AACR, 2021), International Society of Extracellular Vesicles (ISEV, 2022), Canadian Cancer Research Forum (2021) and McGill Fraser Gurd Day of Surgical Research (2019, 2021 and 2022), numerous positive feedback was received. Notably, for the distinguished presentation at American Association of Clinical Oncology conference (2022), I received NOYCIA Oncology Young Canadian Investigator Award (top10 in Canada).

Contribution of Authors

Xin Su, the PhD student himself and his two supervisor Drs. Lorenzo Ferri and Jonathan Cools-Lartigue conceived the ideas, designed the experiments, and directed the study. Xin Su also wrote all this thesis.

Xin Su completed most of the experiments including human pathological sample analysis, tissue culture and maintenance, animal experiments, immunofluorescence stain, NETosis assays, EV isolation and verification, western blots and EV proteomics sample preparation and analysis. Xin Su also analysed and graphed all data for this thesis. Betty Giannias coordinated the project as a lab manager. France Bourdeau helped with animal clone management and animal health.

Ramin Rohannizadeh and Olivia Koufos helped comply patient demographic data. Ariane Brassard and Iqraa Dhoparee-Doomah helped the flow cytometry assays. Alexandra Bartolomucci helped tissue culture. Qian Qiu helped TCGA data analysis. Thupten Tsering helped with the EV TEM staining.

James Tankel, Veena Sangwan, Swneke Donovan Bailey, Julia Valderin Burnier, Lorenzo Ferri and Jonathan Spicer helped with manuscript writing and editing.

List of Figures and tables

Figure R1-R12 are included in the review section.

Figure 1-9, and Figure S1 as well as table 1-3 are included in the research findings sections.

Figure D1 is included in the discussion section

List of Abbreviation

CD11b, Integrin alpha M

CD66b, Carcinoembryonic antigen-related cell adhesion molecule 8

CK7, cytokeratin 7

CM, cell conditioned media

CXCL2, Chemokine (C-X-C motif) ligand 2

CXCL8, Chemokine (C-X-C motif) ligand 8

EAD, oesophageal adenocarcinoma

EV, extracellular vesicles

GEA, gastroesophageal adenocarcinoma

H3Cit, citrullinated histone H3

HR, hazard raio

KD, knockdown

LEC, lymphatic endothelial cell

LN, lymph node

Ly6G, lymphocyte antigen 6 complex locus G6D

LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1

N0, lymph nodes from nodal negative patient

N+neg, cancer free lymph nodes from nodal positive patients

N+met, cancer infiltrated lymph nodes from nodal positive patients

NLR, neutrophil lymphocyte ratio

NE, neutrophil elastase

NEi, neutrophil elastase inhibitor

NETs, neutrophil extracellular traps

MVs, microvesicles

PAD4, peptidyl arginine deiminase 4

PRKD1, Protein Kinase D1

Rab5a, Ras-related protein 5A

Rab27a, Ras-related protein Rab-27A

TMA, tissue microarrays

VAMP7, vesicle-associated membrane protein 7

VEGFC, vascular endothelial growth factor C

Introduction

Cancer treatment paradigms are usually dictated by histology and are organ specific. However, certain behavioural features of cancers, especially solid cancers, transcend histology and are common among different organs. Chief among them is the near ubiquitous development of lymphatic metastases[1]. Regional lymph nodes (LNs) are the first sites of metastasis for nearly all types of solid tumours, including in gastroesophageal adenocarcinoma (GEA), lung adenocarcinoma and melanoma [2, 3]. Lymph node metastases are the most powerful predictor for the development of distant metastasis, the fatal systemic disease stage[4, 5]. Targeting lymph node metastasis, accordingly, presents broad therapeutic implications.

LNs are immunologic organs where immune responses are initiated. One would expect this to represent an inhospitable environment, however, in the context of LN metastasis, this kind of tumour-immune interactions actually favour tumour development and further metastasis into bloodstream and distant organs[6]. The environmental conditions that underpin this counterintuitive observation remain unclear. What is known is that for metastatic colonisation to occur, the conditions within potential metastatic sites, including the LNs, need to be optimised for tumour cell deposition and growth, a process known as pre-metastatic niche formation [4, 5]. This includes lymphangiogenesis and the activation of various kinds of immune cells [6-8].

Neutrophils are part of this milieux. They are the most abundant leukocyte in human body. They are also the primary effector cells implicated in inflammation [9-11] and reside within LNs where they physiologically maintain immune surveillance and present circulating antigens [12]. However, in the setting of cancer, neutrophil related inflammation has long been recognized as one of the hallmarks of cancer and can predict adverse oncologic outcomes [13, 14]. Elevated neutrophil activity is associated with increased tumour proliferation, invasion, escape from dormancy and metastasis. At the clinical level, systemic inflammation is reflected in the ratio of neutrophils to lymphocytes (NLR). Patients with an elevated NLR demonstrate reduced overall and recurrence-free survival. The converse is associated with more favourable survival outcomes and a more robust response to chemotherapy. Moreover, it is also reported that higher LN neutrophils infiltration is associated with worse outcome in cancer patients[7, 8]. However, the detailed cellular mechanism remains poorly defined.

Neutrophils can be implicated in a variety of pro-tumorigenic processes, through the elaboration of cytokines, proteases, and neutrophil extracellular traps (NETs)—extracellular strands of neutrophil derived DNA decorated with antimicrobial peptides. Our group has played a leading role in demonstrating that NETs can sequester circulating tumour cells in distant sites via the NETs [15-18]. Neutrophils exhibit great plasticity to environmental cues and the process of NETs release is believed to be mediated by tumour secretion of numerous kinds of factors, including extracellular vesicles (EVs)[9-11].

EVs are actively secreted by nearly all eukaryotic cells[12, 13]. They have been shown to play important roles in carcinogenesis and cancer progression and are a key player in intercellular communication[14, 15]. Tumour-derived EVs have been shown to be actively uptaken and transported by lymphatic vessels and are able to prepare sentinel LNs for impending

melanoma metastasis[16-18]. Moreover, EVs can polarize neutrophils from an anti-tumour (N1) phenotype to a pro-tumour (N2) phenotype[19]. EV can also directly induce the formation of NETs[20]. However, whether the interplay of EV-NETs has a profundity in the development of LN metastases is entirely unknown.

In the part of comprehensive review of literatures, the detailed biological knowledge regarding topics such as LN physiology and metastasis, inflammation and neutrophils in cancer, EV biology, and the interaction of all these elements will be provided as well as their relevance to clinical oncology practice and biomedical research. It will also be introduced that what is the overall rationale for this project and why the subsequent experiments were conducted, and why certain scientific approaches and methodologies were used in this study.

In the part of research finding, based on with all these previous scientific observations, we hypothesized and eventually demonstrated that EVs released by the primary tumour recruit neutrophils to LNs. This is followed by NETs deposition and the establishment of a local environment favourable for tumour outgrowth. In addition, LN NETs deposition should be associated with reduced survival in human GEA patients. Furthermore, we needed to demonstrate that lymphatic neutrophil accumulation and NETs deposition precedes and is required for LN metastatic outgrowth. Finally, we described the role tumour-derived EVs play in establishing a regional inflammatory microenvironment through the induction of lymphatic endothelial elaboration of CXCL8.metastatic disease.

A comprehensive review of the relevant literature

Lymph node, lymphatic endothelial cells, and lymph node metastasis

Lymph nodes (LNs) are secondary lymphoid organs and are present throughout the human body. A typical LN is usually kidney shape. Each LN is surrounded by fibrous capsule which extends inside a lymph node to form trabeculae. The inside of LN is made up of an outer cortex and an inner medulla, which are both enriched in cells. Through lymphatic vessels, LNs throughout the body are linked to each other and communicate with other parts of immune system and circulation system[21-24].

LNs are major residential sites of lymphocytes that include B and T cells. B cells are mainly located in the outer cortex where they are clustered together as lymphoid follicles, while T cells are mainly found in the paracortex region. The medulla is mostly made up of plasma cells. There are also a population of dendritic cells, macrophages as well as neutrophils. The outer cortex consists of groups of mainly inactivated B cells called follicles. When activated, these may develop into what is called a germinal centre, a specialized microstructure produces long-lived antibody secreting plasma cells and memory B cells. The deeper paracortex mainly consists of the T cells[25-27].

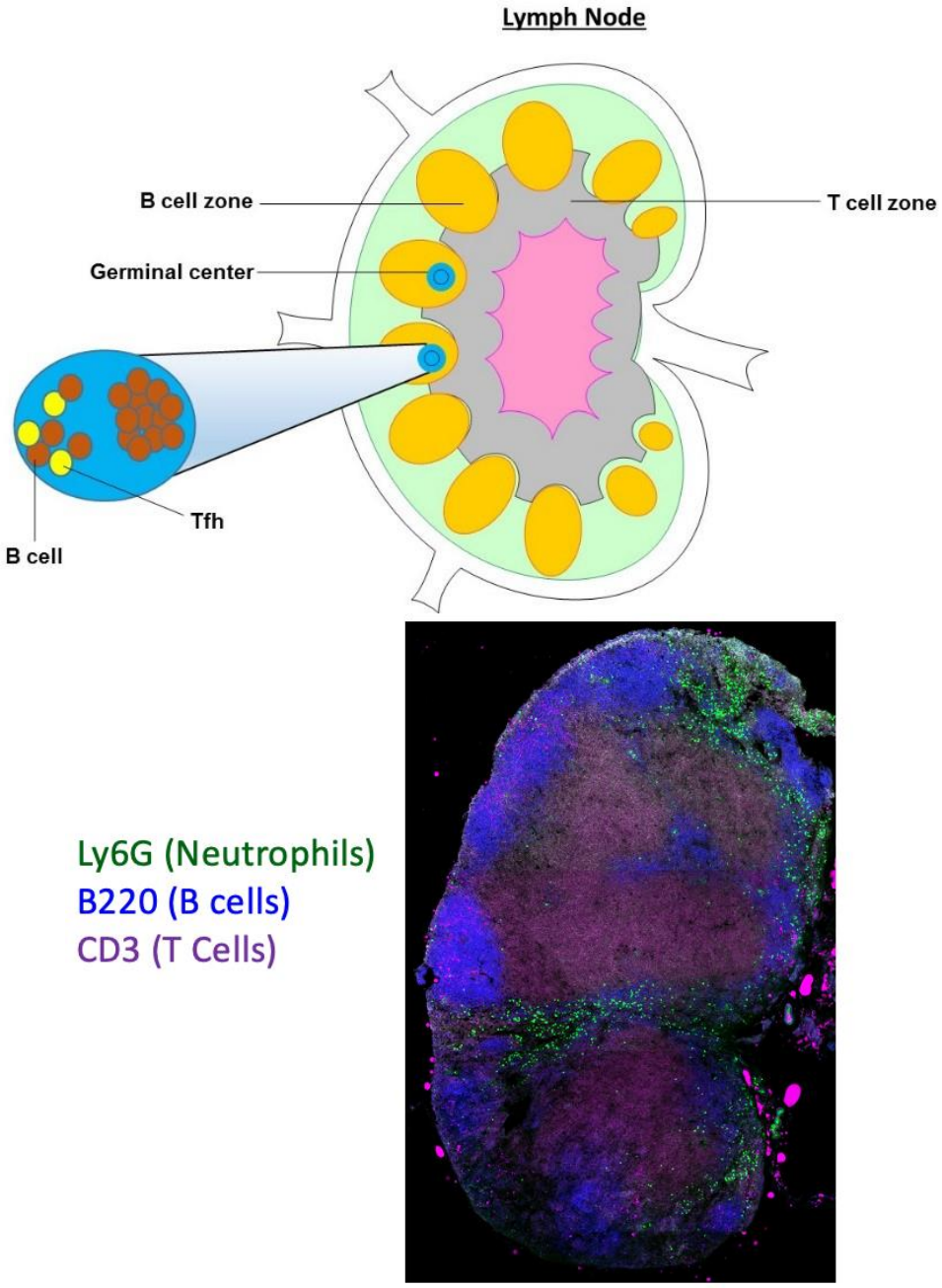

Fig. R1 Immune cells and their locations within LN. Upper: Adapted from Uwadiae, FI, BiteSized Immunology, online article for British Society of Immunology, 2018. Bottom: Immunofluorescence stain of a mouse LN by Xin SU.

LN structure is also characterised by a distinguished lymph flow. The network of lymphatic vessels drains interstitial fluid from tissues or other part of lymphatic system to form lymphatic fluid, as known as lymph. Lymphatic fluid passes through the multiple afferent lymphatic vessels on the convex side to enter LNs. After entering LNs, the lymph filter through the subcapsular sinus, cortex and then into in medulla. On the concave side, medullary sinuses drain lymph into the efferent lymphatic vessels to exit the LN. The specific tissue-to-LN and intra-LN lymphatic flow, as well as the anatomy of LNs, are associated with its physiological functions— they are important for the proper functioning of the immune system, passing microbial organisms and antigens to immune cells and acting as filters for foreign particles including cancer cells[28, 29].

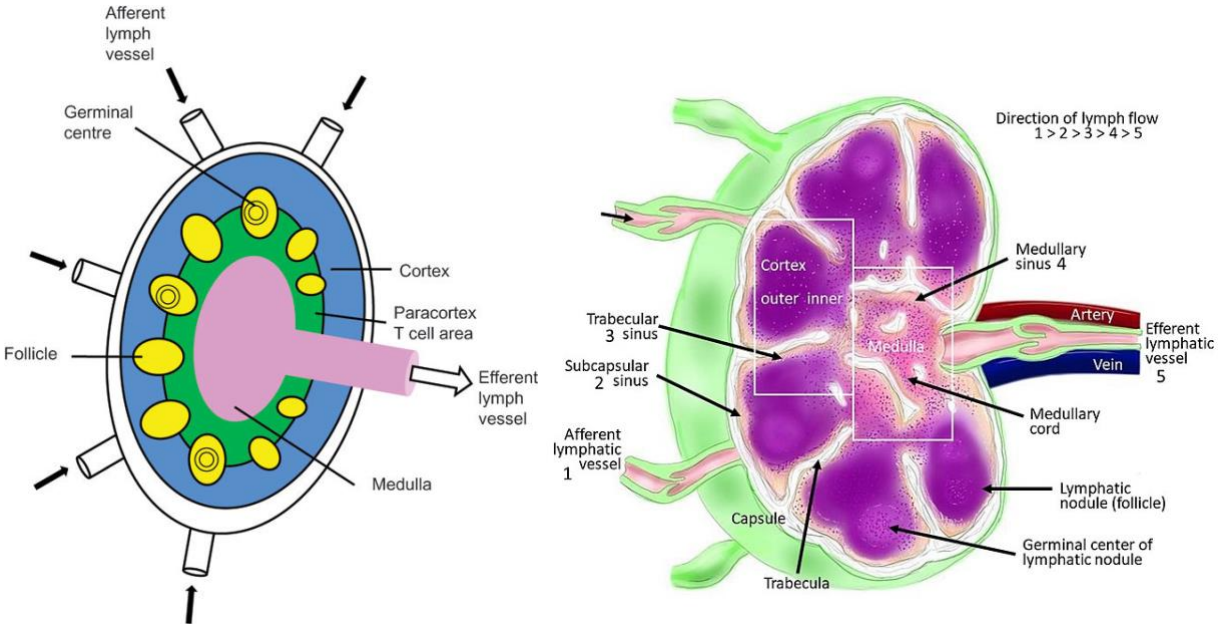

Fig. R2 Illustration showing the lymph flow inside the lymph node. Left: Adapted from Kemeny, D. Cell Mol Immunol 2012[25]. Right: Open access illustration from Wikimedia Commons, Author: Chris Sullivan.

The lymphatic vessels, the channels, and the sinuses within the LN are surrounded and lined by lymphatic endothelial cells (LECs). In human, LECs have a distinct endothelial cell lineage characterized by cellular markers including LYVE-1, Vimentin and Podoplanin[30], which were the markers used later in this thesis for immunofluorescence staining. In addition to their constituting role for the lymphatic structure, LECs also have important functions in substance transport and immunosurveillance. Recently, LN LECs have been reported to archive antigens and directly regulate immune cell properties, thus participating in forming metastatic niches and alerting the response to antitumor immune therapies[31].

Under various kind of pathological condition, the LN cell number, proportion of different kinds of cells, and their function will undergo great changes, usually manifested as swollen LNs. This is often one common symptom of LN metastasis as well[27, 32]. LN metastasis are common problems for patients and the clinician involved in their management. The clinical regime includes identifying LN metastases, surgical removal, pathological evaluation, and other treatments targeting LN metastases. These approaches form the fundamental basis of modern oncology for more than a century[33-36]. Moreover, LN metastasis is one of the most potent predictors for systemic metastasis and worse outcome[37-39].

Surprisingly, regardless of the standard clinical practice and great clinical relevance, the mechanism of LN metastasis, especially the initial events and cellular mechanism associated with LN metastasis, remains largely unknown. Most basic and translational cancer research focus on the topic of carcinogenesis or distant metastasis, which are correspondingly the initial and end point of cancer. It was only until the past decade that there has been more biological research of LN metastasis, this long overlooked field. Emerging evidence suggests that LNs, similar to other metastatic organs, undergo environmental changes which include activation and expansion of

one or more cell populations, which are regulated by signals from primary or circulating tumour cells and in turn facilitated their outgrowth[40-44].

As the predominant and representative matrix cell of LNs, LECs often expand and change their distribution patterns, resulting in formation and branching of lymphatic vessels from pre-existing lymphatic vessel[45, 46]. This phenomenon, known as lymphangiogenesis, is commonly observed in LN metastasis, and is reported to be associated with worse outcome in many types of cancer[47-50]. Lymphangiogenesis can be regulated by many cancer-related factors, among them VEGFC, either derived from cancer cell directly or tumour associated macrophages, is the classic and most potent stimuli[51, 52]. The cancer-induced lymphangiogenesis can result in extensive remodelling of lymphatic network and enhance the attachment of metastatic cancer cells, hence increasing LN metastatic burden[52-54].

Through an elaborate regulatory network of cancer derived factors including soluble cytokines, growth factors as well as extracellular vesicles, the immune cell population can be modulated to facilitate LN metastasis as well. This seems to be a reasonable explanation why LNs could be converted from hostile immune organs into the first sites of metastasis. Lymphocyte, the most abundant immune cells within LNs, can polarise from an anti-tumour phenotype towards a pro-tumour phenotype[55]. For examples, tumour cells can enable the cancer evasion of NK cells, reduce T cell-mediated cytotoxicity, promote exhaustion of effector T cells, and increase the incidence of immunosuppressive regulatory T cells (Tregs)[6, 56-58]. Tumour educated B cells, instead of synthesising anti-tumour antigen specific antibodies, produce tumour promoting antibodies such as HSPA4-targeting IgG, induce lymphangiogenesis and increased the amount of lymph[59-61]. Those phenotypical changes and functional

perturbance of lymphocytes, induced by signalling from cancer cells, facilitate and promote LN metastasis.

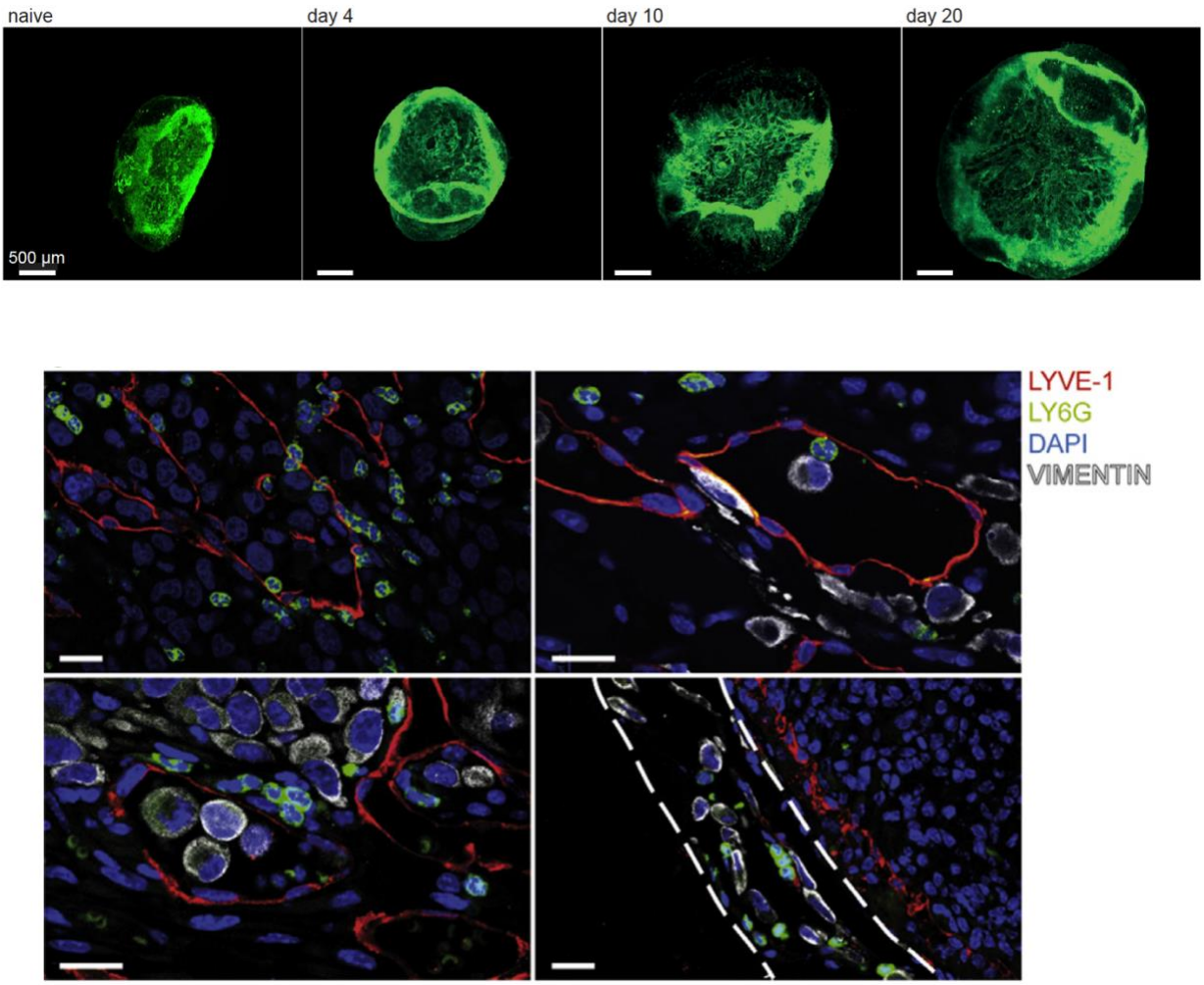

Fig. R3 Representative immunofluorescence of metastatic LN from research papers, indicating environmental change and activation of different types of LN cell. Upper: a time course imaging of tumour draining LNs, showing extensive lymphatic network remodelling and lymphangiogenesis. Adapted from Commerford et al., Cell Reports, 2018. Bottom: The proximality of metastatic melanoma cell and neutrophils within a LN. Adapted from Soler-Cardona et al., Journal of Investigative Dermatology, 2018.

Immune cell of innate immune system, such as macrophages and neutrophils, also contribute to the process of LN metastasis. M2 macrophages, a polarised subtype of macrophage in the milieu of cancer, can prepare LN environment for tumour cell colonization and promote cancer cell invasion into LNs. Various kinds of approaches targeting or depleting macrophages can significantly decrease LN metastatic burden[62-65]. Neutrophils, heedless of their lower numbers in LN, also play an important role in promoting LN metastasis. Melanoma derived CXCL5 can recruit neutrophils into lymphatic system can increase LN metastases[44]. However, comparing to cell biology works on other LN cell types, or the clinical research highlighting the prognostic value of LN neutrophil counts, the clear mechanism how neutrophils can promote LN metastasis remains nearly entirely unknown.

Neutrophil, neutrophil extracellular traps (NETs) and metastasis

Neutrophils are the most abundant type of leukocytes in human body and make up about 40% to 70% of all white blood cells in human blood[66]. They play important roles in anti-infection immunity: when microorganisms, such as bacteria or viruses, invade human body, neutrophils are often first defence to respond[67]. Morphologically, as they belong to the class of polymorphonuclear cells, they are notable for the multilobulated-shaped nucleus[68, 69]. In their cytosol, neutrophils are enriched with granules, which contain proteins that have antimicrobial properties, such as myeloperoxidase in the primary granules, alkaline phosphatase in the secondary granules, and collagenase in the tertiary granules[70]. The life span of neutrophil is relative short, estimated to be less than 24h[71]. Moreover, they become activated to

inflammatory cytokines, mechanic force and various kind of microbes and microbial molecules, making research on neutrophil functions very challenging[72].

Neutrophils can exert their anti-microbial infection roles via different types of biological activities and pathways. The main ones are phagocytosis, degranulation, and the generation of neutrophil extracellular traps (NETs)[73, 74]. Phagocytosis meaning that neutrophils are capable of ingest foreign microbes or microbial particles. When the microbes are internalised as phagosome—the vesicle formed around the engulfed particles or microbes, neutrophil will release pre-stored reactive oxygen species and hydrolytic enzymes into the phagosomes to make phagolysosome[75-77]. Many types of microbes could be killed by this effective mechanism[78].

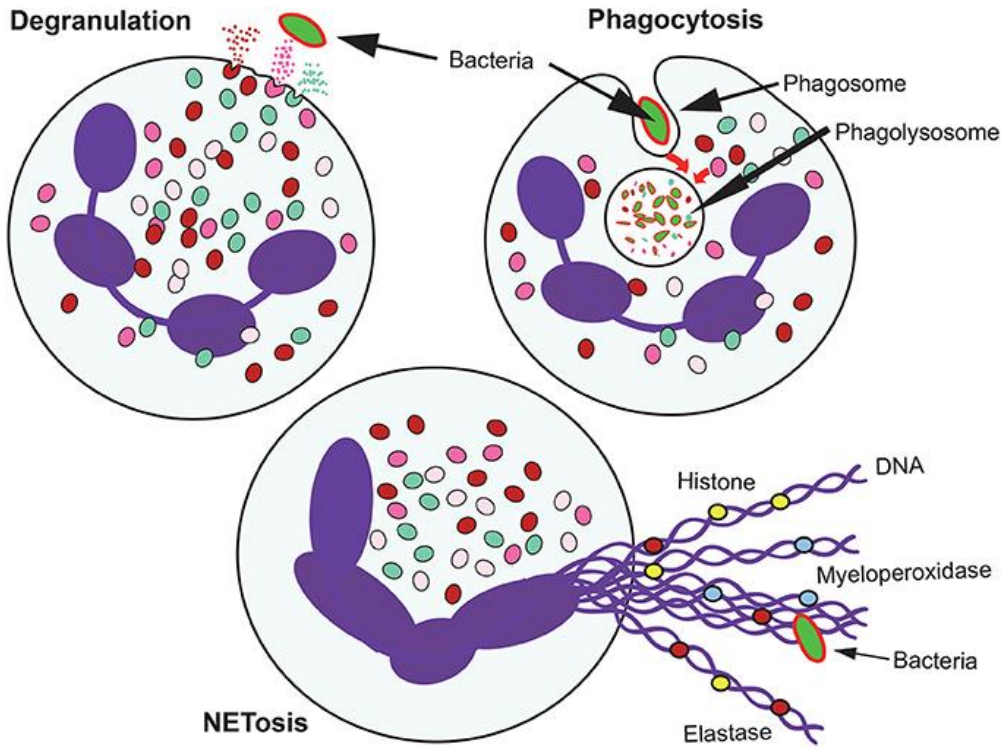

Fig. R4 Main anti-microbial functions of neutrophils. Adapted from Carlos Rosales, Frontiers in Physiology, 2018[67].

Neutrophil can also release the anti-microbial enzymes and proteins stored in granules mentioned previously, directly into extracellular space, a process known as degranulation. These content help combat infection and result in microbe death[79]. Last but not least, neutrophil can also release NETs in response to inflammatory environmental cues. The formation of NETs is also known as NETosis. The web like structure of NETs is composed of DNA and chromatin and decorated with anti-microbial proteins such as serine proteases, neutrophil elastase, and myeloperoxidase[80, 81]. NETs can create a restricted local environment with high concentration of antimicrobial molecules. The web like structure also serves as a physical barrier from further diffusion and migration of microorganisms[82, 83]. Moreover, NETs can serve as a scaffold and augment the function of other immune cells including macrophages[84].

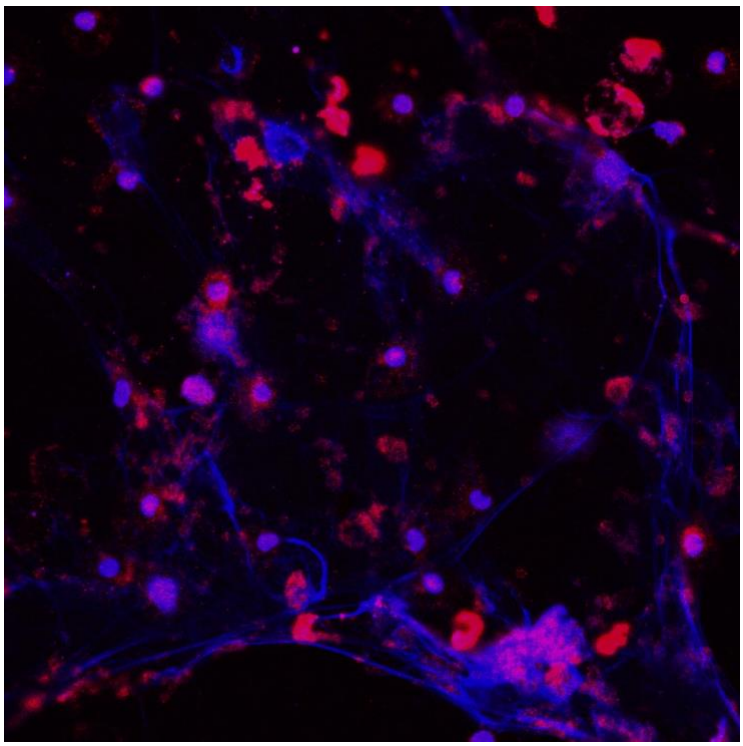

Fig. R5 Immunofluorescence stain of NETs. Imaged by Xin SU, showing the web like structure of DNA (blue) decorated with neutrophil elastase (Red).

Albeit essential for their innate and anti-infection immunity, elevated quantity or activity of neutrophils are also associated with many pathological conditions, especially chronic inflammation, and inflammatory diseases, such as psoriasis, inflammatory bowel disease, atherosclerosis, and rheumatoid arthritis[85-88]. For example, in rheumatoid arthritis, uncontrolled myeloperoxidase release during degranulation can lead to joint tissue damage; Activated neutrophils can release B cell-activating factor thus resulting in B cell proliferation and continuous autoantibody production; Enhanced spontaneous formation of NETs can cause cartilage fibrosis and degradation[89-91]. Thus, many studies and trials targeting neutrophil activation, or those cytokines that recruit neutrophils, as well as NETs depletion, were all able to show therapeutic benefits in chronic inflammatory disease patients[92, 93].

Since the beginning of this century, research on a new pathological role of neutrophils emerged—the discovery of their impacts on promoting cancer progression and metastasis. Inflammation has long been recognized as one of the hallmarks of cancer, facilitating essential functions necessary for tumour progression[94, 95]. Neutrophils are the primary innate effector cells implicated in the formation of an inflammatory milieu. Clinical observations in human cancer patients suggest that elevated PMN activity is associated with adverse oncologic outcomes. This is measured by the ratio of neutrophils to lymphocytes (NLR), which standardizes the metric between individuals. This has been observed in nearly all solid organ malignancies, including gastric and esophageal cancers[96-101]. The drivers of this phenotype, termed “emergency myelopoiesis” are diverse and include many tumour-derived factors such as G-CSF, GM-CSF, IL-1β, IL-6/12 and IL-17. They can result in elevate circulating neutrophils, release of premature neutrophils and increased intratumoral neutrophil infiltration[102-105].

In addition to the quantity changes, neutrophils in the milieu of cancer have a significant quality change as well, manifested by a polarisation from anti-tumour phenotype (N1) to pro-tumour phenotype (N2). This is a similar concept of the M1/M2 polarisation theory of macrophages, highlighting the plasticity of innate immune cells based on tumour regulation and inflammation state[11, 106, 107]. N1 neutrophils exhibit tumour cell cytotoxicity and immunoactivity ability due to their high production adaptive immunity activating factors, such as TNF and CXCL10, which facilitates recruitment and activation of CD8+ T cells[108, 109]. They also release higher amount of reactive oxygen species, to exhibit their killing capability[110]. Moreover, N1 neutrophils are also characterised by their expression of cellular markers such as ICAM-1 and Fas (CD95)[111, 112].

In comparison, N2 neutrophils are characterized by higher expression of pro-tumour factors such as arginase, VEGFA, hepatocyte growth factor (HGF) and reactive nitrogen species[113, 114]. N2 neutrophils can release extracellular matrix degrading enzymes including neutrophil elastase and the family of matrix metalloproteinases (MMPs)into extracellular spaces, hence liberating tumour cells and facilitate their migration[115, 116]. In addition, N2 neutrophils also distinguishably express higher level of immunomodulatory markers such as PD-L1 and Siglecs, in order to hinder anti-tumour immunity and CD8+ T cell cytotoxicity[117-119]. Moreover, N2 neutrophils can also express tumour-capturing molecules. Our lab was the first to discover that neutrophils can capture and interact with circulating tumour cells via Mac-1, thus promoting the outgrowth and metastasis process of tumour cells in liver[120]. In addition, it is feasible to polarize blood-derived primary human neutrophils toward N1- or N2-like phenotypes in vitro. The main driver of N1 polarisation is INF- β while N2 is mainly driven by TGF- β[121,

122], which further consolidates the concept of the plasticity of neutrophils and the potential to modulate their phenotypes in oncological research and therapeutical attempts.

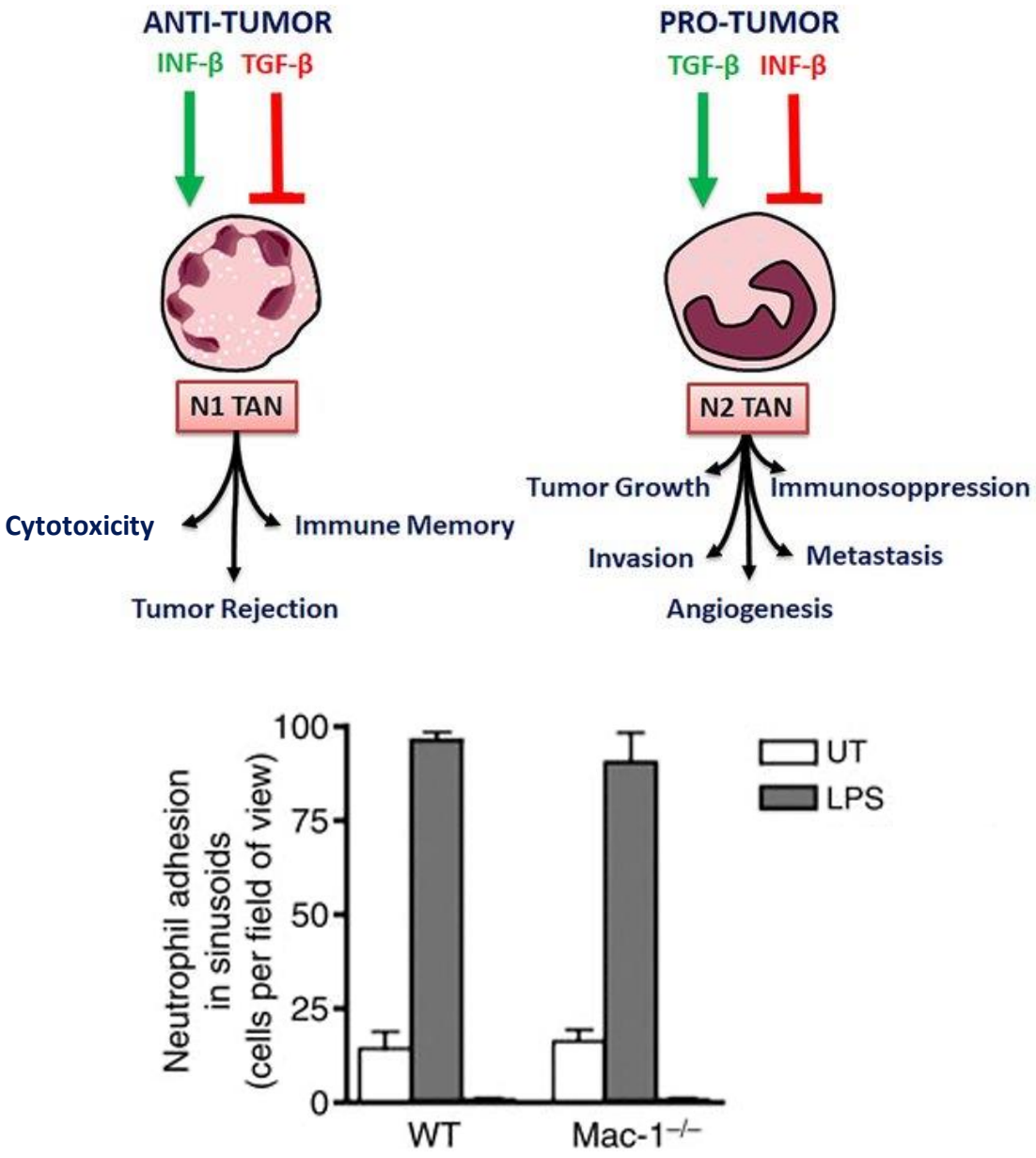

Fig. R6: The polarisation of neutrophil phenotypes and the pro-tumour roles of neutrophils. Upper: Illustration showing the N1/N2 phenotypes of tumour associated

neutrophils (TANs), their functions, and the conditions to induce these two phenotypes. Adapted from Masucci MT et al., Frontiers in Oncology, 2019[106]. Bottom: Neutrophils can increase tumour cell adhesion in liver sinusoids in mice via Mac-1, while Mac-1 knockout abolished this phenotype. UT: untreated, LPS: LPS stimulation to mimic inflammatory states. Adapted from Spicer J et al., Cancer Research, 2012[120].

Notably, NETs also have a profound impact on promoting cancer progression and metastasis. Our group has previously shown that under conditions of systemic inflammation, the elaboration of NETs by tissue infiltrated neutrophils can promote the development of distant metastasis in murine models of lung cancer[123, 124]. Follow this direction, there has been several ground-breaking reports: cancer cells have the ability to directly induce NETs formation to support metastasis[125], and this is also the mechanism for cancer cells to resist radiation therapy and form persistent diseases[126]. Moreover, NETs forming during chronic inflammation can awaken dormant cancer cells, leading the disease relapse. In addition, NETs have great immunosuppressive ability: the PD-L1 expression on NETs can promote T cell exhaustion in the tumour microenvironment [127] while the TLR4 ligands on NETs can interact with regulatory T cells, which contributes to carcinogenesis in non-alcoholic steatohepatitis, a chronic inflammatory state in liver[128]. The immunosuppressive impact of NETs offers as a potential determining factor in immunotherapy failure and could be an alternate target to exploit[129, 130]. Furthermore, NETs can also implicate in chemotherapy resistance. On this topic, we recently published a review paper suggesting that NETs and the various kind of proteins on NETs can be involved in oncological events including epithelial-to-mesenchymal transition, drug detoxification, and angiogenesis, which all contribute to dissatisfactory treatment

results[81]. Taken together, ours and others' works have highlighted NETs as an important metastasis driver and a promising therapeutic target in the prevention of metastatic development.

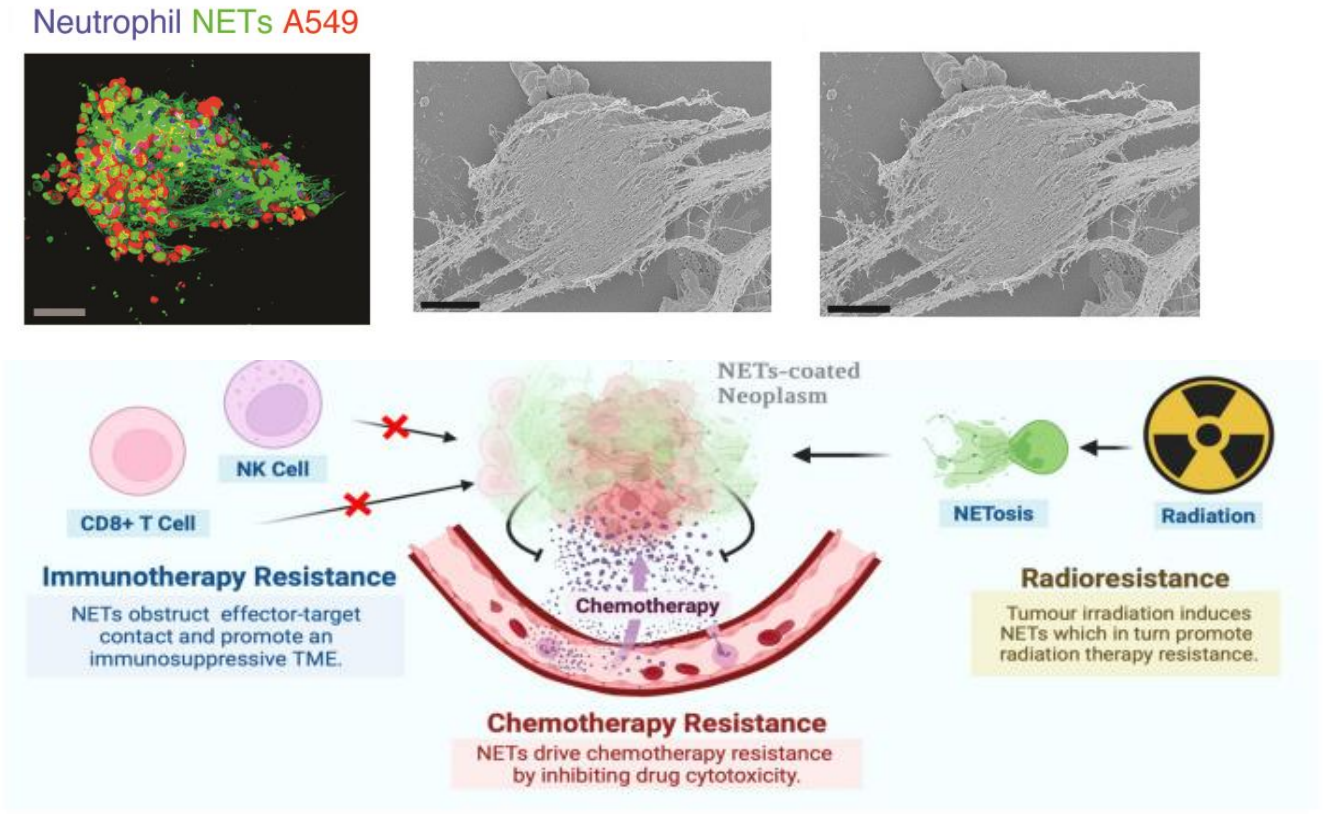

Fig. R7 Neutrophil extracellular traps (NETs) can capture circulating cancer cells, promote cancer progression and metastasis, and lead to resistance and failure in multiple kinds of therapies. Upper: Our confocal imaging and scanning electron microscopy demonstrating cancer cells (red) become trapped within webs of extracellular DNA (green) in proximity to neutrophils (blue). Adapted from Cools-Lartigue J et al., 2013, Journal of Clinical Investigation. Bottom: Our illustration showing Involvement of NETs in resistance to systemic and local anti-cancer therapies. Adapted from Shahzad et al., 2022, Cancers.

Clinically, a strong association between neutrophil, LN metastasis and compromised outcome has been reported. Numerous research showed that blood NLR can predict LN metastasis and this correlation is nearly universal among solid tumours, similar to the association of blood NLR and poor survival[131-136]. In addition, neutrophil level in primary tumour is correlated with the level of neutrophils in draining LNs, and high intratumoral neutrophil count can predict LN metastasis[137-139]. This indicates the dynamic correlation among multiple sites in cancer-bearing individuals. Most importantly, high LN neutrophil level is associated with disease progression and reduced overall survival[7, 139]. This is strong evidence stating that the LN immune profile is inseparable with systemic disease status and outcome. This serves as a rationale to the centuries old clinical practice—treating LN metastasis.

Although the pro-tumour roles of neutrophils and NETs have been extensively reported in various kinds of cancers and in multiple metastatic organs and sites, whether this is a possible mechanism implicated in LN metastasis remains undefined. One group showed that melanoma derived CXCL5 recruited high amounts of neutrophils and increased lymphangiogenesis. In this work, neutrophils, lymphatic vessels, and melanoma cells stayed in close proximity, indicating the possibility of interaction between these elements[140]. However, this work used xenograft model of human melanoma cells and immune-deficient mouse model, thus it is hard to draw conclusion regarding the chances of spontaneous occurrence of this phenotype and the role of intrinsic tumour-immune reaction. Moreover, unlike NLR, whether this mechanism is true to other types of cancers is unproved.

In conclusion, compared to the numerous works on other LN cells and clinical evidence, it is unfortunate to say that the cellular mechanism of a potential neutrophil-mediated LN metastasis remains entirely unknown. That was the motivation and objective of this thesis.

Extracellular vesicles (EVs) and metastasis

Extracellular vesicles (EVs) are lipid membrane bound vesicles secreted into the extracellular space[141, 142]. This term includes a heterogeneous group of vesicles with a range of different sizes. The main and most studied subtypes of EVs are microvesicles (MVs, can range from 100 nm up to 1μm in diameter), exosomes (typically 30–150 nm in diameter), and apoptotic bodies (vary in size from 50 nm up to 5000 nm in diameter. The size of most apoptotic bodies tends to be on the larger side)[143, 144].

Different subtypes of EVs have very different biogenesis and release pathways[141, 144, 145]. Exosome are formed by inward budding of the membrane of early endosomes, which mature into multivesicular bodies (MVBs). Some of MVBs can fuse with the cell's plasma membrane to release its content, including exosomes[12, 141]. Since MVBs are involved in the endocytic and trafficking functions of the cell's material, a putative physiological function is the protein sorting, recycling, and discharging from cells[146, 147]. Late McGill professor Rose Johnstone was the first scientist to discover and name exosomes, as early as in the 1980s[148]. She also investigated the unique pathway of exosome generation, which distinguishes them from other types of vesicles[149].

On the other hand, MVs are formed through direct outward budding of the cell membrane[150]. The mechanism of MV generation is not yet well known. It might require cytoskeleton participation and energy consumption[151]. The genesis and uptake of MVs also depends on the physiological state of the organism body and microenvironment around the cells, both for the donor and recipient cells[152]. The apoptotic bodies, evident by their name, are

released into the extracellular space by apoptotic cells. They tend to be on the larger side of the EVs[153].

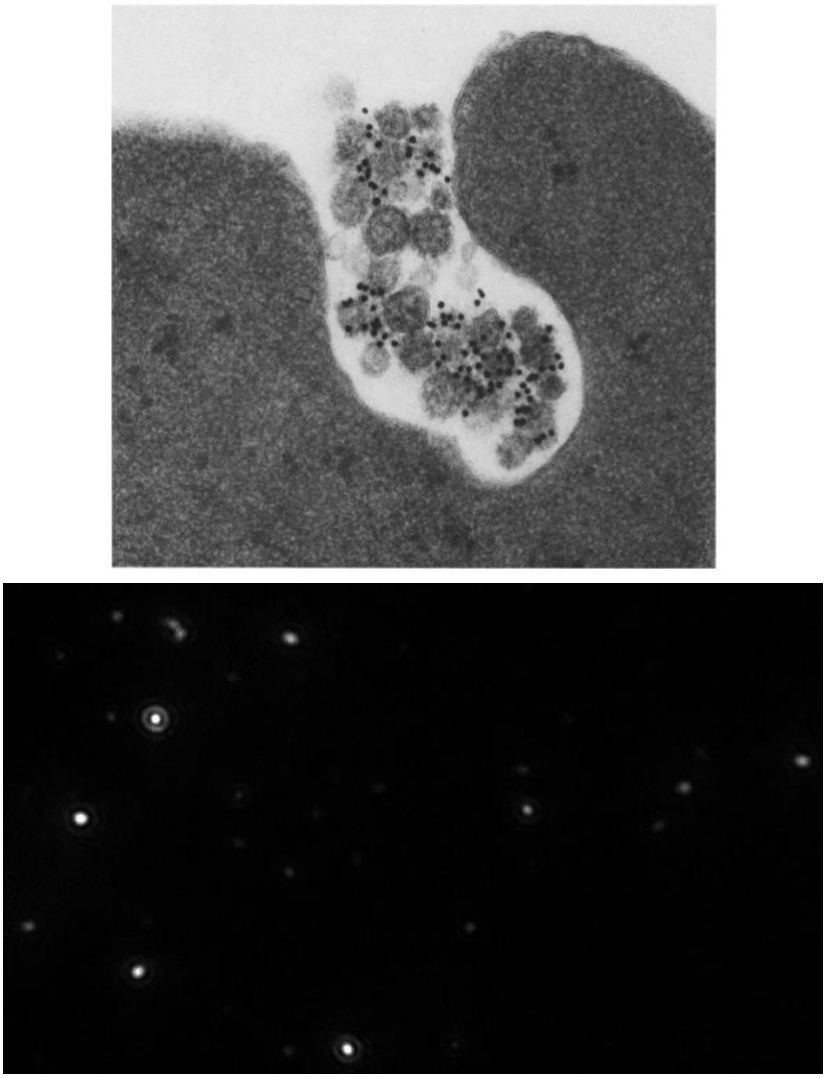

Fig. R8 Morphology of EVs and the presentation in bio-fluid. Top: Electronic microscopic view of exosomes leaving the reticulocyte. Adapted Rose Johnstone et al., Cell, 1983. Bottom: Nanoparticle tracking assay by Xin SU showing the extracellular vesicles, mainly exosome, suspending and detectable in the plasam from tumour-bearing mice.

The contents of EVs are composed of nucleic acids, proteins, peptides, and lipids, though the proportion and enrichment of each content might vary between different subtypes of EV[154, 155]. The lipid membrane that encloses EVs is also decorated with all kinds of proteins and receptors, similar to cell membrane[156, 157]. The fascinating part about EVs is that they can secreted by nearly all eukaryotic cells and they are present in every kind of biofluid, including plasma/serum, bile, urine, and cerebrospinal fluid[158-161]. The ubiquitous characters of EV underly its essential participation in the pathogenesis of many diseases, also render their accessibility for clinical detection biomedical research[162].

The physiological function of extracellular vesicles is still largely unknown. As mentioned previously, exosomes might be responsible for proteins transportation from cell, as the example of Rose Johnstone's works demonstrating that exosomes can carry iron-binding proteins to leave reticulocytes during maturation of red blood cells. This also highlighted the roles of exosomes in developmental biology[148, 163]. For apoptotic bodies, it is not hard to understand that they are capable of sealing proteins and other materials from dying cells as a way of removal of cell debris[145, 164]. In addition to the traditional "protein recycling" roles, recently it has been reported that EVs has great roles in maintaining normal functions of the immune systems[165, 166]. For example, EVs can regulate the formation of the immunological synapse between T-cells and antigen-presenting cells (APC), promote the development of an immune response[167]. Moreover, it has been reported that MVs can facilitate the crosstalk between stem and injured cells thus promoting tissue repair[168]. Even though there is no systemic and defined conclusion on the physiological roles of EVs, it is clear that EV is a way of communication between cells: They are intercellular messenger and they can modulate cell signalling. The EV content and surface marker are the information that they are carrying. EVs

exert their effects by both receptor ligand signalling as well as by uptake in recipient cells. Through this, they can horizontally transfer their cargo to distant sites[169, 170].

Comparing to the relative unknown side of EVs in normal condition, there are increasingly abundant research focusing on how EVs contribute to different kinds of disease[171, 172]. For example, EVs released by endothelium under atherosclerotic conditions can improves plaque stability and lead to coronary artery disease progression[171, 173]. In neural system, EVs can carry the misfolded forms of proteins associated with Alzheimer's, Parkinson's, and the prion diseases, thus leading to the further spread of disease to other brain regions[174, 175]. Another example is the involvement of EVs in autoimmune disease: just as EVs can maintain normal immune balances, they can also participate in breaking this balance[176, 177]. For example, in systemic Lupus Erythematosus, EVs containing surface antigens can bind to circulating immunoglobulins thereby causing the accumulation of immune complexes. This will lead to inflammation and tissue damage[178].

A unique character of EVs is that not only do they participate in the progression of many diseases, but they can also indicate the severity of certain diseases as they are detectable biomarkers[17, 141, 162]. Monitoring the number of circulating EVs or the level of a certain EV content is part of liquid biopsy—the concept of sampling and analysis of non-solid biological tissues, including blood and other blood fluid, with the benefit of being non-invasive and repeatable[179, 180].

Most notably, EVs play a great role in orchestrating nearly all major oncological events in cancer development and progression, from carcinogenesis and tumour cell growth to metastasis and treatment resistance[147, 169, 170]. EVs from cancer cells may induce or promote neoplasia in pre-neoplastic epithelial cells by inducing mutations and reprogramming

stemness with the RNA contents they carry [181-183]. After carcinogenesis, EVs can also facilitate epithelial to mesenchymal transition (EMT), angiogenesis and extracellular matrix remodelling[184-187]. Moreover, EVs can neutralising treatment agents by expressing the same therapeutic targets on their surfaces (for example, the CD20 protein on EVs secreted by B cell lymphoma can bind to anti-CD20 antibodies). Thus, EVs can promote cancer cell resistance to various antibodies[188, 189]. Notably, by horizontally transferring miRNAs, EVs can also spread drug-resistance properties between cancer cell populations[190, 191].

EVs have a profound role in promoting metastasis, especially in preparing pre-metastatic niche in distant organs—a process of environmental changes and matrix cell activation in potential metastatic sites before the arrival to disseminating cancer cells[192, 193]. Based on their surface integrin profiles, EVs from different types of tumours showed different organotropism—an affinity to a certain organ for metastasis[194]. After travelling to the potential metastatic sites, EVs can cause cellular activation and biochemical changes in the recipient cells such as Kupffer cell in liver, alveolar cells in lung and endothelial cells in brain[195-197]. This premetastatic modulation creates a fertile environment for the upcoming disseminating cancer cells, supporting their outgrowth and eventually the new metastases formation.

Irrespective of the relatively limited number of reports, the roles of EVs in preparing LN premetastatic niche are already established. EVs can be actively and rapidly uptaken by lymphatic system[18]. Moreover, the level of EVs in lymph is higher than in plasma[17]. After arriving at sentinel LNs, EVs can induce great changes in the LN environment. EVs can lead to lymphangiogenesis and lymphatic network remodelling, either through direct interact with LEC or inducing macrophages to secrete lymphangiogenic VEGFC[52, 198-200]. In addition, EVs

also has an immunosuppressive effect in LNs by inducing apoptosis in antigen specific CD8+ T cells[201]. Moreover, EVs also exert pro-LN-metastasis effects in peri- and post-metastatic behaviours: they can increase the cancer cell adhesion[198] and surprisingly, EVs from LN metastatic cells can influence back to the primary cancer cells, increasing their metastatic capacity[202].

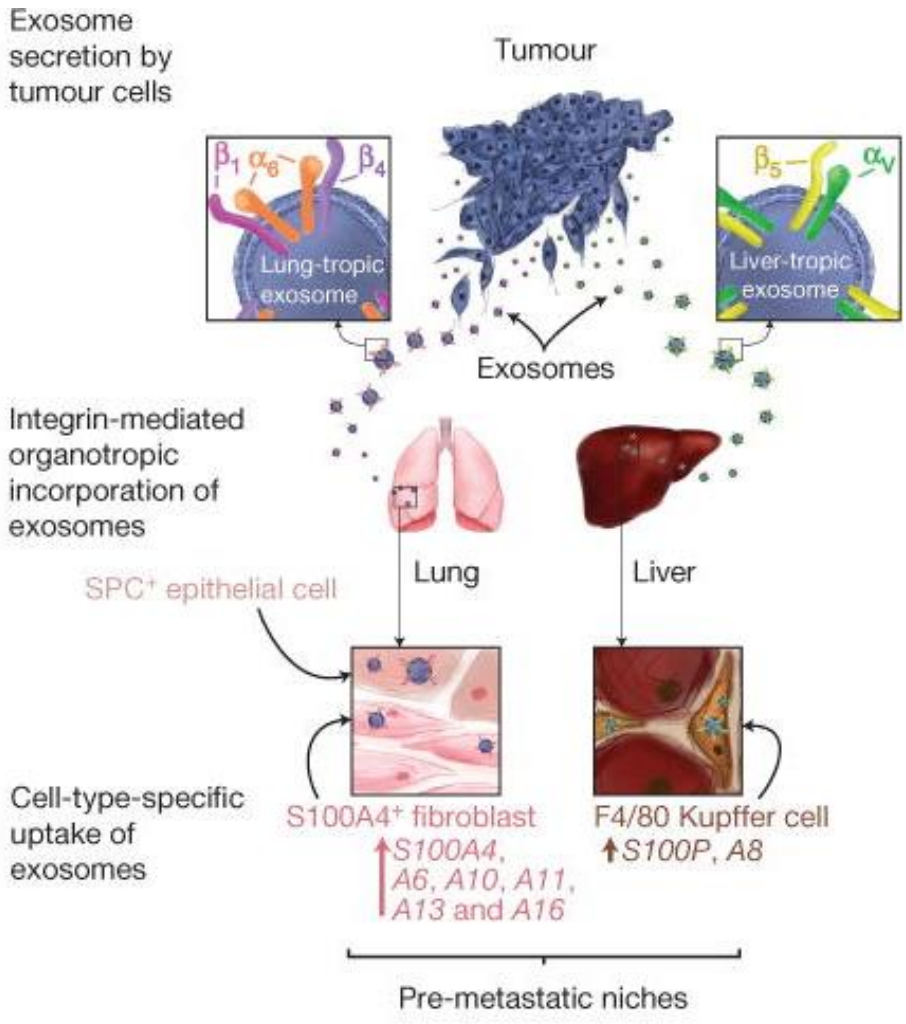

Fig. R9 Tumour exosome integrins determine organotropic metastasis by activating specific matrix or immune cells in the potential metastatic site. Adapted from Hoshi et al., Nature, 2015[203].

Taken together, these environmental changes induced by EVs result in an increased LN metastasis burden: the immunogenic, cancer-hostile organs, have been converted into the first sites of metastasis. However, here a new research question and a new knowledge gap have been raised: EVs has dramatic impact on promoting metastasis in many organs including LN, and neutrophils show pro-tumour phenotype in many organs too. However, does the interplay of EVs and neutrophils can promote LN metastasis? This is entirely unknown.

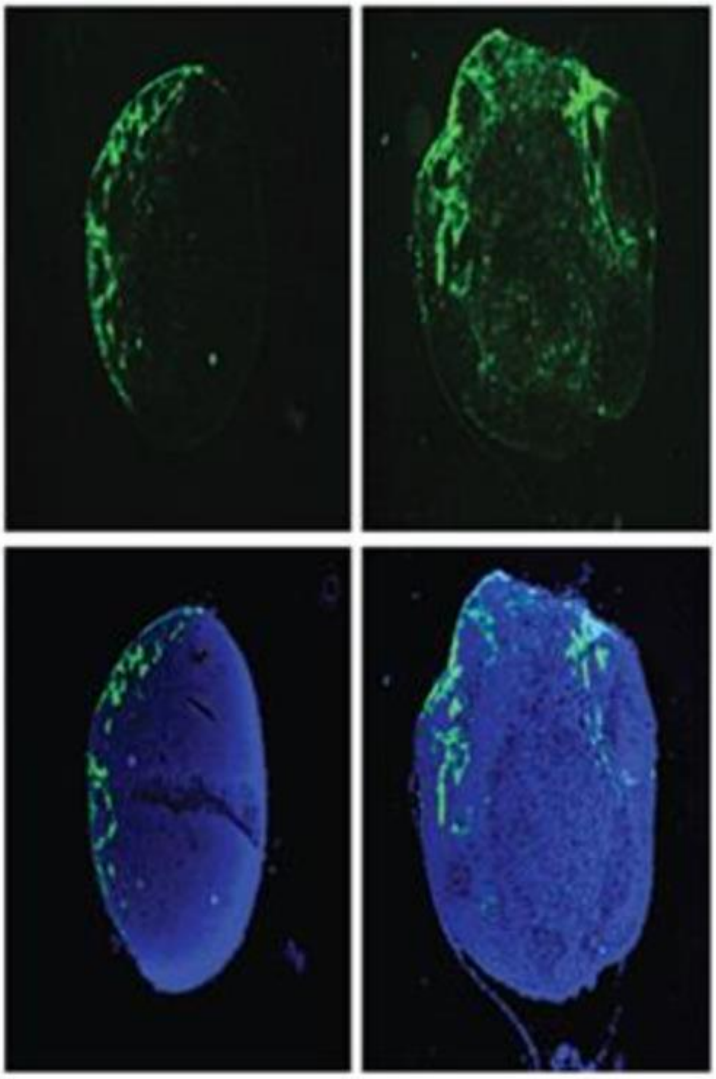

Fig. R10 EV prepare LNs for metastasis. After pre-treatment of EVs, the metastatic burden of melanoma cells was significantly in mouse LNs. Adapted from Joshua Hood et al., Cancer Research 2012[16].

Neutrophil plasticity in the tumour microenvironment and the regulations of EVs

Neutrophils can display great plasticity. This means that they have the abilities to change their morphology and biological functions in different conditions, including tumour and tumour related inflammation. In this context, neutrophil actually represent a spectrum of cells, and the naming include terms such as N1/N2 polarised neutrophils, tumour-associated neutrophils, and polymorphonuclear neutrophil myeloid–derived suppressor cells (PMN-MDSCs). These classification methods are all based on the phenotypes of neutrophils, because of few identified classification/surface markers[9, 11, 204]. Nevertheless, the key concept is that neutrophils can exhibit very diverse even contradictory roles in different environments.

It is widely accepted that cancer demonstrated great similarity to chronic inflammation or unhealed wound[11, 205, 206]. This similarity is multi-dimensional. First, unsolved inflammation such as inflammatory bowel disease or chronic hepatitis, either viral hepatitis or non-alcoholic fatty liver disease, could lead to cancers. Next, after tumorigenesis, cancer still preserved the phenotypical mimicking of inflammation, characterised by active matrix degradation, abundant angiogenesis and most importantly, the infiltration of various kinds of immune cells including neutrophils[95, 207]. In addition to attracting neutrophils by their rapid growth and necrosis, tumour cells are actually taking active efforts to recruit and exploit tumour cells[106], and this regulation is also multi-dimensional.

First, tumour cells secrete high level of pro-myelopoiesis factors, including traditional colony stimulating factors G-CSF, GM-CSF, or novel factors such as sRAGE.[208-210] These factors either directly work on bone marrow or bone matrix cells to switch the haematopoiesis towards a myelopoiesis side—generating more neutrophils and monocytes. Moreover, tumour derived factors also disturb the process of normal myelopoiesis, resulting in the release of premature, abnormal neutrophil into circulation[102, 103, 105]. Second, tumour cells also release neutrophil chemoattractant such as CXCL4, CXCL8 (IL-8), CXCL12 and complements, thus neutrophils are actively recruited to and retained at primary tumour or metastases[211-215].

Once infiltrated into tumours, as tumour associated neutrophils, their phenotypes and functions can be changed dramatically as well. Many types of tumour cells overexpress growth factors that can modulate neutrophil phenotypes, among them TGF-β is the most thoroughly studied one[216]. TGF-β can lead to the N2 polarisation of circulating neutrophil. Like mentioned previously in the neutrophil section, instead of releasing tumour cytotoxic ROS or activating adaptive immune system, N2 neutrophils release cancer cell nurturing enzymes and angiogenic VEGFA[117, 121, 217]. Furthermore, cancer cells can impact NETs formation as well. Cancer cells can release molecules such as High mobility group box 1 protein (HMGB1), IL-8 and TGF-β, especially under stress conditions, such as post-radiotherapy or postoperative infectious complications. This result in extensive NETs formation[19, 126, 218-220]. Notably, circulating neutrophils from cancer patient are also more prone to form NETs, compared to neutrophil from health controls. This is possibly due to the pre-exposure to high level of G-CSF in cancer patients[221, 222].

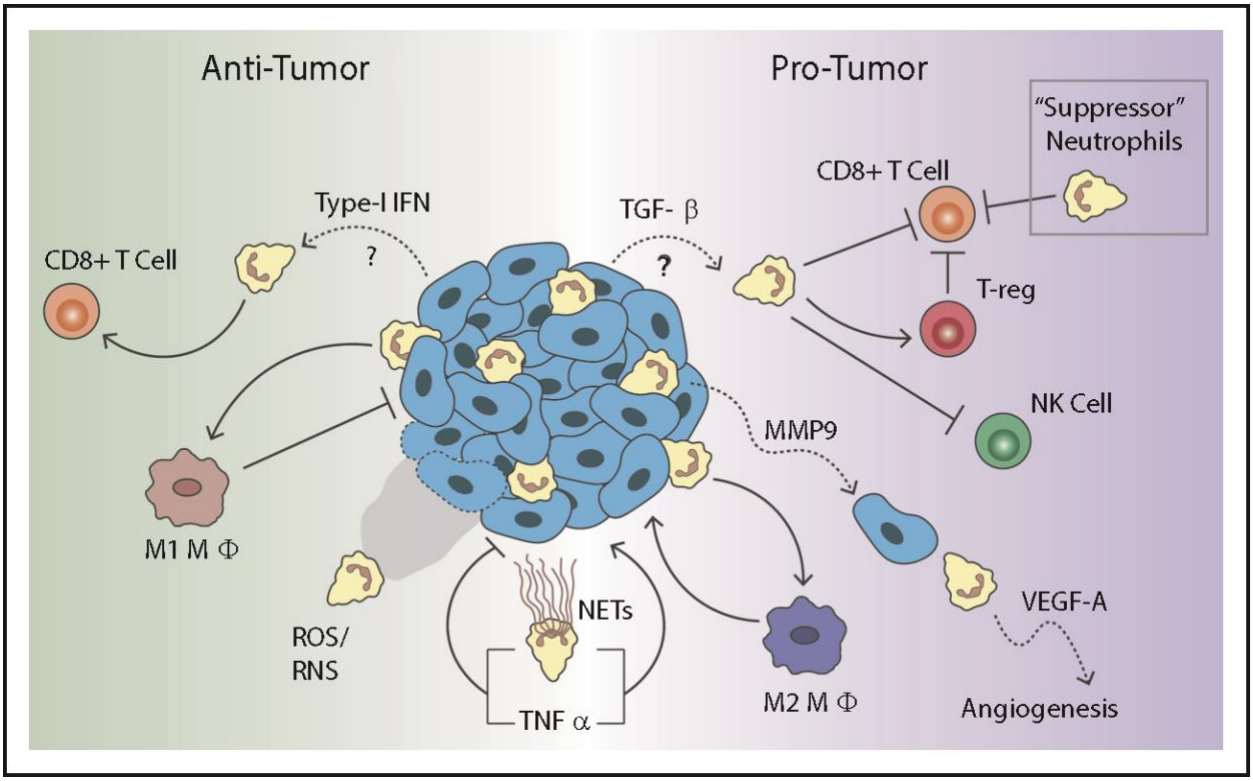

Fig. R11 Neutrophil plasticity in the tumor microenvironment. Under the regulation of different kinds of cytokines, neutrophil can exhibit either anti- or pro-tumour phenotype and conduct opposite functions. Adapted from Giese et al., Blood, 2019[11].

In addition to soluble factors such as cytokines, which have been thoroughly studied, there is emerging evidence suggesting the roles of EVs in regulating neutrophil plasticity. For example, mesenchymal stromal cells in bone marrow, especially under the reprogramming impact of tumour cells, can secrete EVs to promote myeloid-biased multipotent hematopoietic progenitor expansion, thus favouring the neutrophil production and elevate the blood level of neutrophils[223, 224]. In addition, it is reported that cancer exosomes can induce alveolar epithelium to secreted CXCL2 and CXCL5 to recruit neutrophils, thus forming inflammatory

premetastatic niche[196]. Moreover, in the tumour microenvironment, tumour EVs can modulate neutrophil phenotypes as well: gastric cancer derived exosomes can induce N2 polarization of neutrophils to promote gastric cancer cell migration[19]. Furthermore, tumour cell derived EVs can induce the formation of NETs, thus increasing the incidence of cancer-associated thrombosis, a common complication in cancer patients[20]. Interestingly, in this report, neutrophils are pre-exposed to G-CSF, indicating the synergy of neutrophil regulating cytokines and EVs. Nevertheless, similar to cytokine, EVs can impact and regulating neutrophil plasticity, including their expansion marrow, their motility to tumour sites, their pro-tumour phenotype polarisation, and finally, their propensity of form NETs.

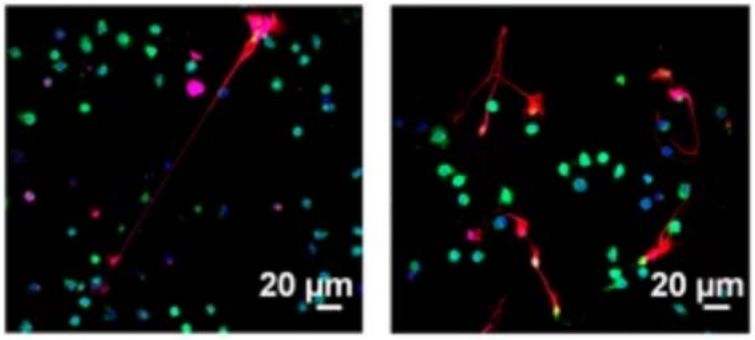

Fig. R12 In vitro mouse NETs formation induced by cancer EVs. Immunofluorescence staining for DNA (blue), Ly6G (green) and citrullinated histone (red). These neutrophils were pre-treated with G-CSF. Adapted from Zhang et al., Scientific Reports, 2017.

Given the established role of neutrophil, NETs and EVs in promoting cancer metastasis in many organs, and the certainty that EVs can modulate neutrophil function towards a pro-tumour polarity, whether this link contributes to the process of LN metastasis is still unknown. It is reasonable and necessary to investigate:

1. As tumour-derived, mobile, and lymphatic affinitive factors, can EVs can be retained in LNs and impact local environment?

2. As LN resident cells, can neutrophils be regulated and modulated by EVs from primary tumour?

3. Can interplay between neutrophil and EVs lead to the formation of NETs, since NETs is a pro-tumour molecule generated under tumour associated inflammation?

4. Can this kind of LN NETs formation and local inflammation can promote LN metastasis?

5. Will disruption of any element in this inflammatory chain, either EVs, neutrophils or NETs, show therapeutic potentials in treating LN metastasis?

With all these hypothesis and objectives, we conducted the experiments in this thesis.

Body of the thesis

Methodology

Patients

This study investigated surgical lymph node of 175 patients with stage I to IV gastroesophageal adenocarcinoma for the TMA study (Table 1), and the 10 patients for plasma EV proteomics study (Table 3) who were treated at the McGill University Health Centre (MUHC), Montréal, Canada. All the studies were conducted according to the relevant regulatory standards, upon approval by the Research Ethics Board Office at McGill University.

TMA construction, immunofluorescence (IF) staining and analysis

Tissue microarrays (TMAs) were constructed from the surgical blocks used for pathologic evaluation. Three pathologists constructed 8 TMA blocks using 1 mm cores punched from formalin-fixed and paraffin-embedded tissue blocks. After sectioning of TMA blocks and IHC staining, a pathologist verified that each core contained the lymph node tissue of interest and whether there was LN metastasis. TMA slides were stained at the histopathology core of Research Institute of McGill University Health Centre (RI-MUHC) using primary antibodies including anti-NE (MAB91671100; R&D Systems), anti-CD66b (392902; BioLegend), anti-H3Cit (ab5103; abcam) and anti-cytokeretin 7(ab9021; abcam). To visualize these antibodies, OPAL kit (PerkinElmer) fluorophores were used and prepared according to the manufacturer's

protocols. The slides were counterstained with DAPI (D21490; Invitrogen) and cover slipped. TMA slides were then digitalized at 20× magnification using Axio Scan Z.1. whole slide scanner (Zeiss). HALO image analysis software (Indica Labs) was used to quantify the number of $CD66b^{+}NE^{+}$ positive cells as neutrophil and the area covered by NETs(H3Cit).

Quantification of neutrophil and lymphocyte counts in peripheral blood

Circulating NLR of patients was calculated from clinical laboratory blood tests performed on the date of diagnosis. A cut off NLR value of 4 was used to assign patients to into low or high groups. LN NLR was determined via IF staining of TMAs as described before.

Cell Lines

B16-F10, H59, A549 and BEAS-2B cells were all maintained in corresponding media suggested by American Type Culture Collection (ATCC) with 10% heat inactivated FBS. Cells used for in vitro EV production were maintained in media with 10% heat inactivated FBS depleted of bovine EVs by ultracentrifugation at 100,000g for 14 hours.

Animals

C57BL/6 (Charles River Laboratories) and PAD4 knockout ($PAD4^{-/-}$; gift of Alan Tsung, The Ohio State University Comprehensive Cancer Centre, Columbus, Ohio, USA) mice were used for all experiments at 7–10 weeks old. 250k B16F10-GFP, H59-GFP, B16F10,

B16F1, B16F10-scramble RNA, or B16F10-Rab27 KD cells were injected subcutaneously into the flank of these mice, and tumour growth was monitored twice a week using a calliper. Blood was collected by saphenous vein bleeding. All experiments were performed with the Veterinary Authority of the Institutional Animal Care and Use Committee at the RI-MUHC under protocol no. 7469 and 8210. For the neutrophil depletion, experimental group mice received intravenous injection of 100µg anti-Ly6G (1A8, BE0075-1; BioXCell) on day 0, 2, 6, 8, 10, 12 post tumour inoculation, and 100µg of anti-Rat-kappa light chain (MAR18.5, BE0122; BioXCell) on day 1, 5, 7, 11, 13 post tumour inoculation. Control group only received anti-Rat-kappa light chain injection. For the knockout experiments, PAD4–/– mice were compared to age and gender matched C57BL/6 mice. For the NEi treatment, experimental group mice received daily gavage of 2.2 mg/kg of neutrophil elastase inhibitor Sivelestat (NEi, ab146184; abcam) resuspended in saline. Control mice received saline gavage.

Flow cytometry

Mouse blood were prepared by lysing red blood cell in BD Pharm Lyse lysing solution (555899; BD Pharm) and washed in PBS. Mouse LN were minced and filtered through 40µm cell strainers (087711; Fisher Scientific) and washed in PBS. Cell suspensions were blocked with Fc-block (553141; BD) and incubated with the following primary antibodies: Viability Dye Eflour780 (65-0865-14; eBioscience), anti-CD11b-BV510(101263; BioLegend) and anti-Ly6G-AF647(127610; Biolegend). Data acquired on a BD LSRFortessa™ Cell Analyzer cytometer was analyzed using FlowJo software (Tree Star).

Lymph node immunofluorescence staining, imaging, and analysis

Mouse Ipsilateral inguinal lymph node of the tumour side was dissected after sacrificing and fixed in 1% paraformaldehyde, dehydrated in 30% sucrose, and embedded in Optimal Cutting Temperature (OCT) compound. 15 μm OCT tissue sections were stained with primary antibodies including: anti-H3Cit (ab5103; abcam), anti-Ly6G-AF647 (127610; Biolegend), anti-SOX-10-AF488 (NBP2-59621AF488; Novus Bio),anti-LYVE-1 (AF2125; R&D Systems), anti-Ly6G-FITC (11-9668-80; Biolegend), anti-MIP2/CXCL2 (500-P130; PeproTech) and secondary antibodies including: goat-anti-rabbit-AF568 (A11011; Invitrogen), donkey-anti-goat-AF488 (A110555; Invitrogen), donkey-anti-goat-AF568 (A11057; Invitrogen), donkey-anti-rabbit-AF647 (A-31573; Invitrogen).

Human lymph node block from consented lung adenocarcinoma patients was retrieved from Department of Pathology of MUHC. 4μm paraffin tissue slides were de-paraffined, antigen-retrieved in pH6 sodium citrate buffer and stain with primary antibodies including: anti-LYVE-1 (AF2089; R&D System), anti-CXCL8 (MAB208; R&D System) and secondary antibody including: donkey-anti-goat-AF568 (A11057; Invitrogen) and donkey-anti-mouse-AF488(A-21202; Invitrogen).

The slides were counterstained with DAPI (D21490; Invitrogen) and cover slipped. Negative controls were stained with secondary antibody only or isotypes conjugated to the same fluorophore. The slides were imaged with LSM 780 confocal microscope at the molecular imaging core of RI-MUHC. ImageJ Software (NIH) was used to analyse the fluorescence area of GFP, Ly6G, H3Cit, SOX-10 and the relative fluorescence area of CXCL2 to LYVE-1.

EV isolation labelling and mouse injection

Cells were cultured in media supplemented with 10% EV-depleted FBS. Supernatant fractions collected from 48–72 h cell cultures were pelleted by centrifugation at 300g for 10 min. The supernatant was centrifuged at 20,000g for 20 min. EVs were then harvested by centrifugation at 100,000g for 70 min. The EV pellet was resuspended in 20 ml of PBS and collected by ultracentrifugation at 100,000g for 70 min. For fluorescence labelled EVs, supernatant was concentrated using Amicon Ultra-15 Centrifugal Filter Units with 100 KDa filter size, incubated with 1.0 mM CM-DiI or 50 μM CFSE for 2 hours before this first 100,000g centrifugation.

For the EV pre-treatment experiment (Fig. c, d), 10 μg EV in 30 μL PBS was injected into the ipsilateral footpad every other day before tumour inoculation. For the EV location experiments, 10 μg CM-Dil labelled EV were injected 24 hours before sacrificing mice and LN dissection. For the EV treatment in no-tumour bearing mice, 10-30 μg EV in 30 μL PBS was injected into the ipsilateral footpad every other day before sacrificing.

Lentiviral infection and screening procedure

B16F10 cells were transfected with Rab27a-mouse shRNA and scramble RNA lentiviral particles purchased from Origene according to the manufacturer's instructions. Briefly, B16F10 cells were plated in 24-well plate at 50,000 cells per well (day zero). At day 1, cells were infected with lentiviral vectors in the presence of 8 μg/ml polybrene. Media was changed at day3 and from day 4, cells were treated with 0.5 μg/ml puromycin for 10 days to generate the stable

knockdown cell line. The sequence of the Rab27a shRNA is CTGGATAAGCCAGCTACAGATGCACGCGT.

Western Blot

25 µg of EVs or cell lysates was mixed with Laemmli SDS sample buffer (Bio-Rad), incubated 10 min at 95°C, and cooled to 4°C. Electrophoresis was performed on Mini-PROTEAN TGX Gels (Bio-Rad). Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Blocking was performed 30min at room temperature (RT) in 5% Tris-buffered saline (TBS) milk, primary in 5% TBS bovine serum albumin (BSA), overnight at 4°C (shaking), and secondary (HRP-conjugated;Bio-Rad) in 5% TBS BSA, 30min at RT (shaking). The following primary antibodies were used: anti-ITGA6 (1:250, 3750; Cell Signaling), anti-Alix (1:250, 2171; Cell Signaling), anti-BiP (1:500, 3183; Cell. Signaling), anti-TSG101 (1:500, ab125011; abcam), anti-GAPDH (1:1000, MA5-15738; Invitrogen) and anti-Rab27a (1:500, 69295; Cell Signaling).

Nanosight analysis of EVs

EV density and size distribution were assessed by nanoparticle tracking analysis (NanoSight; Malvern Instruments). 5 µl of isolated EVs was diluted in 500 µl of PBS to achieve a uniform particle distribution that was analyzed in three to five sequential measurements at 37°C. For the Rab27a KD experiments, 500ul of FBS-free cell conditioned media from control and Rab27a KD cell lines were used.

Transmission electron microscope (TEM) analysis of EVs

Negative stain was performed in the following way: 5 µl sample solution was adsorbed to a glow-discharged carbon-coated copper grid (Canemco & Marivac), washed with deionized water, and stained with 5 µl 2% uranyl acetate. The samples were imaged at RT using Tecnai Spirit electron microscope at the Facility for Electron Microscopy Research of McGill University.

LEC culture and immunofluorescence

LEC were purchased and cultured MV2 media (C-22022) from in PromoCell on Collagen I coated tissue culture vessels and were treated with 10 µg/ml EV or 5 µg/ml Lipopolysaccharides (LPS, Sigma-Aldrich) for 24 hours. The LECs monolayer was washed in PBS after cocultures was done. After 15 min of fixation n 2% paraformaldehyde, RT, LEC were stained with anti-Podoplanin-AF647 and anti-CXCL8-AF488 (IC208G; R&D System). Negative controls were stained with isotypes conjugated to the same fluorophore. The slides were imaged with LSM 780 confocal microscope at the molecular imaging core of RI-MUHC. ImageJ Software (NIH) was used to analyse the fluorescence area of CFSE and the relative fluorescence area of CXCL8 to DAPI.

Human peripheral blood neutrophil isolation

Human neutrophils were isolated as previous described[124]. Briefly, blood was diluted in PBS and layered over Lymphocyte Separation Media (Wisent Bioproducts). After centrifugation at 800 g for 30 min at RT, the pellet containing neutrophils and red blood cells (RBCs) was collected and resuspended in 3% Dextran (Wisent) and left at RT to sediment RBCs. After 30min, the supernatant is collected and centrifuged at 450 g at 4°C for 5 min. Remaining RBCs in the neutrophil rich pellet are then lysed using BD Pharm Lyse Lysing buffer. The obtained pellet is then washed and resuspended in cold RPMI media (Wisent Bioproducts. Purity and viability of the obtained neutrophils were verified through Methylene blue (Stem cell Technologies) and Trypan blue (Wisent) staining respectively.

Boyden Chamber Neutrophil migration assay

LEC were treated with 10 μg/ml A549/BEAS-2B EV, 5 μg/ml LPS, 100nm Liposome control (Encapsula Nanoscience) of same number of particles (determined by NTA) or PBS of the same volume in MV2 for 24 hours. The next day, fresh MV2 media was changed. 72 hours later. The conditioned media was collected, centrifuged at 450g at 4°C for 5min to pellet down cells. The supernatant was then transfer to the bottom chamber of the transwell. 1 million neutrophil was layered in the top chamber and leave for migration. 24 hours later, migrated neutrophil in the bottom chamber was resuspended and count by Methylene blue stain. This assay was repeated 3 times with triplicates.

ELISA of CXCL8

The A549/BEAS-2B cell lines were incubated in corresponding media with 0.5% FCS for 72 hours to collect cell conditioned media (CM). The LEC CM were collect in the same was as the boyden chamber experiments. CXCL8 amount in the CM were incubated with fluorescence multiplex ELISA kit from RayBioTech and quantified using Q-analyser from the same manufacturer.

LEC CM induced NETs formation

LEC CM was collected in the same way as in the boyden chamber experiments and transferred to Nunc Lab-Tek II Chamber Slide (Thermo Scientific), 200k neutrophils were added to the CM and NETs formation was performed at 37°C. After 24 hours, the supernatant was discarded and the slides were gentle washes, fixed with 4% PFA for 15min and stained with DAPI, anti-MPO-FITC (ab11729; abcam), anti-H3Cit (ab5103; abcam), anti-CD66b-APC (130-122-966; Miltenyl Biotech) and then goat-anti-rabbit-AF568 (A11011; Invitrogen). The slides were imaged with LSM 780 confocal microscope at the molecular imaging core of RI-MUHC. Percentage of NETosing neutrophils (H3Cit positive neutrophils) were quantified.

EV induced NETs formation in health control and GEA patients

Health control or GEA patient neutrophils were isolated as mentioned before. Every 200k neutrophil were treated with 10 μg/ml A549 EV and incubated at 37°C for 4 hours. After 4 hours, SYTOX Green (Invitrogen) was added to a final dilution of 1:1000 and fluorescence

intensity were acquired Tecan Infinity F200. Proneness to NETose was quantified as the relative fluorescence intensity of SYTOX Green of EV treated wells to untreated wells. Representative images were acquired using EVOS cell imaging systems (Thermo Scientific).

Patient plasma EV isolation, mass spectrometry and proteomics analysis

1ml of plasma for each patient was retrieved from the biobank platform of RI-MUHC. The plasma was thawed, 350 µl thromboplastin (292273; Thermo Scientific) was added and the mixture was incubated at room temperature for 1 hour. 20ml PBS was added to dilute the mixture, followed by serial ultracentrifugation of 20,000g for 20 min to deplete the cell debris and the supernatant then and 100,000g for 70 min. The EV pellet was resuspended in 20 ml of PBS and collected by ultracentrifugation at 100,000g for 70 min. The final EV pellet was suspended and lysated in 50-70 µl RIPA Lysis and Extraction Buffer (89900; Thermo Scientific).

For the proteomic analysis of EVs, liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed at the clinical proteomic platform at RI-MUHC. The raw file was processed by Scaffold software (Proteome Software), FunRich and Graphpad.

Statistical analysis

Data were expressed as the mean ± SEM. Data were analyzed using Prism software (GraphPad Software, Inc.). Statistical significance between two groups was assessed using a Mann Whitney t test. When more than two value sets were compared, we used one-way

ANOVA, Kruskal-Wallis test or Brown-Forsythe ANOVA test. $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$ was considered statistically significant.

The Kaplan-Meier curves in Fig.2c were generated using Prism software and the Kaplan-Meier curves in Fig. 5a were generated using RStudio (Version 3.6.3)[225]. Higher and lower expression levels were stratified based on median expression levels. Statistical analysis was performed using log-rank tests, and HRs were calculated.

Research Findings

Lymphatic NETs are associated with reduced survival in gastroesophageal cancer patients

We have previously demonstrated that neutrophil accumulation and NETs deposition promotes the development of metastasis at distant organ sites[123, 124, 222]. Here, we sought to determine if this process was conserved in respect to lymphatic metastasis in GEA patients. Accordingly, we characterized the pattern of lymphatic neutrophil recruitment and NETs deposition within pathologic LN samples from patients with GEA. Surgical samples from 175 patients were made into tissue microarrays (TMAs) (Table 1) and immunofluorescence was performed. The TMA was constructed from surgical resection specimens and thus comprised both patients with (N+) and without (N0) LN metastasis. Thus, within an N+ patient, not all nodes harbour metastatic cancer. Those that do are designated N+met, while those that do not are designated N+neg. Conversely, within an N0 patient, all regional nodes are free of cancer.

Table 1 Demographic and clinical characteristics of patients with gastroesophageal adenocarcinoma from in the TMA study

Patients with gastroesophageal adenocarcinoma in the TMA study (n = 175)	
Mean Age, years	68.08
Sex, male	78.28%
Neoadjuvant Treatment, yes	54.48%
Clinical Stage 1	21.65%
Clinical Stage 2	31.85%
Clinical Stage 3	36.94%
Clinical Stage 4	9.55%

Clinical T1	12.10%
Clinical T2	21.65%
Clinical T3 & 4	66.24%
Clinical N, yes	55.69%
Clinical M, yes	10.82%
Pathologic Stage 1	18.07%
Pathologic Stage 2	22.29%
Pathologic Stage 3	51.20%
Pathologic Stage 4	8.43%
Pathologic T1	14.94%
Pathologic T2	19.55%
Pathologic T3 & T4	65.51%
Pathologic N0	25.28%
Pathologic N1	19.54%
Pathologic N2	26.43%
Pathologic N3	28.75%
Pathologic M, yes	8.82%
Recurrence, yes	39.65%
Grade, poor	52.35%
Grade, moderate	41.76%
Grade, well	5.88%
Lymphovascular Invasion, yes	70..00%
Average Overall Survival (days)	1213
Average Disease-Free Survival (days)	1109

Samples were stained with neutrophil markers CD66b and neutrophil elastase (NE), NETs marker citrullinated histone H3 (H3Cit) and GEA epithelial marker cytokeratin 7 (CK7). We found that neutrophils were present and progressively recruited to regional LNs in N+ patients (Fig. 1a). By quantification, we demonstrated that cancer positive nodes (N+met) have significantly higher neutrophil counts compared to negative nodes (N+met vs. N+neg, 137.0 vs. 51.77, $p = 0.0033$) (N+met vs. N0, 137.0 vs. 21.23, $p<0.0001$) (Fig.1b). Notably, the neutrophil count in N+neg is significantly higher than within N0 (51.77 vs. 21.23, $p<0.0001$). This indicates that LN neutrophil recruitment may occur prior to lymphatic neoplastic invasion.

Figure 1

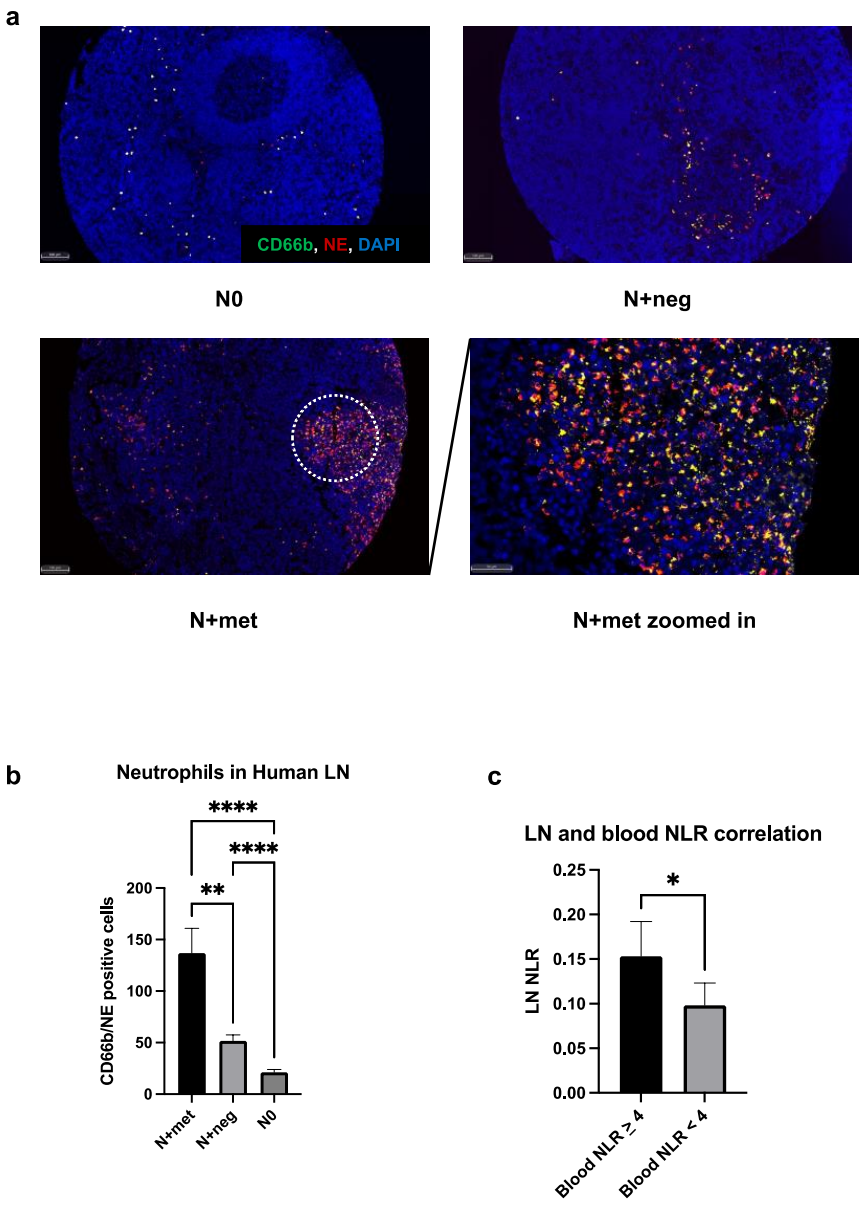

Figure 1: Neutrophils are recruited to regional lymph nodes in gastroesophageal adenocarcinoma patients. (a) Representative images of lymphatic involvement by neutrophils depicting a node negative patient (N0, top left), negative lymph node in a node positive patient (N+neg, top right), and a positive lymph node (N+met, bottom left). Scale bars represent 100μm.

White circle indicates the zoomed area shown in bottom right. Scale bars represent 50μm. (b)Quantification of lymphatic neutrophils (CD66b/NE positive cells) in positive nodes(N+met), negative nodes in node positive patients (N+neg), and node negative patients (N0). (c) Comparison of lymph node neutrophil-lymphocyte ratio (LN NLR) with patient with high blood NLR (≥4) and patients with low blood NLR (<4). Data shown as mean ± SEM. n = 175; **, P < 0.01; **** P < 0.001 by Brown-Forsythe ANOVA test.

In addition, we assessed the relationship between the peripheral blood neutrophil-lymphocyte ratio (NLR), a standardized method of neutrophil quantification[96, 97], and LN NLR (Fig. 1c). We found that patients with a blood NLR>4, which has previously been defined as elevated, also have a significantly higher LN NLR (0.02430 vs. 0.01374, p= 0.0451). This result suggests the dynamic correlation between lymphatic neutrophil accumulation and systemic inflammation.

Having shown that neutrophil accumulation within regional LNs can occur alongside tumour LN infiltration, we next sought to determine if NETs deposition was also taking place within the LN. Mirroring the same trend shown in nodal neutrophil infiltration, both N+met and N+neg nodes have higher levels of NETs deposition compared to N0 nodes (Fig. 2a, b). (0.05134 versus 0.1663, p = 0.0372) (0.05134 versus 0.1330, p = 0.0401) Moreover, we found that increased LN NETs quantity was significantly associated with reduced overall survival, both in N+met (hazard ratio [HR], 2.633, 604 versus 1415.5 days, p = 0.0014) and N+neg (hazard ratio [HR], 1.680, 688 versus 1161 days, p = 0.03) LNs (Fig. 2d).

Figure 2

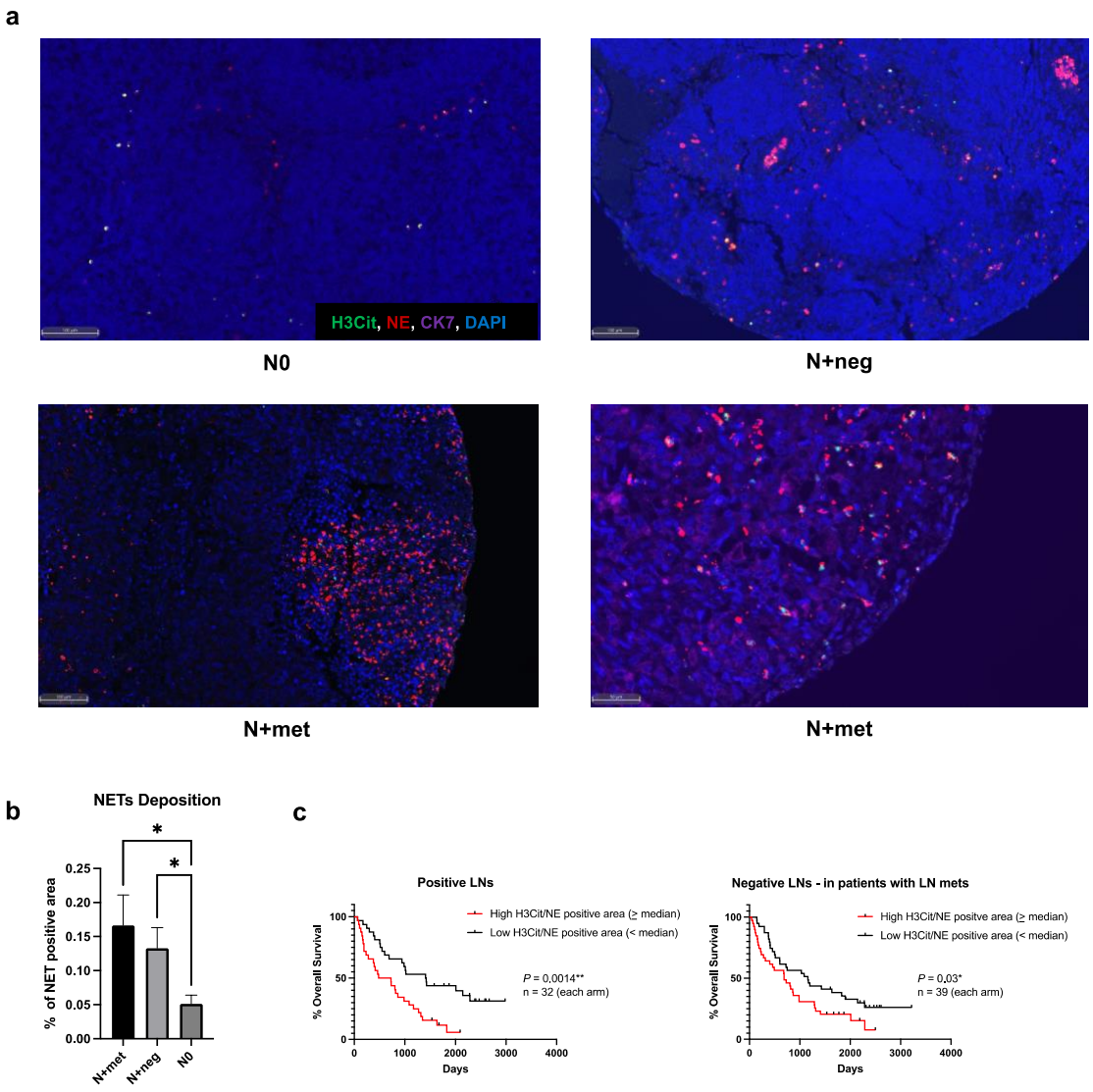

Figure 2: Elevated lymphatic NETs deposition is associated with poor survival. (a) Representative images of lymphatic involvement by NETs depicting a node negative patient (N0, top left), negative lymph node in a node positive patient (N+neg, top right), and a positive lymph node (N+met, bottom left). Scale bars represent 100µm. Zoomed details of NETs are shown in bottom right. Scale bars represent 50µm. (b)Quantification of lymphatic NETs (% of NETs

positive area) in positive nodes(N+met), negative nodes in node positive patients (N+neg), and node negative patients (N0). Data shown as mean ± SEM. n = 175; *, $P < 0.05$ by Brown-Forsythe ANOVA test. (c) Kaplan-Meier survival curves comparing survival of gastroesophageal patients with low versus high levels of median lymphatic NETs positive area, in both tumour positive and negative lymph nodes. P value by Log-rank (Mantel-Cox) test.

Considered collectively, this data suggests a correlation between lymphatic neutrophil accumulation, NETs deposition, and ultimately survival in patients with GEA. This points to a pro-tumorigenic role of neutrophils and NETs in the progression of regional lymphatic disease. In addition, our data suggests that potential changes favourable for lymphatic metastasis may occur prior to nodal neoplastic ingress.

In vivo metastasis reveals the time course of nodal neutrophil recruitment and NETs deposition

Based on our observations in GEA patients with respect to nodal NET deposition, we next established an animal model to investigate the kinetics of LN NET deposition. The aim of this was demonstrate that neutrophil accumulation and NETs deposition within LNs precedes overt metastasis formation.

In order to achieve this, the flank of C57bl/6 mice were injected with different epithelial tumour cells (H59 and B16 F10) and sacrificed at different time points in order to reflect both pre metastatic and post metastatic settings. Subsequently, the tumour draining ipsilateral inguinal LNs were resected and analysed. First, the LN neutrophil population was assessed by flow

cytometry (Fig. 3a). A significant increase in the percentage of viable neutrophils (Viability Dye eFlour780^{-}CD11b^{+}Ly6G^{+}) in premetastatic LNs in all tumour bearing mice was found. At this stage, LNs were devoid of neoplastic cells. At the post metastatic stage, a relative drop (H59) or stability (B16F10) of neutrophil numbers was observed. This indicated the possibility of neutrophil death, possibly through NETosis, the process of NETs formation in the post metastatic stage.

This was confirmed by immunofluorescence microscopy (Fig. 3b). Compared to non-tumour bearing LNs, we found significantly higher neutrophil infiltration (Ly6G area) and NETs deposition (H3Cit area) in both pre-metastatic and post-metastatic LNs (Fig. 3c). To confirm our results, the same experiment was repeated with non-GFP tagged cell lines. This was done in order to prove that the observed phenotype was not caused by the immunogenic effects of GFP (Fig. 3e). We also assessed the neutrophil percentage in peripheral blood on a time course and found that systemic neutrophilia only occurred after LN metastases had formed (Fig. 3d). This suggests that local neutrophil recruitment precedes general neutrophilia.

The observation that NET accumulation and NET deposition precede overt lymphatic metastasis suggests that cues from the primary tumour drive the formation of a favourable neutrophil/NET rich environment. In order to demonstrate this, we introduced a low-grade cell line (B16F1) which does not induce LN metastasis in our animal model. Along these lines, we did not observe increased LN neutrophil infiltration or NET deposition as seen in the high-grade counterpart B16F10 (Fig. 3f, g).

Figure 3

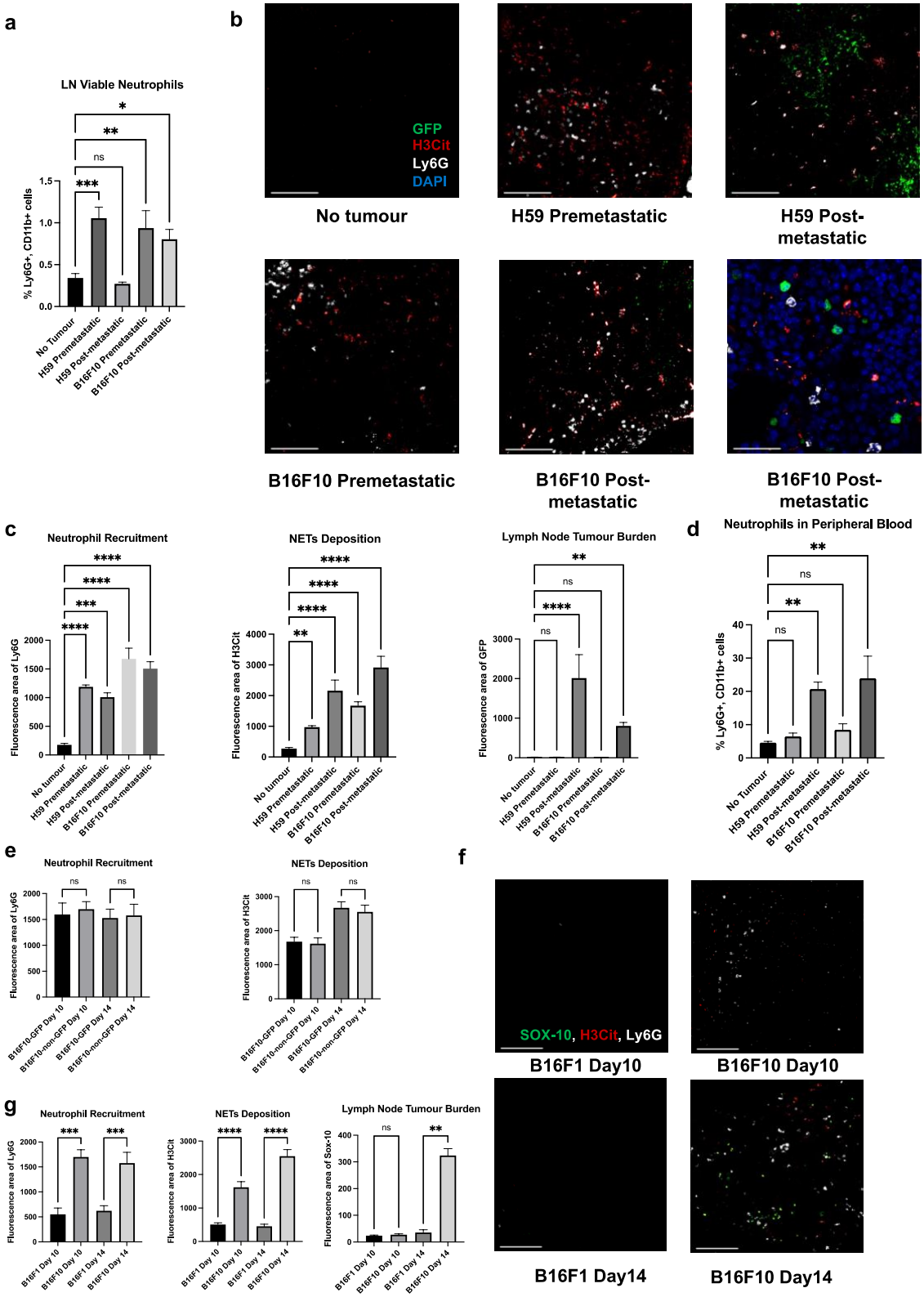
a
LN Viable Neutrophils
% Ly6G+, CD11b+ cells
No Tumour
H59 Premetastatic
H59 Post-metastatic
B16F10 Premetastatic
B16F10 Post-metastatic
b
GFP
H3Cit
Ly6G
DAPI
No tumour
H59 Premetastatic
H59 Post-metastatic
B16F10 Premetastatic
B16F10 Post-metastatic
B16F10 Post-metastatic
c
Neutrophil Recruitment
Fluorescence area of Ly6G
NETs Deposition
Fluorescence area of H3Cit
Lymph Node Tumour Burden
Fluorescence area of GFP
d
Neutrophils in Peripheral Blood
% Ly6G+, CD11b+ cells
e
Neutrophil Recruitment
Fluorescence area of Ly6G
B16F10-GFP Day 10
B16F10-non-GFP Day 10
B16F10-GFP Day 14
B16F10-non-GFP Day 14
NETs Deposition
Fluorescence area of H3Cit
f
SOX-10, H3Cit, Ly6G
B16F1 Day10
B16F10 Day10
B16F1 Day14
B16F10 Day14
g
Neutrophil Recruitment
Fluorescence area of Ly6G
NETs Deposition
Fluorescence area of H3Cit
Lymph Node Tumour Burden
Fluorescence area of Sox-10
B16F1 Day 10
B16F10 Day 10
B16F1 Day 14
B16F10 Day 14

Figure 3: The dynamic of nodal neutrophil recruitment and NETs deposition in mouse model. (a) Percentage of neutrophils within among viable LN cells on a time course by flow cytometry. (b) Representative images of tumour draining lymph nodes on a time course for both H59 lung cancer and B16F10 melanoma. Scale bars represent 100μm. Zoomed details of NETs are shown in bottom right. Scale bars represent 20μm. (c) Quantification of the area of lymphatic neutrophils (Ly6G), NETs (H3Cit) and metastasis (GFP) in no tumour, pre and post metastatic nodes for both H59 and B16F10. (d) Percentage of neutrophils within blood leukocytes on a time course by flow cytometry. (e) Comparison of the ability of lymphatic neutrophil recruitment and NETs deposition between GFP tagged and wild type B16F10 cells. (f) Representative images of tumour draining lymph nodes on a time course B16F1 and B16F10 melanoma cells. Scale bars represent 100μm. (g) Quantification of the area of lymphatic neutrophils (Ly6G), NETs (H3Cit) and metastasis (Sox-10) in no tumour, pre and post metastatic nodes for both B16F1 and B16F10. Data shown as mean ± SEM. n = 10; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; **** $P < 0.001$ by One-Way ANOVA.

Neutrophil depletion and NETs inhibition abrogates LN metastasis

Based on this experiment, we hypothesized that NETs play a central role in facilitating LN metastasis. Therefore, we sought to demonstrate this through a number of experiments demonstrating reduced LN metastasis in the absence of NETs.

First, we began with the approach of systemic neutrophil depletion. As published previously[226], the experimental group received intravenous injection of anti-Ly6G (1A8) (100 μg/mouse) and anti-Rat-kappa light chain (MAR18.5) (100 μg/mouse) while the control group

only received anti-Rat-kappa light chain injection during 14 days of H59 tumour incubation. When sacrificed on day 14, flow cytometry showed significant reduction in circulating neutrophils (Fig. 4b), suggesting significant depletion (8.359% vs. 2.755, p=0.023). When we assessed the LN, a significantly decrease in neutrophil infiltration, NET deposition, and metastatic burden was noted (Fig. 4c, d) (neutrophil 2276 vs 236, NETs 2109 vs 294, tumour 1155 vs 229.3, all $p<0.0001$). The observation that systemic neutrophil depletion could abrogate LN neutrophil recruitment corroborates the association between elevated circulating and lymphatic neutrophils observed earlier.

We similarly demonstrated that NET deposition is required for the effective establishment of LN metastases. This was achieved through the use of *pad4*$^{-/-}$ knockout mice which are unable to form NETs, or through administration of Sivelestat, which prevents NET formation. Peptidyl arginine deiminase 4 (PAD4) catalyses histone hypercitrullination during NET formation. This modification is required for histone decondensation, and its inhibition prevents NET formation[227]. By comparing the tumour draining LNs from wildtype (WT) and *pad4*$^{-/-}$ mice, we observed the same trend of diminished neutrophil recruitment, NETs formation and metastatic burden as was seen with neutrophil depletion (Fig. 4e, f) (B16F10, neutrophil 2123 vs. 854.6 $p<0.0001$, NETs 2195 vs. 831.6 $p<0.0001$, tumour 1061 vs. 302.8 $p= 0.0026$) (H59, neutrophil 1701 vs. 278.1, NETs 1849 vs. 208.9, tumour 1837 vs. 174.7, all $p<0.0001$).

Figure 4

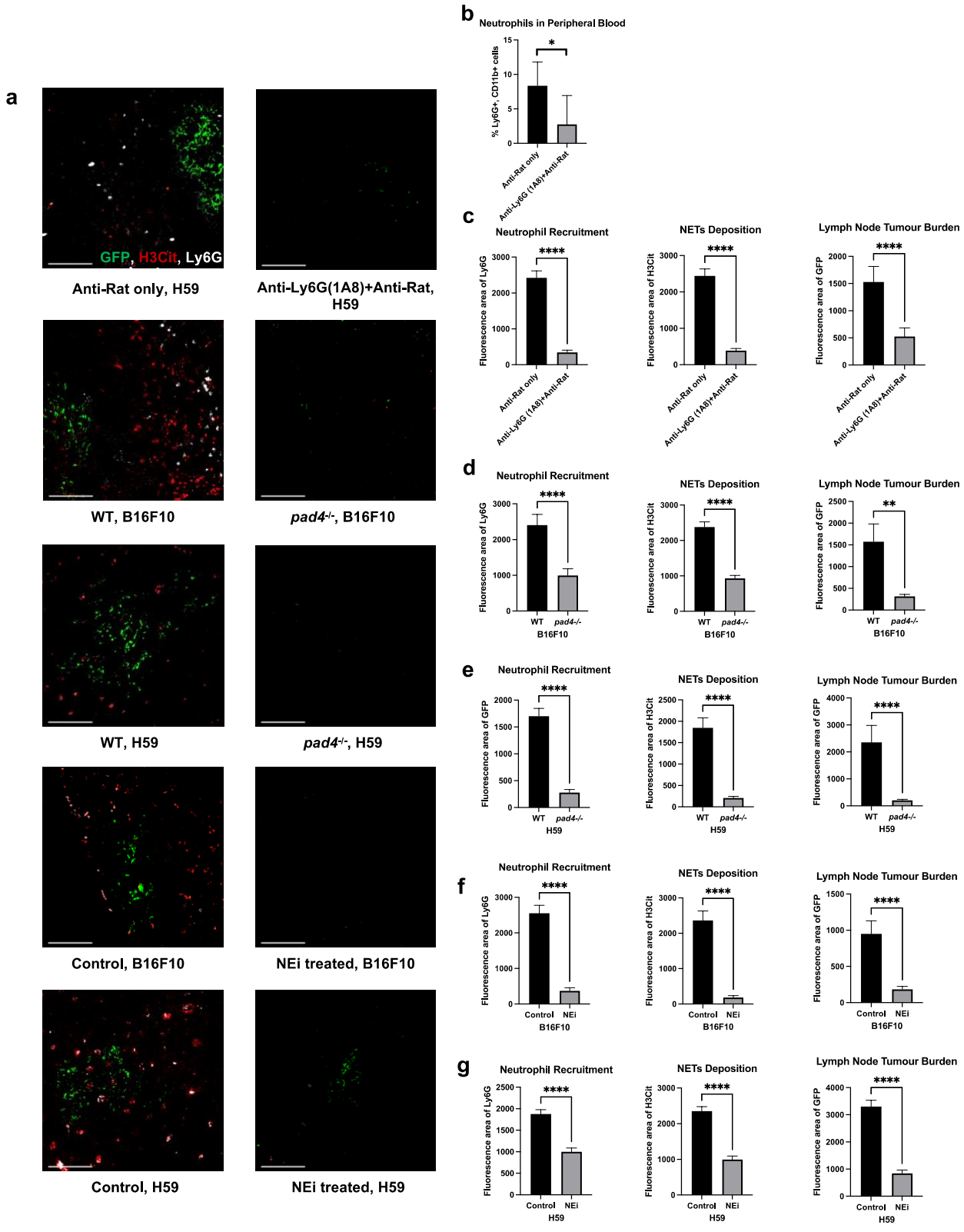
a
GFP, H3Cit, Ly6G
Anti-Rat only, H59
Anti-Ly6G(1A8)+Anti-Rat, H59
WT, B16F10
pad4-/-, B16F10
WT, H59
pad4-/-, H59
Control, B16F10
NEi treated, B16F10
Control, H59
NEi treated, H59
b
Neutrophils in Peripheral Blood
% Ly6G+, CD11b+ cells
Anti-Rat only
Anti-Ly6G (1A8)+Anti-Rat
c
Neutrophil Recruitment
NETs Deposition
Lymph Node Tumour Burden
Fluorescence area of Ly6G
Fluorescence area of H3Cit
Fluorescence area of GFP
****
d
Neutrophil Recruitment
NETs Deposition
Lymph Node Tumour Burden
WT
pad4-/-
B16F10
**
e
Neutrophil Recruitment
NETs Deposition
Lymph Node Tumour Burden
H59
f
Neutrophil Recruitment
NETs Deposition
Lymph Node Tumour Burden
Control
NEi
B16F10
g
Neutrophil Recruitment
NETs Deposition
Lymph Node Tumour Burden
H59

Figure 4: Neutrophil depletion and NETs inhibition abrogates LN metastasis. (a) Representative images of tumour draining lymph node on day 14 post tumour inoculation for both H59 and B16F10, comparing wildtype/no treatment to neutrophil depletion, pad4 knockout and NEi treatment. Scale bars represent 100μm. (b) Flow cytometry on blood leukocyte indicates sufficient depletion of neutrophils. (c)-(g) Quantification of the area of lymphatic neutrophils (Ly6G), NETs (H3Cit) and metastasis (GFP) in day 14 lymph nodes for both H59 and B16F10, comparing wildtype/no treatment to neutrophil depletion, pad4 knockout and NEi treatment. Data shown as mean ± SEM. n = 10; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; **** $P < 0.001$ by Mann-Whitney t test.

In order to highlight the susceptibility of NETs to pharmacologic blockade, mice were treated with daily gavage of the neutrophil elastase inhibitor Sivelestat (NEi). This is an orally bio-available compound currently under several clinical trials for autoimmune and respiratory diseases[228, 229]. It inhibits the function of neutrophil elastase, a key regulator and component of NETs[230]. NEi treatment also significantly decreased the LN NET deposition and metastatic disease burden (Fig. 4f, g). (B16F10, neutrophil 2444 vs. 243.6, NETs 2014 vs. 130.1, tumour 812.9 vs. 130.2, all $p<0.0001$) (H59, neutrophil 1701 vs. 278.1, NETs 1849 vs. 208.9, tumour 3305 vs. 841.7, all $p<0.0001$)

Collectively, these results suggest that recruitment of neutrophils and the subsequent deposition of NETs within LNs are necessary for the establishment of nodal metastasis. Disruption of NETs deposition alone through neutrophil depletion or pharmacologic blockade was sufficient to prevent the formation of clinically detectable LN disease.

EVs are essential for LN NETs formation and metastasis

In the previous experiment, we demonstrated that lymphatic NETs deposition occurs prior to, and was required for the ingress of neoplastic cells within LNs, it is possible that signalling pathways prompting neutrophil infiltration and NET deposition originated from the primary tumour. Tumour-derived factors including EVs have profound effects on distant organ sites and play an essential role in the formation of a pre-metastatic niche; a fertile microenvironment within target organs favouring neoplastic implantation[194]. EVs have been shown to preferentially accumulate within lymphatic endothelium, thus serving as a biologic link between the primary tumour and lymphatic microenvironment[17, 18]. To support this hypothesis, we queried The Cancer Genome Atlas Program (TCGA) data with respect to expression of EV machinery (Table 2)[231-233]. This showed that the expression of EV synthesis and secretion related genes in esophageal adenocarcinoma (EAD) tissue are significantly upregulated in patients with nodal disease (Fig. 5a) (Rab5a 16.61 vs. 18.96 p=0.0361, PRKD1 0.2598 vs.0.4705 p=0.0240, VAMP7 17.23 vs. 20.91 p= 0.0019). Moreover, high expression of VAMP7 is correlated with poor overall survival in EAD patients (hazard ratio [HR] 2.31, 495 versus 1599 days, p = 0.008) (Fig. 5b).

Table 2 Demographic and clinical characteristics of patients with oesophageal adenocarcinoma from TCGA

Patients with esophageal adenocarcinoma from TCGA (n =80)	
Mean Age, years	66.2
Sex, male	13.75%

Clinical Stage 1	2.50%
Clinical Stage 2	3.75%
Clinical Stage 3	7.50%
Clinical Stage 4	6.25%
Clinical Stage, NA	80.00%
Clinical T1	1.25%
Clinical T2	2.50%
Clinical T3	13.75%
Clinical T, NA	82.50%
Clinical N, yes	12.50%
Clinical M, yes	6.25%
Pathologic Stage 1	12.50%
Pathologic Stage 2	27.50%
Pathologic Stage 3	33.75%
Pathologic Stage 4	6.25%
Pathologic Stage, NA	20.00%
Pathologic Stage, I IIA	18.75%
Pathologic Stage, IIB IV	61.25%
Pathologic T0	1.25%
Pathologic T1	26.25%
Pathologic T2	13.25%
Pathologic T3 & T4	43.75%
Pathologic T, NA	15.00%
Pathologic N0	25.00%
Pathologic N1	46.25%
Pathologic N2	6.25%
Pathologic N3	6.25%
Pathologic N, NA	16.25%
Pathologic M, yes	6.25%
Average Overall Survival (days)	539
Status, dead	41.25%

Figure 5

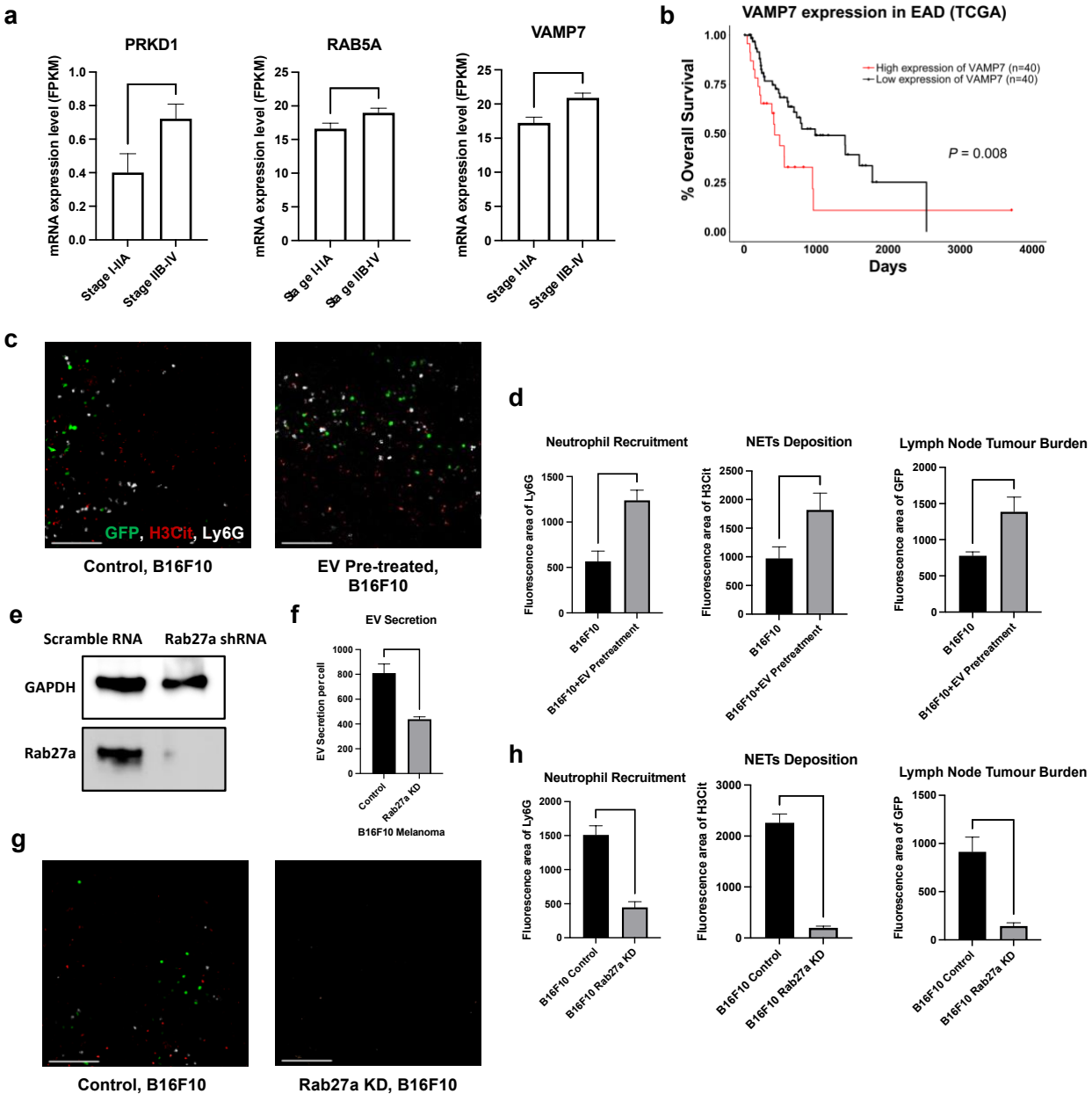

Figure 5: Extracellular vesicles are essential for LN NETs formation and metastasis. (a) Statistical analysis of TCGA mRNA expression data of EV synthesis related genes in primary esophageal adenocarcinoma tissue. n = 15 (Stage I-IIA), n = 65(Stage IIb-IV). (b) Kaplan-Meier

survival curves comparing survival of patients with esophageal adenocarcinoma with low versus high levels of median VAMP7 expression. (c) Representative images of tumour draining lymph node on day 14 post tumour inoculation for B16F10, with or without pre-treatment of EVs. Scale bars represent 100μm. (d) Quantification of the area of lymphatic neutrophils (Ly6G), NETs (H3Cit) and metastasis (GFP) in day 14 lymph nodes for B16F10, with or without pre-treatment of EVs. (e) Representative Western Blot images indicating the knockdown of Rab27a expression in B16F10 cell. (f) Nanoparticle Tracking Analysis (NTA) indicates Rab27a knockdown in B16F10 cell leads to decreased EV secretion. (g) Representative images of tumour draining lymph node on day 14 post tumour inoculation for B16F10, comparing cells infected with control lentivirus contain scramble RNA or Rab27a shRNA. Scale bars represent 100μm. (h) Quantification of the area of lymphatic neutrophils (Ly6G), NETs (H3Cit) and metastasis (GFP) in day 14 lymph nodes for B16F10, comparing cells infected with control lentivirus contain scramble RNA or Rab27a shRNA. Data shown as mean ± SEM. n = 10; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; **** $P < 0.001$ by Mann-Whitney t test.

To validate that EVs from the primary tumour are necessary for lymphatic neutrophil recruitment and NETs deposition, we pre-treated mice with cancer cell-derived EVs before tumour inoculation (10μg B16F10 EV per mouse, footpad injection every other day, 3 times) (Fig.S1). We found preexposure to cancer EVs significantly increase nodal neutrophil recruitment and NET deposition (Fig. 5c, d). (Neutrophil 568.3 vs. 1238 p=0.0006, NETs 974.1 vs. 1821 p =0.0261, tumour 779.0 vs. 1387 p=0.0132).

To further confirm the role of EVs, we knocked down (KD) the expression of Rab27a, a key gene involved in EV secretion[234]. The knockdown approximately halved EV secretion

levels (Fig. 5f) (787.0 vs. 450.0 p=0.0022). We thereby repeated the tumour inoculation with the Rab27a-KD cell line and observed abrogated LN neutrophil recruitment, NETs deposition and significantly decreased LN metastasis (Fig. 5g, h) (neutrophil 1508 vs. 447.1, NETs 2259 vs. 199.8, tumour 914.4 vs.144.0, all $p<0.0001$). Taken together, these data suggest EVs are essential for LN NETs formation and metastasis.

EV-Lymphatic endothelium interaction results in LN NETs deposition

EVs often exert their biological effects through their uptake into recipient cells and have been shown to preferentially accumulate within lymphatic endothelium[17]. We reconfirmed this finding as the majority of fluorescent EV localised in the cytosol of lymphatic endothelial cells (LECs) in mouse LNs (Fig. 6a). In vitro, LECs exhibit the same active uptake of EVs (Fig. 6b). Surprisingly, we found LECs dramatically enriched A549 lung cancer EVs compared to benign bronchial epithelial cell BEAS-2B EVs, as quantified by the relative area of CFSE-EV to DAPI (Fig. 6c) (0.7436 vs. 0.02310, $p<0.0001$). Thus, malignant EVs appear to specifically accumulate within lymphatic endothelium.

Figure 6

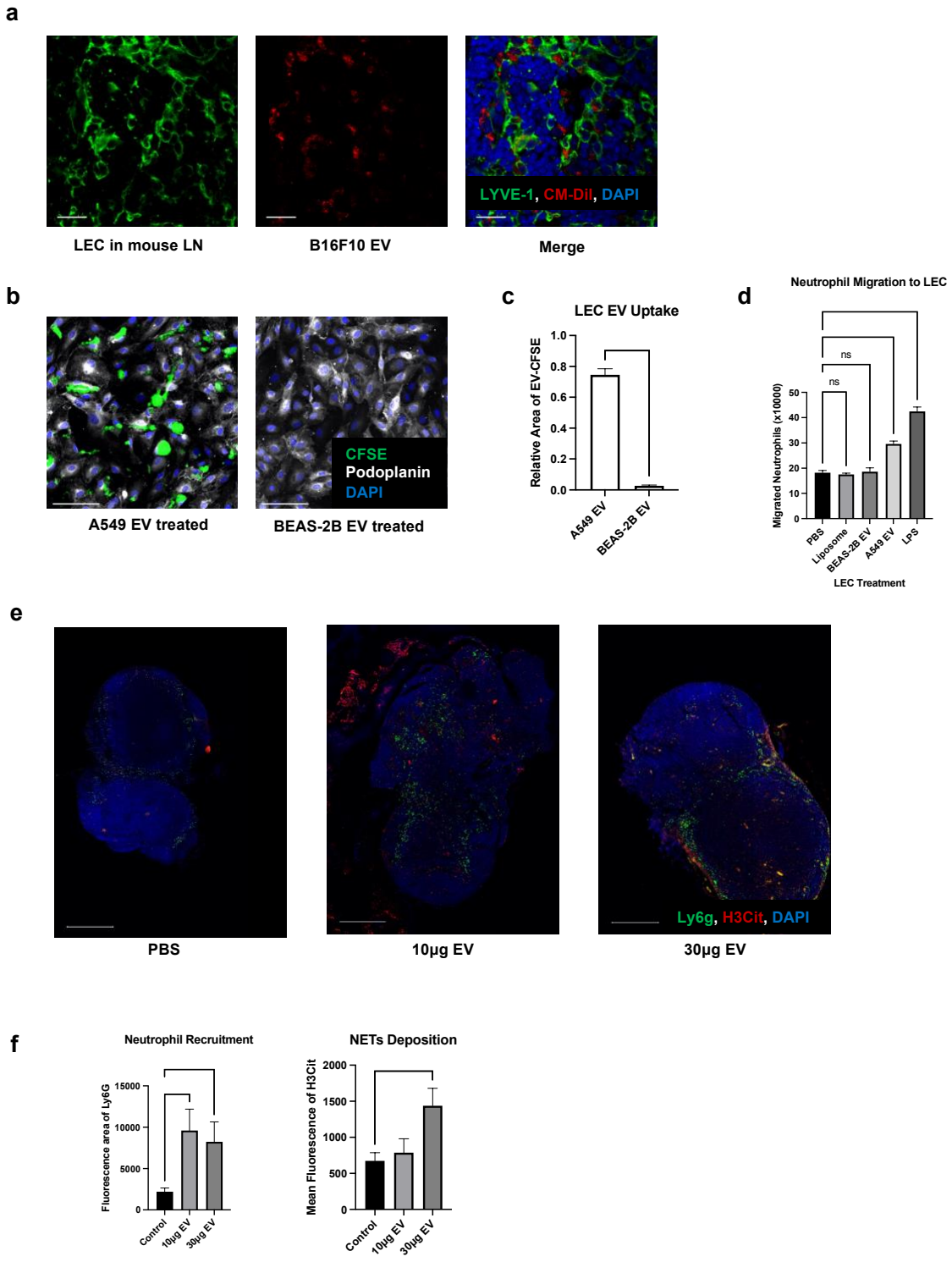
a
LYVE-1, CM-DiI, DAPI
LEC in mouse LN
B16F10 EV
Merge
b
CFSE
Podoplanin
DAPI
A549 EV treated
BEAS-2B EV treated
c
LEC EV Uptake
Relative Area of EV-CFSE
1.0
0.8
0.6
0.4
0.2
0.0
A549 EV
BEAS-2B EV
d
Neutrophil Migration to LEC
Migrated Neutrophils (x10000)
ns
ns
50
40
30
20
10
0
PBS
Liposome
BEAS-2B EV
A549 EV
LPS
LEC Treatment
e
Ly6g, H3Cit, DAPI
PBS
10μg EV
30μg EV
f
Neutrophil Recruitment
Fluorescence area of Ly6G
15000
10000
5000
0
Control
10μg EV
30μg EV
NETs Deposition
Mean Fluorescence of H3Cit
2000
1500
1000
500
0
Control
10μg EV
30μg EV

Figure 6: Lymphatic endothelial cells are the LN recipient cells of EVs and regulate neutrophil recruitment and NETs formation. (a) Representative images of draining lymph nodes after footpad B16F10 EV injection. Scale bars represent 20μm. (b) Representative images of LEC after A549 and BEAS2B EV treatment. Scale bars represent 100μm. (c) Quantification of the EV uptake (relative fluorescence area of CFSE to DAPI) in LEC. (d) Boyden chamber assay of neutrophil migration towards conditioned media from treated LEC. (e) Representative images of draining lymph nodes after footpad PBS or different dose of B16F10 EV injection, indicating the subsequent neutrophil recruitment and NETs deposition. Scale bars represent 500μm. (f) Quantification of the area of lymphatic neutrophils (Ly6G), NETs (H3Cit) in draining lymph nodes after footpad PBS or different dose of B16F10 EV injection. n = 5 Data shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; **** $P < 0.001$ by Mann-Whitney t test or One-Way ANOVA.

The consequences of LEC EV uptake were subsequently assessed. We performed Boyden chamber experiments to assess the migration of neutrophils towards the conditioned media (CM) from LEC pre-exposed to PBS (vehicle control), liposome (particle control), BEAS-2B EVs (benign EV control), A549 EVs and LPS (positive control). We found A549 cancer EVs treated LEC could significantly induce neutrophil transwell migration while benign EVs or liposomes - treated LECs could not (Fig. 6d) (11.36 vs. 24.93×10^6 neutrophils, $p=0.0489$). The finding of EV mediated neutrophil accumulation and NET deposition was subsequently confirmed *in vivo*. B16F10 EV were footpad injected into non-tumour bearing mice. We found that EV administration alone is sufficient to drastically increase LN neutrophil infiltration as well as the

amount of NETs (Fig 6e, f) (10μg, neutrophil 12.95 VS.28.14 P=0.0026, NETs 15.57 vs. 18.00 ns; 30μg, neutrophil 12.95 vs. 26.00 p=0.0050, NETs 15.57 vs. 27.78 p=0.0093).

Lymphatic secretion of CXCL8/2 induces LN Neutrophil infiltration and NETs formation

Having demonstrated that tumour EVs are sufficient to induce lymphatic neutrophil migration and NET deposition, we next sought to elucidate the mechanism by which this occurs. First, LECs were co-cultured with EVs from A549 lung cancer cells or BEAS-2B bronchial epithelial cells and conditioned media (CM) was generated. CM was then subjected to multiplex ELISA of common neutrophil chemokine. LEC production of CXCL8 was radically increased after A549 EV treatment (Fig. 7b) (1865 vs. 4447 pg, p=0.0004). A549 cells themselves also secreted more CXCL8 compared to BEAS-2B (Fig. 7a) (91652 vs. 16316pg, p <0.0001). We also performed immunofluorescence to detect LEC derived CXCL8. We observed a significant increase in CXCL8 synthesis in the LECs after A549 EV treatment (Fig. 7c), quantified by relative area of CXCL8 to DAPI (5.500 vs. 20.40 p= 0.0003). We also demonstrated the LEC expression of CXCL8 in surgical lymph node samples from lung adenocarcinoma patients.

As mice do not express CXCL8 but rather express its analogue CXCL2[235], we examined the latter's distribution pattern in mouse LNs. We found CXCL2 colocalised well with LEC and were in proximity to LN neutrophils (Fig. 7e). The level of CXCL2 was also extensively increased in pre-metastatic condition or after EV injection (Fig. 7f), quantified by relative area of CXCL2 to LYVE-1 (control vs. B16F10 premetastatic 0.07592 vs. 0.4462, p= 0.0058; control vs. EV 10μg 0.07592 vs. 0.8199, p <0.0001), as well as the number of

neutrophils infiltrated into LNs, quantified by area of Ly6G (Fig. 7g) (control vs. premetastatic B16F10 3.500 vs. 10.88, p= 0.0420; control vs. EV 10µg 3.500 vs. 17.00, p=0.0002).

Figure 7

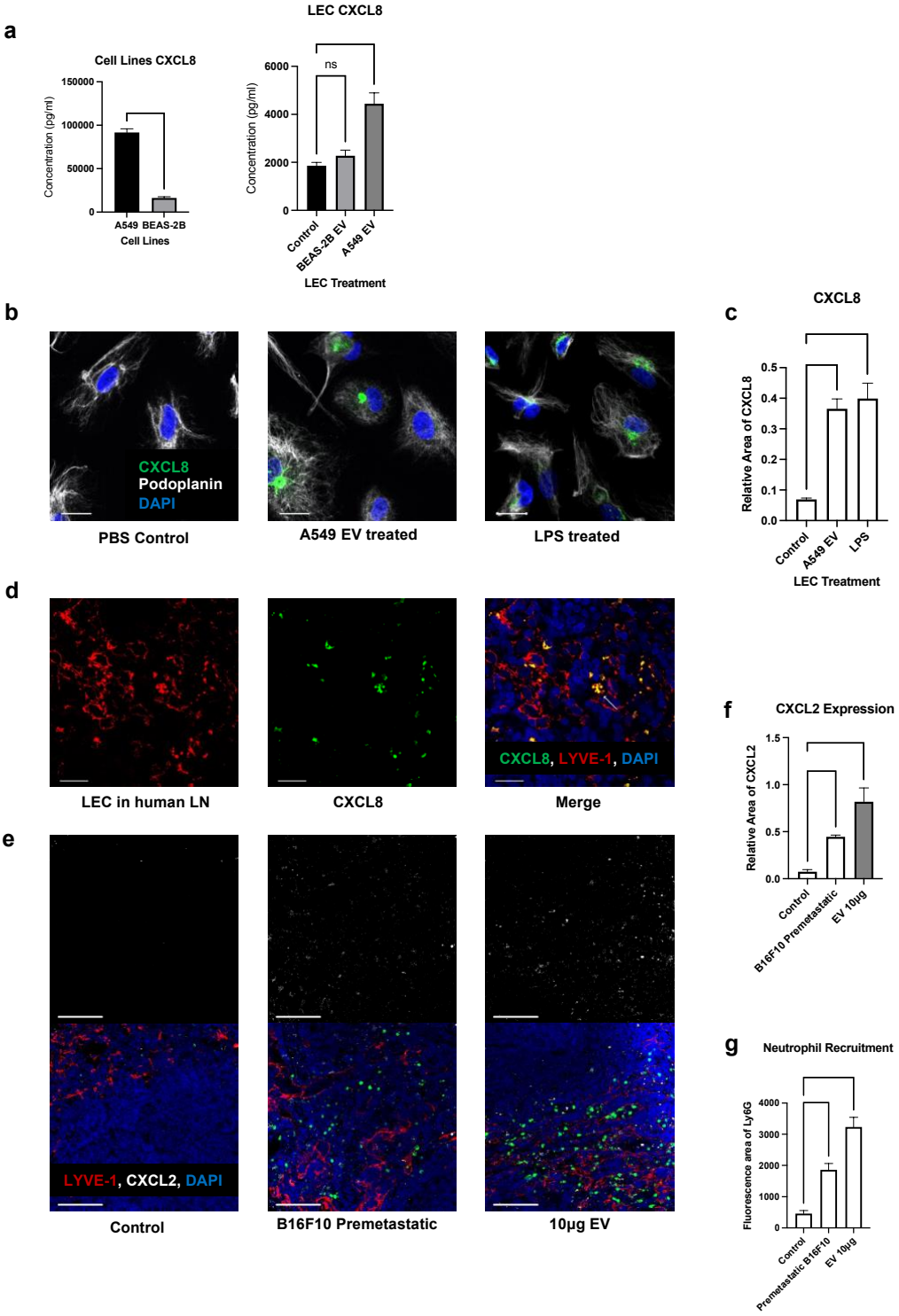

Figure 7: Lymphatic endothelial cells secrete neutrophil chemoattractant and NETs inducer upon EV uptake. (a) ELISA of CXCL8 in conditioned media of A549, BEAS-2B and treated LEC. (b) Representative images of LEC expressing CXCL8 after A549 EV and LPS treatment. Scale bars represent 20μm. (c) Quantification of CXCL8 expression in LEC (relative fluorescence area of CXCL8 to DAPI). (d) Representative images of LEC expressing CXCL8 in positive nodes of lung cancer patients. Scale bars represent 20μm. (e) Representative images of LEC expressing CXCL2 and recruiting neutrophils mouse LNs. Scale bars represent 100μm. (f) and (g) Quantification of CXCL2 expression (relative fluorescence area of CXCL2 to LYVE-1) in LEC and neutrophil recruitment (Ly6G) in mouse LN. n = 5. Data shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; **** $P < 0.001$ by Mann-Whitney t test, One-Way ANOVA or Kruskal-Wallis test.

In addition to a common chemoattractant of neutrophils, CXCL8 is also a potent NETs inducer[236]. To consolidate the conclusion that LEC secretion of CXCL8 can result in NETs formation, we exposed healthy control-derived neutrophils to A549 EV treated LEC CM, with or without CXCL8 blocking antibody. We found A549 EV treated LEC CM sufficiently increased the percentage of neutrophils that were undergoing NETs formation and CXCL8 blockade abrogated the phenotype (Fig. 8a, b) (control vs. A549 EV 7.415% vs. 79.63%, $p<0.0001$; control vs. A549 EV+CXCL8 Ab 7.415% vs. 24.64% ns; A549 EV vs. A549 EV+CXCL8 Ab 79.63% vs. 24.64%, $p<0.0001$). Furthermore, because it was reported that EVs can directly lead to the release of NETs[20], we simultaneously exposed GEA patient or healthy control derived neutrophils to A549 EVs and we found GEA patient derived neutrophils are more likely to form NETs (Fig 8 d,e), quantified by the relative fluorescence of SYTOX green to untreated

neutrophils (39.71 vs. 23.36 p= 0.0040). The increased propensity of NETosis in response to EVs in cancer patient indicates the profundity of neutrophil preexposure to other tumour derived molecules and the potential synergy of EVs and cytokines[221].

Figure 8

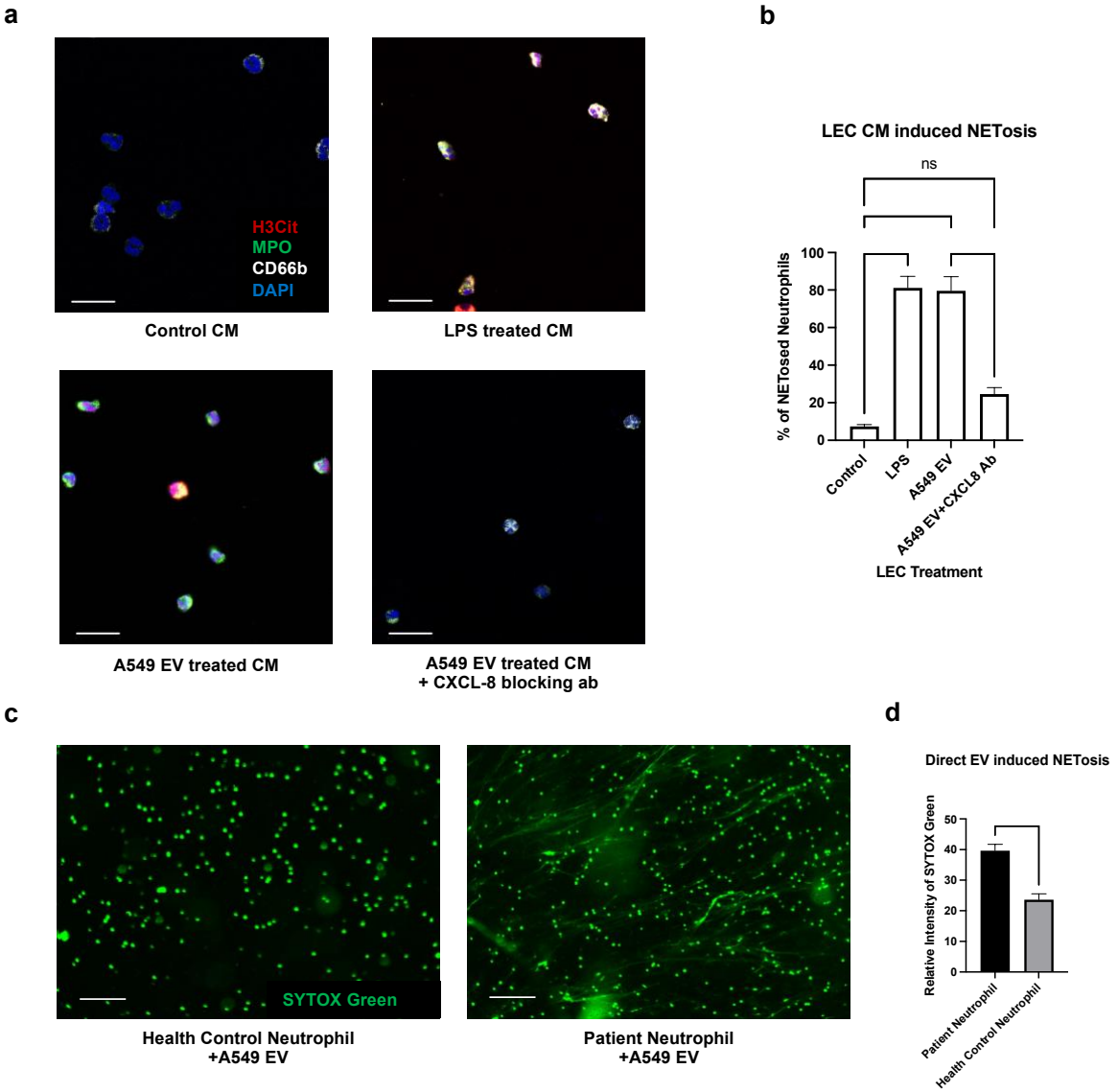

Figure 8: Lymphatic CXCL8 secretion upon EV uptake leads to NETs formation. (a) Representative images of neutrophil treated with conditioned media from treated LEC treated. Scale bars represent 20μm. (b) Quantification of LEC CM induced NETosis (% of H3Cit positive neutrophils). (c) Representative images of indication different proneness to NETose of neutrophil from health control and GEA patients. Scale bars represent 100μm. (d) Quantification of propensity of neutrophil to form NETs induced by EV (Relative fluorescence intensity of SYTOX Green treated to untreated neutrophil) from health control and GEA patients). Data shown as mean ± SEM.**, $P < 0.01$; ****, $P < 0.001$ by Kruskal-Wallis test or Mann-Whitney t test.

Clinical proteomics revealed distinct EV populations of patient plasma

Finally, we sought to access and analyse EV protein contents to further understand the mechanism of EV promoted LN metastasis. We isolated EVs from 10 GEA patient plasma (4 patients without LN metastasis as N0 and 6 with LN metastasis as N+, Table 3). These plasma samples were collected at the timepoint of diagnosis, thus reflecting the baseline status of treatment naïve patients. We compared the protein cargo in EVs by liquid chromatography–tandem mass spectrometry (LC-MS/MS). EV origin of sample preparations was verified by the identification of 71 out of 100 most-frequent proteins found in exosomes from the ExoCarta database[237] and in total we identified 1308 proteins (Fig. 9a). EV proteins are largely overlapped, yet not identical when comparing N0 to N+ samples (Fig.9b). We found 179 proteins significantly more abundant in N+ patients. In comparison, only 9 proteins are significantly upregulated in N0 patients, which are mostly immunoglobins (Fig. 9c). This

indicates that EV populations from patients with different LN metastasis status are distinct (Fig.9d).

Table 3 Demographic and clinical characteristics of patients with oesophageal adenocarcinoma for plasma EV proteomics study

Patients with esophageal adenocarcinoma from TCGA (n =10)	
Mean Age, years	67.5
Sex, male	90%
Clinical Stage 1	0%
Clinical Stage 2	0%
Clinical Stage 3	90%
Clinical Stage 4	10%
Clinical T1	10%
Clinical T2	0%
Clinical T3	90%
Clinical N, yes	40%
Clinical M, yes	20%

We used DAVID functional analysis to cluster the proteins upregulated in N+ patients and analyse the pathway in which these proteins are implicated. The results showed a significant enrichment in several pathways in nuclear protein translation, viral transcription, and viral process, indicting the possibility of acute/innate inflammatory reaction mimicking viral infection. Moreover, we specifically quantified total protein spectra count of those upregulated proteins in N+ sample. We found a higher EV expression of acute inflammation related proteins such as HSPD1(23.00 versus, $p = 0.0381$) and HSP90AB1(41.50 versus 77.50, $p = 0.0381$), and onco-proteins such as MAPK3 (6.500 versus 16.00, $p = 0.0190$), indicating the possibility of these EVs in inducing acute non-specific inflammation and tumour cell proliferation and progression.

Figure 9

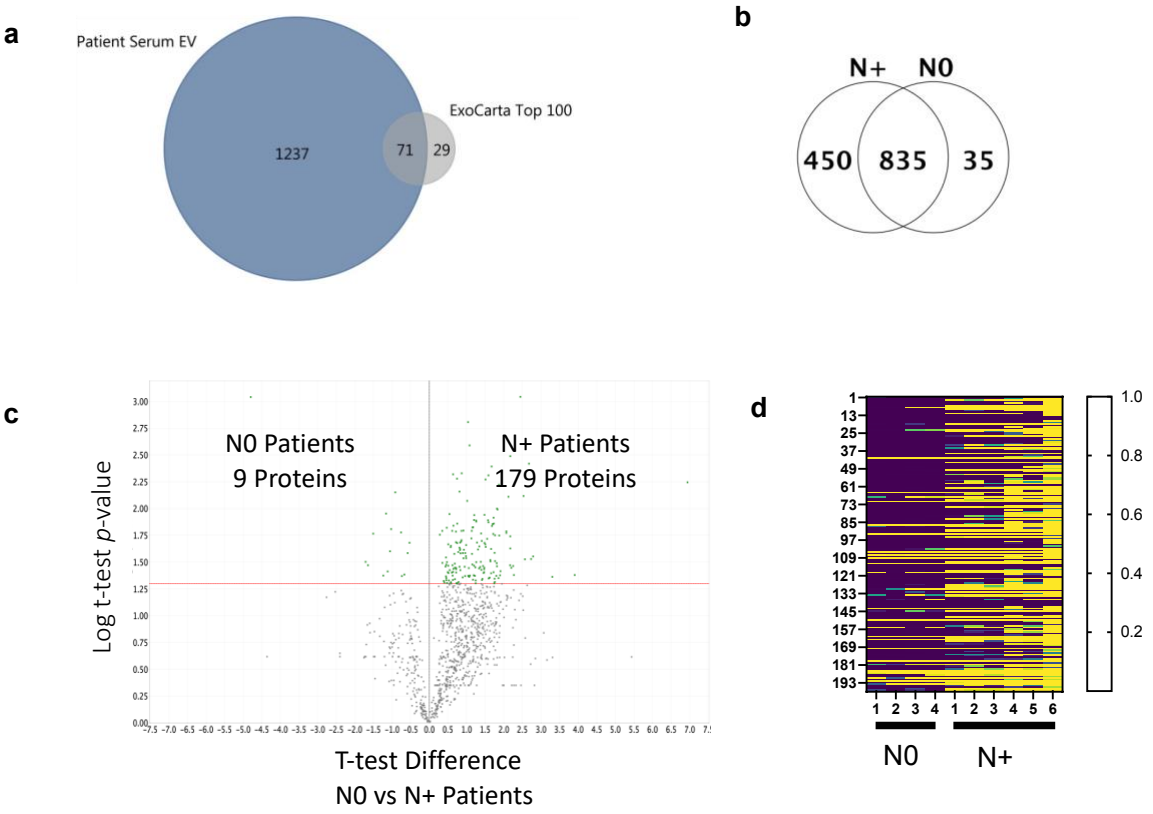
a
Patient Serum EV
ExoCarta Top 100
1237
71
29
b
N+
N0
450
835
35
c
N0 Patients
9 Proteins
N+ Patients
179 Proteins
Log t-test p-value
T-test Difference
N0 vs N+ Patients
d
N0
N+
e
nuclear-transcribed mRNA catabolic process
translation
SRP-dependent cotranslational protein targeting to membrane
viral transcription
translational initiation
rRNA processing
cell-cell adhesion
small GTPase mediated signal transduction
viral process
Others
f
HSPD1
HSP90AB1
MAPK3
Total Spectra
N0
N+

Figure 9: Proteomic and bioinformatic analysis of EV proteins from gastroesophageal cancer patient with or without lymph node metastasis. (a) Venn diagram showing the overlap between identified proteins from plasma EVs and ExoCart Top 100 EV markers. (b) Venn diagram showing the identified proteins from plasma EVs between GEA patients with or without LN metastasis. (c) Volcano plot of proteins differentially expressed between EVs from gastro-oesophageal cancer patients with or without LN metastasis. Significant threshold line $P < 0.05$. (d) Heat map of the proteins of different expression (T-test $P<0.05$) in GEA patients with or without LN metastasis. (e) DAVID analysis of the Reactome pathways associated to the proteins upregulated in the EVs from patient with LN metastasis versus patient without LN metastasis. (f) Representative quantification of total protein spectra counts for innate inflammation associate proteins like HSPD1 and HSP90AB1, or onco-protein such as MAPK3. N=4 for N0 patients and N=6 for N+ patients. *, $P < 0.05$ by Mann-Whitney t test.

Figure S1

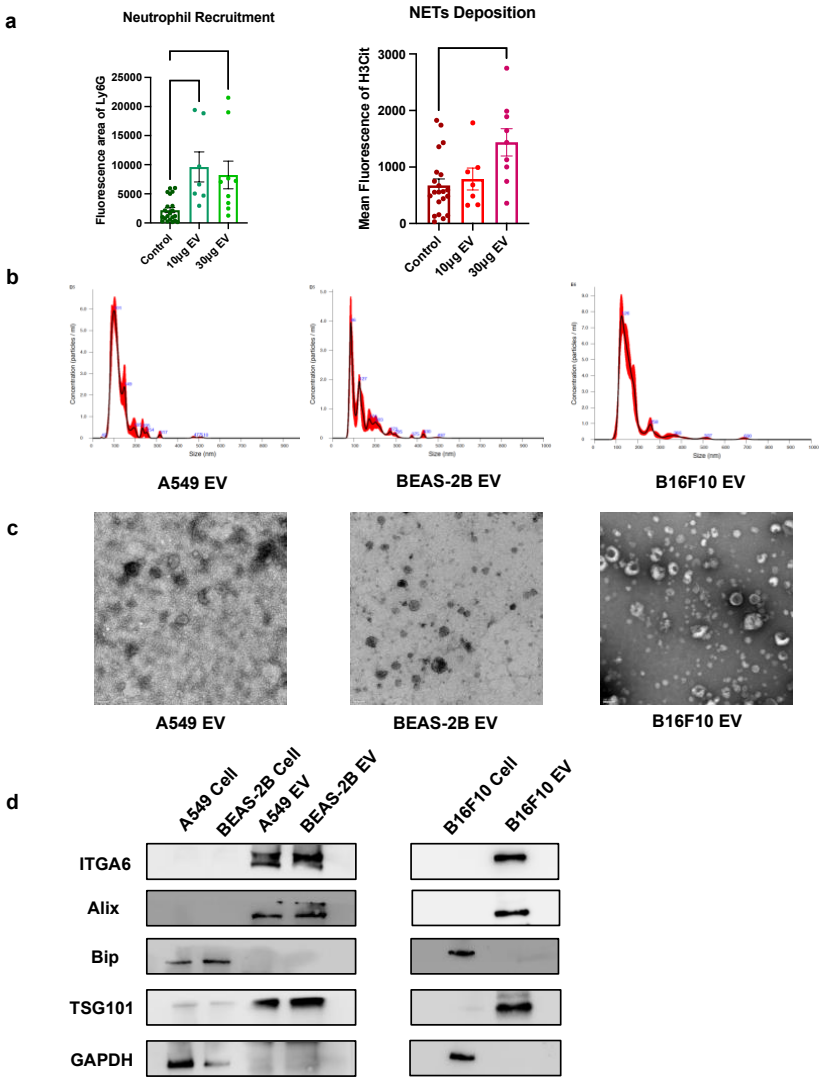

Figure S1: Validation of EV isolation. (A) Nanoparticle tracking assay (NTA) showing the size distribution of EVs. (B) Representative transmission electron microscopy (TEM) images of EVs. Scale bars represent 100nm. (C,D) Representative Western Blot images of EV and cell lysates.

Comprehensive scholarly discussion of all the findings

LN metastasis is nearly ubiquitous among epithelial cancers and portends poor survival[238]. Treating regional LNs via resection forms the basis for modern surgical oncologic treatment approaches. Regional LNs are removed and often trigger the implementation of systemic therapies when they harbour cancer[239]. The commonality of LN metastasis among cancers presents a broadly applicable opportunity for treatment.

In this current study we set out to demonstrate that similar to conditions of systemic inflammation, that a regional inflammatory state can promote the development of LN metastasis. We were able to demonstrate that lymphatic neutrophil accumulation and NETs deposition occurs within tumour LNs in GEA patients. Furthermore, we highlighted lymphatic NETs levels as a poor prognostic indicator in these individuals. Interestingly, while NETs levels were highest in LNs that had been infiltrated with cancer (N+met), even negative regional nodes (N+neg) demonstrated elevated NETs compared to LNs harvested from patients without nodal metastasis (N0). This finding suggests that regional lymphatic neutrophil accumulation and NETs deposition can occur without the presence of neoplastic cells. Because NETs levels were highest in N+met, we surmised that lymphatic neutrophils and NETs present a favourable environment for tumour implantation. Accordingly, neutrophils infiltrate LNs and deposit NETs prior to the arrival of neoplastic cells. This led us to hypothesize the presence of a regional inflammatory environment within tumour lymphatic beds. In further support of this, lymphatic neutrophil accumulation was most pronounced in patients with an elevated circulating NLR, a marker of inflammation. This would explain the a priori inflammatory lymphatic infiltrate. In order to prove this hypothesis, it was necessary to demonstrate several phenomena. First, it was necessary

to show that regional lymphatic neutrophil accumulation and NETs deposition occurs prior to the arrival of tumour cells. Second, we had to demonstrate that lymphatic neutrophils and NETs promote LN metastasis. Finally, we had to elucidate that the signalling cascade responsible for regional inflammation.

In this current study, we demonstrated that the distinct premetastatic phase within LNs is characterized by lymphatic neutrophil infiltration and subsequent deposition of NETs. Using an animal model of LN metastasis, we were able to demonstrate the kinetics of nodal inflammation with respect to tumour implantation. Two cell lines were employed to demonstrate common biology among neoplasms as previously described. Flank injection of B16F10 melanoma cell lines or H59 Lewis lung carcinoma cell lines resulted in LN metastasis at 14 days. However, in both models, a distinct premetastatic period defined by LN infiltration with Ly6G positive cells was observed. This was accompanied by the deposition of H3Cit at days 7-10. To validate the identity of presumed neutrophils and NETs, neutrophil depletion using Ly6G depletion antibody administration completely abrogated the presence of Ly6G positive cells or extracellular deposition of H3Cit. Further supporting the presence of lymphatic NETs is the finding that PAD4 knockout mice were unable to deposit these structures. Treatment of mice with NEi had the same result. As was seen in the human data, neutrophil infiltration and NETs deposition were highest infiltrated nodes and their presence was associated with an increase in the circulating NLR.

What was similarly clear from this data is that any intervention which diminished lymphatic NETs deposition was associated with reduced metastasis, strongly favouring the hypothesis that NETs play a supportive role for tumour cells. To further support this notion of LN inflammation, the lymphatic metastasis assay was repeated with two different cell types.

Mice were flank injected with either B16 F10 melanoma cells, or the non-metastatic melanoma cell line B16 F1. The inguinal LN was harvested at same two time points (day 10 and day 14). B16F1 cells failed to induce lymphatic neutrophil infiltration and NETs deposition at day 7 or 14. Along these lines no metastases were observed. This finding suggests that lymphatic NETs may be required for efficient neoplastic colonization of LNs.

Furthermore, the data presented thus far suggests that neoplastic cells at the primary tumour site are able to induce regional lymphatic inflammation. In the study by Wang et al., the authors highlighted tumour associated neutrophils (TAN) within early gastric cancer resection specimens as independent risk factors for LN metastasis[137]. Similarly, Hiramatsu et al. demonstrated a similar association between neutrophil numbers in tumour draining LN and patient outcome[8]. The data presented thus far suggests that neoplastic cells at the primary tumour site are able to induce regional lymphatic inflammation. This results in nodal infiltration of neutrophils followed by the deposition of NETs. The signals responsible for this phenotype were subsequently elucidated. In the data presented here, regional lymphatic inflammation by neutrophils appears to have increased as a result of inflammatory mediators in the form of EVs possibly originating from within the primary tumour. The observation of a distinct pre-metastatic phase has important implications when considering pathologic staging of neoplasia. LN staging in contemporary clinical practice is binary; nodes are either infiltrated with cancer or not. This study, along with previous work on the prognostic profundity of looking art immune compartment in pathological samples, suggest LN infiltration represents a spectrum of disease[240-242].

Along these lines, we have also demonstrated that EVs derived from the primary tumour play a critical role in establishing an pre-metastatic niche in the LN. Tumour derived EVs have

been shown to rapidly disseminate through the lymphatic system and into regional LNs, particularly during infection[18]. The observation that EVs can be rapidly taken up by LECs followed by transport to the luminal aspect of the vessel and rapid concentration within LN has led to the suggestion that lymphatic flow and uptake of EV from the periphery represents a rapid means of information exchange that can precede the arrival of migrating cells. We posit an analogous mechanism taking place between the primary tumour site and regional lymphatics. In further support of this, patients with cutaneous melanoma demonstrate lymphatic fluid enriched in melanoma EV[17]. Therefore, analysis of lymphatic composition may permit the differentiation of patients with early as opposed to advanced disease.

In keeping with previous studies, neutrophil depletion abrogated both the deposition of presumed NETs and abrogated the formation of nodal metastasis all together[123, 124, 243]. We and others have previously shown a pivotal role for neutrophils in the development of metastasis[81, 120]. In the current study, neutrophils demonstrated widespread NETs deposition once infiltrated into LNs. This led to the formation of an environment permissive to tumour outgrowth. Indeed, NETs have been shown to play a pro-tumorigenic role through direct effects on tumour cell proliferation, invasion, and escape from dormancy[124, 125, 243, 244]. Furthermore, NETs themselves have been shown to induce local immunosuppression through the extracellular elaboration of NET associated PD-L1[127]. Thus, it is feasible to surmise that NETs supported tumour outgrowth through a variety of mechanisms, including but not limited to sequestration within NETs, enhanced adhesion, migration, proliferation, and local immune suppression. The necessity of NETs was concretely demonstrated however, through the effect of both PAD4 inhibition and NE inhibition on tumour outgrowth.

In *vivo*, EV were sufficient to induce nodal neutrophil ingress and likewise appeared to be necessary for the formation of metastasis. A similar observation was made by peer researchers focusing on the macrophage recruitment[52], lymphangiogenesis[245] and immunosuppression[198] within LN microenvironment. Indeed, EVs serve as mediators of communication between primary tumour cells and LN cells, resulting in premetastatic changes that create a permissive condition for subsequent tumour cell colonisation. Our work highlights the essential role of EVs in mediating this NETs induced LN metastasis as EV knockdown in cancer cell lines abrogated the process of LN metastasis. Moreover, while the process of NETs formation is previously reported to be regulated by both cytokines, such as CXCL8 and G-CSF[221, 236], as well as EVs[20], we are the first to simultaneously study and link the regulation of the CXCL8 axis and EVs on NETs formation.

EVs induced the production of CXCL8 leading to neutrophil migration and NETs deposition both in vitro and in vivo. This was confirmed through CXCL8 inhibition which diminished much of this phenotype. CXCL8 has been long been studied as a pro-inflammatory cytokine that predominantly activates and recruits neutrophils[213]. Earlier work also characterised the role of CXCL8 as a NETs inducer and aggravator in chronic inflammatory diseases. Future works could focus on the synergy between tumour-derived soluble factors and EVs and investigate the plasticity of neutrophil function in the milieu of cancer from a cancer secretome mindset.

To further dissect the signalling molecules within those EVs secreted from primary tumour and to further understand the mechanism of EV regulated LEC secretion of CXCL8, we subject GEA patient serum to clinical proteomic and bioinformatic analysis. As the plasma sample was taken from treatment naïve patients, the results demonstrated their baseline status

and the key process driving the phenotype of LN metastasis. Initially our goal was to identify one or several molecules that are directly regulating the LEC secretion of CXCL8, or to identify several biomarkers. However, we were challenged with several obstacles. Firstly, the introduction of rabbit thromboplastin greatly decreased the background artefact from prothrombin. Unfortunately, this process introduced new contamination of several other rabbit plasma proteins as they are inevitable impurities during the manufacture of rabbit thromboplastin. In addition, as many rabbit proteins and human proteins are homologues, it is impossible for software to distinguish them, which impaired the confidence of focusing on one single protein.

Moreover, as these are all patient plasma, the only difference is the LN metastasis status, thus the majority of EV proteins are overlapped between two groups, thus, it is important to compare and study them as a cluster of proteins and one possible way of doing this is by locating these differently expressed proteins into biological pathways. Our analysis using functional annotation software demonstrated EV proteins that are upregulated in N+ group are associated in biological pathways mimicking viral inflammation, activation of innate immune system and non-specific inflammation, comparing to N0 groups, which contain more immunoglobins in EVs, indicating the possibility of anti-tumoral and adaptive immunity. Such polarity difference was unreported previously and further consolidated our findings that LN metastasis is promoted by regional inflammation induced by primary tumour derived EVs. Future direction of this work includes development of better methods of purification and isolation of plasma EVs as well as introducing health control samples to facilitate the identification of LN metastasis driving factors as well as potential biomarkers.

The detailed cascade of this novel regional inflammation model is demonstrated in this following graphic abstract (Fig.10).

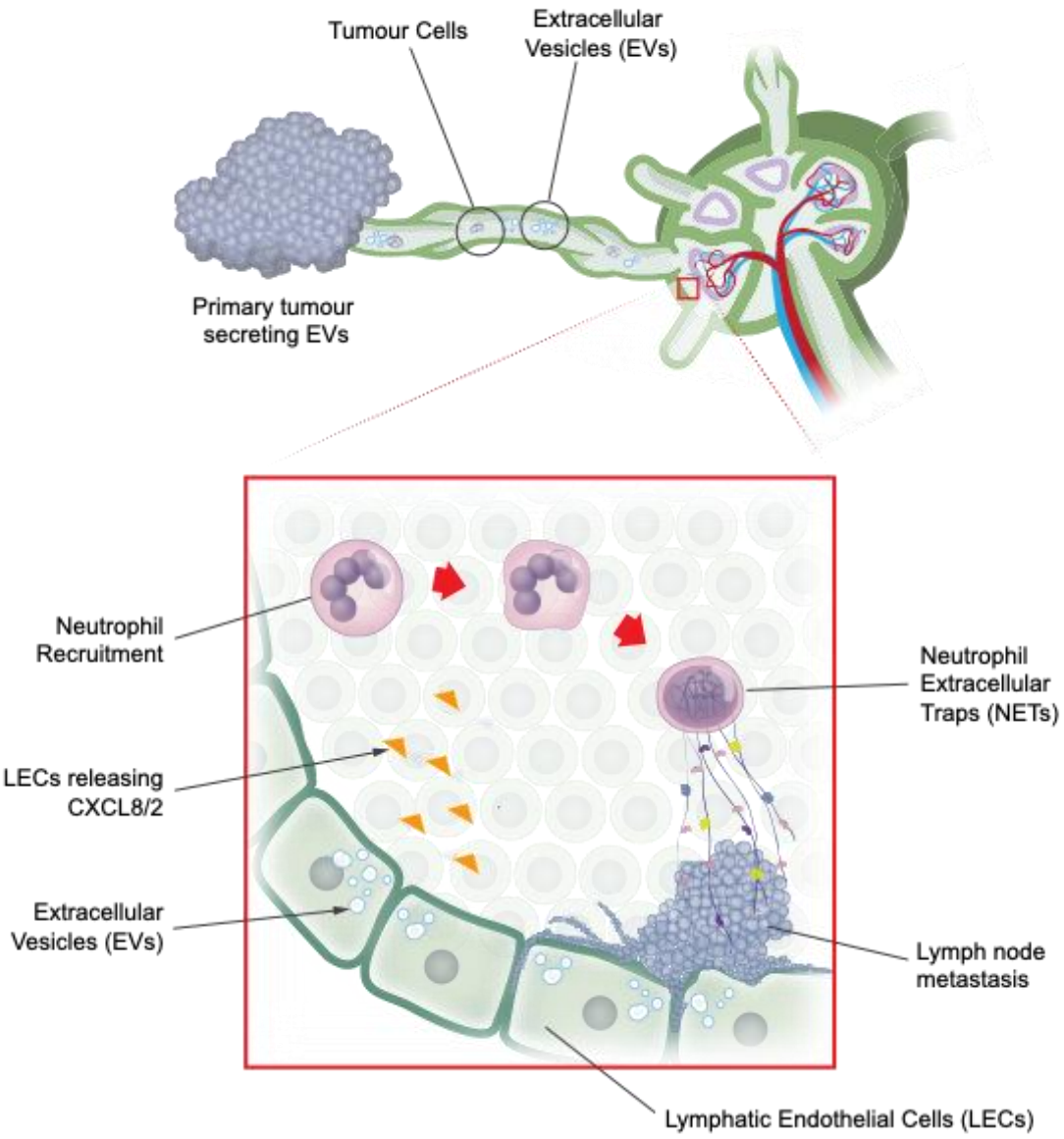

Figure D1: Illustrative demonstration of the lymph nodes preparation for tumour metastasis by extracellular vesicles and neutrophil extracellular traps. Primary tumour constantly secretes EVs which were uptaken by lymphatic endothelial cells. LEC subsequently secretes CXCL8 or CXCL2 upon EV reception, which are both neutrophil chemoattractant and potent NETs induced. The following neutrophil recruitment and NETs formation lead to increased lymph node metastasis burden.

Final conclusion and summary

In sum, the data presented sets a scene wherein tumour derived EVs accumulate within lymphatic endothelium. This results in a local chemotactic gradient promoting neutrophil influx and NET deposition mediated by LEC secreted CXCL8/2. Furthermore, locally infiltrated neoplastic cells may themselves attract additional neutrophil ingress and NETs release through the direct elaboration of CXCL8 and through the direct ability of EVs to induce NETosis. This results in a local microenvironment that is permissive and favourable to tumour outgrowth. This was the case in the murine models employed, as well as the in vitro LEC culture model. Moreover, LEC expression of CXCL8 was also confirmed in human surgical LNs.

In addition, we performed extensive blocking assays through inhibition of NETs formation (by PAD4 knockout and NEi administration), depletion of neutrophil, and inhibition of EV biogenesis (Rab27a knockdown in cancer cells), as well as CXCL8 blockade in in vitro model. These assay not only demonstrated that every element of this inflammatory cascade is essential, but also provide new therapeutic target for anti-cancer treatment. These are novel and promising discoveries because there are numerous clinical trials undergoing targeting neutrophils, NETs, CXCL8 or EVs. These alternate therapeutic vulnerabilities could be further developed into solution to clinical issues such as chemotherapy and radiation therapy resistance, cancer related inflammation (emergency myelopoiesis) and immunotherapy failure.

Notably, using patient derived TMA, we illustrated that there was a clear association between lymphatic NETs deposition and reduced survival in GEA patients. The finding that this may occur across malignant types may highlight NETs as an LN metastasis promoter as well as prognostic factor. The most valuable part is that this is based not on histology but host

inflammatory status instead, as our clinical proteomic data on GEA patient plasma EVs also suggested the immune polarisation towards innate immune system activation. Given that there is no treatment practice to date that target host inflammatory status, the results of this study are particularly germane. This work adds significantly to the growing body of evidence that highlights neutrophils and their effector mechanisms as potent facilitators of tumour progression. NETs represent an attractive therapeutic target as they are amenable to pharmacologic inhibition and appear to be involved throughout the metastatic cascade. Additional studies focusing on targeting this effector mechanism and further investigating the EV contents are needed.

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