The Role of Neutrophils in Cancer Progression

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THESIS ABSTRACT (English)

High circulating neutrophil to lymphocyte ratio (NLR) has been shown to be associated with poor oncologic outcomes. This raises the question as to whether neutrophils play a causative role in driving tumor progression. Accordingly, the first aim of this project was to determine if high NLR in cancer patients is associated with tumor-promoting neutrophils. In this aim, we also studied the involvement of NETosis in lymph node metastasis.

It has been shown that tumor-associated neutrophils (TANs) exhibit phenotypic diversity and plasticity. However, most of these studies were in animals using aggressive cancer cell lines. Therefore, in the second aim, we sought to identify how in cancer patients, neutrophil phenotypes change when cells migrate from bone marrow to blood and then to tumor, and how tumor influences neutrophil phenotypes.

Aim 1: Determine the association of NLR with neutrophil phenotypes in patients with gastro-esophageal adenocarcinoma

Methods: Tissue microarrays consisting of tumor core, tumor periphery, healthy stomach, and metastatic and non-metastatic lymph nodes were constructed using post-operative samples from 175 patients with gastro-esophageal adenocarcinoma. Immunohistochemistry (IHC) and immunofluorescence (IF) imaging were used to quantify the number of neutrophils/lymphocytes and the level of expression of arginase (Arg1) and neutrophil elastase (NE) in neutrophils. Low-density (LDNs) and high-density (HDNs) neutrophils were also isolated from blood samples of patients and immunophenotyped. LDN is known to be a tumor-promoting phenotype of neutrophils.

Results: Tumor NLR and percentage of circulating LDNs were higher in patients with elevated blood NLR ($\geq$ 4). LDNs exhibited higher expression of CD66b, Arg1, and CXCR2 than HDNs. High expression of these biomarkers has been reported in other studies to promote pro-tumor activities of neutrophils. High NLR in tumor core was associated with decreased survival, while

NLR in peripheral area had no such association. Patients with elevated blood and tumor NLR demonstrated higher expression of Arg1 and NE in TANs. It has been reported in the literature that Arg1 suppresses anti-tumor immunity, and NE promotes tumor cell proliferation. Patients with nodal metastasis showed a higher number of neutrophils and neutrophil extracellular trap (NET) formation in their lymph nodes compared to those without nodal metastasis. In our study, higher NET formation, in both involved and uninvolved lymph nodes, was associated with poor survival.

Conclusion: We showed that high blood and tumor NLRs were associated with neutrophils with tumor-promoting phenotypes in blood and tumor. We also demonstrated that nodal neutrophils and NETs may act as pre-metastatic promoter niche in lymph nodes of cancer patients.

Aim 2: Determine the influence of tumor microenvironment on neutrophil phenotype

Methods: To compare neutrophil phenotypes in different body compartments, post-operative samples were collected from bone marrow, peripheral blood, human esophageal tumor, and healthy esophagus from 21 patients with esophageal adenocarcinoma. Neutrophils were immunophenotyped by flow cytometry using a panel of 12 biomarkers. Peripheral blood neutrophils (PBNs) from healthy volunteers were also treated with different cancer cell-conditioned media (CM) using four human esophageal cancer cell lines.

Results: TANs acquired an activated phenotype evidenced by increased CD66b, CD11b, and CD54 and decreased CD62L and CD16 expressions. TANs had a lower expression of NE in internal flow cytometry staining, which could be due to the degranulation of TANs. This assumption was supported in our study by increased Arg1 and NE positive areas in tumor samples compared to healthy tissue (using IF imaging). Increased CXCR4 and decreased CD62L expressions in TANs showed that TANs were aged compared to neutrophils in healthy tissue. CXCR2 expression decreased in TANs, probably due to the ligation of CXCR2 with proinflammatory factors in tumor. *In vitro* treating PBNs with CM resulted in the activation of PBNs.

Conclusion: We showed that TANs acquired an activated phenotype compared to neutrophils isolated from other body compartments.

RÉSUMÉ DE THÈSE (French)

Il a été démontré dans la littérature qu'un rapport élevée de neutrophiles/lymphocytes (NLR) est associé à de mauvais résultats oncologiques. Cela soulève la question de savoir si les neutrophiles jouent un rôle causal dans la progression de la tumeur. En conséquence, le premier objectif de ce projet était de déterminer si un NLR élevé chez les patients cancéreux est associé à l'existence des neutrophiles favorisant la tumeur. Dans ce but, nous avons également étudié l'implication de la NETosis ganglionnaire dans les métastases.

Il a été aussi démontré que les neutrophiles associés aux tumeurs (TAN) présentent une diversité phénotypique et une plasticité. Cependant, la plupart de ces études portaient sur des animaux, utilisant des lignées cellulaires cancéreuses agressives. Par conséquent, dans le deuxième objectif, nous avons cherché à identifier comment, chez les patients cancéreux, les phénotypes des neutrophiles changent lorsque les cellules migrent de la moelle osseuse vers le sang puis vers la tumeur, et comment la tumeur influence les phénotypes des neutrophiles.

Objectif 1: Déterminer l'association du NLR avec les phénotypes neutrophiles chez les patients avec adénocarcinome gastro-œsophagien

Méthodes: Microarrays tissulaires constituées du noyau et de la périphérie de la tumeur, d'un estomac sain, et de ganglions lymphatiques métastatiques et non métastatiques ont été construites des échantillons postopératoires de 175 patients. L'imagerie par immunohistochimie (IHC) et immunofluorescence (IF) ont été utilisées pour quantifier le nombre de neutrophiles/lymphocytes et le niveau d'expression de l'arginase (Arg1) et celle de l'élastase des neutrophiles (NE) dans les neutrophiles. Des neutrophiles de faible densité (LDN) et de haute densité (HDN) ont également été isolés à partir d'échantillons sanguins de patients et ont été immunophénotypés. Le LDN est connu pour être un phénotype des neutrophiles favorisant la tumeur.

Résultats: Le NLR tumoral et le % de LDN circulants étaient plus élevés chez les patients avec un NLR sanguin élevé (≥ 4). Les LDNs présentaient une expression plus élevée de CD66b, Arg1 et CXCR2 que les HDNs. Il a été rapporté que l'expression élevée de ces biomarqueurs favorise les activités pro-tumorales des neutrophiles. Un NLR élevé dans le noyau de tumeur était associé à une diminution de la survie, tandis que le NLR dans la zone périphérique de tumeur n'avait pas une telle association. Les patients avec un NLR sanguin et tumoral élevé ont démontrés une expression plus élevée d'Arg1 et de NE dans les TANs. Il a été rapporté que Arg1 supprime l'immunité anti-tumorale, et NE favorise la prolifération des cellules tumorales. Les patients présentant des métastases ganglionnaires ont montré un nombre plus élevé de neutrophiles et de formation de pièges extracellulaires des neutrophiles (NETs) dans leurs ganglions lymphatiques par rapport à ceux sans métastase ganglionnaire. Une formation plus élevée de NET, à la fois dans les ganglions lymphatiques positives et négatives, était associée à une faible survie.

Conclusion: Nous avons montré que des NLR sanguins et tumoraux élevés étaient associés à des neutrophiles favorisant la tumeur. Nous avons également démontré que les neutrophiles ganglionnaires et les NETs ganglionnaires peuvent agir comme une niche de promoteur pré-métastatique dans les ganglions lymphatiques.

Objectif 2: Déterminer l'influence du microenvironnement tumoral sur le phénotype des neutrophiles

Méthodes: Pour comparer les phénotypes des neutrophiles dans différents compartiments corporels, des échantillons postopératoires ont été prélevés de la moelle osseuse, du sang périphérique, de la tumeur œsophagienne humaine et de l'œsophage sain de 21 patients. Les neutrophiles ont été immunophénotypés par cytométrie en flux à l'aide d'un panel de 12 biomarqueurs. Les neutrophiles du sang périphérique (PBN) de volontaires sains ont également été traités avec différents milieux conditionnés par des cellules cancéreuses (CM) en utilisant quatre lignées cellulaires de cancer de l'œsophage humain.

Résultats: Les TANs ont acquis un phénotype activé mis en évidence par une augmentation des CD66b, CD11b et CD54 et une diminution des expressions de CD62L et CD16. Les TANs avaient une expression plus faible de NE démontré par cytométrie de flux, ce qui pourrait être dû à la dégranulation des TANs. Cette hypothèse était étayée par une augmentation des zones positives pour Arg1 et NE dans la tumeur par rapport aux tissus sains (en imagerie IF). Une augmentation de CXCR4 et une diminution des expressions de CD62L ont démontré que les TANs étaient âgés par rapport aux neutrophiles dans les tissus sains. L'expression de CXCR2 a diminué dans les TANs, probablement en raison de couplage de CXCR2 avec des facteurs pro-inflammatoires dans la tumeur. Le traitement *in vitro* des PBNs avec le CM a entraîné l'activation des PBNs.

Conclusion: les TANs ont acquis un phénotype activé et pro-tumoral par rapport aux neutrophiles isolés d'autres compartiments corporels.

List of Abbreviations

Antibody-dependent cell-mediated cytotoxicity (ADCC)
Antigen-presenting cells (APCs)
Arginase 1 (Arg1)
Bone marrow (BM)
Bone marrow neutrophils (BMNs)
Bone morphogenic protein (BMP)
Cancer stem cells (CSCs)
Chronic obstructive pulmonary disease (COPD)
Conditioned medium (CM)
Cystic fibrosis (CF)
Deep vein thrombosis (DVT)
Dendritic cells (DCs)
Disease-free survival (DSF)
Extracellular matrix (ECM)
Fetal bovine serum (FBS)
Fluorescence minus one (FMO)
G protein-coupled receptors (GPCR)
Gastro-esophageal cancer (GEC)
Granulocyte colony-stimulating factor (G-CSF)
Granulocyte–monocyte progenitors (GMPs)
Granulocytic myeloid derived suppressor cells (G-MDSCs)
Hematopoietic stem cells (HSC)
Hepatocellular carcinoma (HCC)
High mobility group box 1 (HMGB1)
High-density neutrophils (HDNs)
Histocompatibility complex (MHC)
Histone H3 Citrulline (H3Cit)
Immunofluorescence (IF)
Immunohistochemistry (IHC)
Intercellular adhesion molecule 1 (ICAM-1)
Interleukin-6 (IL-6)
Interleukin-8 (IL-8)
Low-density neutrophils (LDNs)
Lymphocyte separation media (LSM)
Lymphovascular invasion (LVI)
Macrophage-1 antigen (Mac-1)
Major histocompatibility complex (MHC)
Matrix metalloproteinase-9 (MMP-9)
Median fluorescence intensity (MFI)
Monocytic myeloid derived suppressor cells (M-MDSCs)
Myeloid derived suppressor cells (MDSCs)
Myeloperoxidase (MPO)
Natural Killer Cells (NK Cells)
Neutrophil elastase (NE)
Neutrophil extracellular traps (NETs)
Neutrophil-to-lymphocyte ratio (NLR)
Non-small-cell lung carcinoma (NSCLC)
Nuclear factor kappa B (NF-κB)
Overall survival (OS)
P-selectin glycoprotein ligand-1 (PSGL-1)
Pancreatic ductal adenocarcinoma (PDAC)

Peptidyl arginine deiminase 4 (PAD4)

Peripheral blood neutrophils (PBNs)

Phosphate-buffered saline (PBS)

Phosphatidylinositol 3-kinase (PI3 K)

Programmed death-ligand 1 (PD-L1)

Reactive oxygen species (ROS)

Red blood cells (RBCs)

Regularity T-Cells (Tregs)

Squamous cell carcinoma (SCC)

T helper type 2 (Th2)

T-distributed Stochastic Neighbor Embedding (tSNE)

Tissue inhibitor of metalloproteinases (TIMP)

Tissue microarrays (TMAs)

Toll-like receptors (TLRs)

Transforming growth factor beta 2 (TGF-β2)

Tumor-associated neutrophils (TANs)

Uniform Manifold Approximation and Projection (UMAP)

Vascular endothelial growth factor (VEGF)

White adipose tissue (WAT)

Contribution of Authors

I wrote the entire thesis with editorial input from my supervisors, Dr Ferri and Dr Jonathan Cools-Lartigue.

Chapter 2: I am the first author in this manuscript (chapter). I carried out all experiments and analyses, except immunohistochemistry/immunofluorescence staining, which was performed by the Histopathology Core at the Centre for the Translational Biology (CTB), RI-MUHC (Glen Site). We studied eight tissue microarrays (TMAs) containing post-operative tissues, and these TMAs were constructed and validated by MUHC pathologists: Drs Sophie Camilleri-Broët, Victoria Marcus, Marianne Samir Makboul, and Duc-Vinh Thai. The clinical and pathologic data of patients were collected by Ms Emma Lee, Research Administrator at the Thoracic Surgery, MUHC. Ms Olivia Koufos, our Laboratory Research Assistant, helped me with organizing the patient data. This chapter was conceptualized by my supervisor, Dr Ferri, and the idea was further developed by me. Drs Jonathan Cools-Lartigue, Jonathan Spicer, Veena Sangwan, and Roni Rayes contributed invaluable comments and scientific inputs that helped me to plan the experiments and shape this chapter.

Chapter 3: I am the first author in this manuscript (chapter). I carried out all experiments and analyses. Ms Olivia Koufos, our Laboratory Research Assistant, helped me with post-operative sample preparation for flow cytometry analysis. This chapter was conceptualized by my supervisor, Dr Ferri, and the idea was further developed by me. Drs Jonathan Cools-Lartigue, Jonathan Spicer, Veena Sangwan, and Roni Rayes contributed invaluable comments and scientific inputs that helped me to plan the experiments and shape this chapter.

INTRODUCTION

There is an increasing amount of evidence showing that inflammation is a predisposition of cancer progression, implicating the crucial role of immune cells, including neutrophils, in cancer development, prognosis, and therapy [1, 2]. Peripheral blood Neutrophil-to-lymphocyte ratio (NLR) has been proposed to be an independent and reliable marker for cancer-associated inflammation [3, 4]. A prevalence of neutrophils over lymphocytes could be a sign of decreased immune surveillance, and high peripheral blood NLR in cancer patients is shown to be associated with poorer prognosis and survival, emphasizing the important function of neutrophils in cancer progression [5]. Although a significant number of inflammatory cells that infiltrate into tumors are indeed neutrophils, until recently tumor-associated neutrophil (TAN) was considered to be less significant in cancer progression compared to the other tumor-infiltrated immune cells, such as macrophages and lymphocytes [6]. This presumed lack of significance of neutrophils in cancer progression was based on the assumption that neutrophils are short-lived in tumors and possess a low level of mRNA in order to be able to express significant volumes of bioactive molecules [6]. However, in the last two decades, TAN has gained enormous attention and is now considered to be an important component of the tumor microenvironment in cancer development [6-14]. Increasing numbers of studies demonstrated the crosstalk between tumor cells and TANs, and the response of TANs to different cues from the tumor microenvironment to produce and release a high volume of various cytokines, chemokines, and bioactive molecules stored in their granules [6-14]. This suggests TANs can affect the tumor microenvironment by acting on other immune cells and tumor cells, highlighting the critical role of TANs in orchestrating the immune response to cancer. TANs are heterogenous and exhibit a wide functional diversity and plasticity, acquiring a spectrum of phenotypes ranging from anti-tumor to pro-tumor activities [15-19]. Having a better understating of different neutrophil subsets and phenotypes in cancer patients is therefore crucial in developing novel treatment strategies to fight cancer.

In this project, we aim to shed some light on neutrophil diverse phenotypes and plasticity in patients with gastro-esophageal cancer. Based on neutrophil protein expression, we investigated different phenotypes in circulating and tumor-infiltered neutrophils, and we established the relationship of these phenotypes with NLR and patient prognosis and survival. We also compared neutrophil phenotypic characteristics in bone marrow, peripheral blood, and tumor environment, and investigated the influences of tumor cells on neutrophils' protein expression. The outcome of this project could improve our understanding of the mechanisms underlying neutrophil plasticity and factors that promote or inhibit certain neutrophil phenotypes in cancer patients. The knowledge gained from this project could contribute to developing novel neutrophil-based therapeutic approaches, where neutrophils subsets can be employed as prognostic and/or diagnostic tools. The project could also contribute to developing novel treatments in cancer patients through altering neutrophil phenotypes to eliminate or promote specific neutrophil subsets.

The project studied neutrophils in the context of gastro-esophageal cancer that is the fastest rising malignancy in the western world. It has a poor prognosis with an overall 5-year survival of only 20% and a high rate of recurrence, which is a limiting factor to survival in this cancer. This highlights the need to develop novel prognostic and therapeutic approaches to inhibit gastro-esophageal cancer progression and systemic recurrence. In this project, we studied neutrophils in human tissue/blood samples obtained from cancer patients and healthy volunteers. Neutrophils cannot be frozen and stored, and because of this limitation, to date data, on the role of neutrophils in cancer progression have mostly been obtained from animal neutrophils and animal models, mostly mice, using aggressive cancer cell lines. There are differences in neutrophil function and protein expression between human and mouse, and therefore functional aspects and phenotypic diversity of TANs in human are mostly still unclear and poorly understood.

The project is divided into two aims (chapters), and the rationale, hypothesis, and objectives for each aim are described below.

Aim 1: Determine the association of neutrophil-to-lymphocyte ratio (NLR) with neutrophil phenotype in gastro-esophageal cancer patients

Rationale: As mentioned, high peripheral blood NLR, and in a few studies, high tumor NLR have been shown to be indicators of poor oncologic outcomes—conversely patients whose NLR normalized with therapy demonstrated improved prognosis. However, it is not yet clear whether the association between NLR and poor patient outcome is simply due to a higher prevalence of neutrophils over lymphocytes. Or, it is more complex in that, and high NLR may, indeed, indicate the existence of the pro-tumor types of neutrophils in cancer patients.

Hypothesis: *High blood and tumor NLRs are associated with the tumor-promoting phenotype of neutrophils in cancer patients.*

The objectives of this aim are to determine:

- The correlation between blood NLR and circulating low-density neutrophils (LDNs) fraction, a pro-tumor phenotype of neutrophils, and immunophenotyping of LDNs in cancer patients
- The relationship between blood NLR and tumor NLR
- The association of NLR within tumor, non-diseased tissue, and lymph nodes with patient survival
- The association of tumor and blood NLRs with the expression of arginase (Arg1) and neutrophil elastase (NE) in tumor-associated neutrophils (TANs)
- The association of neutrophil extracellular traps (NETs) in tumor and lymph nodes with survival and lymphatic metastasis

Aim 2: Determine the influence of tumor microenvironment on neutrophil phenotype

Rationale: Based on *in vivo* and *in vitro* studies, neutrophils displayed diversity and plasticity in phenotypic characteristics from tumor-promoting to tumor-inhabiting phenotypes. Under Aim 1,

we established the association between NLR and neutrophil phenotype, as well as the correlation of neutrophil phenotype (based on protein expression) and outcome/survival in gastro-esophageal cancer patients. In Aim 2, we went a step further and analyzed the influence of the human tumor microenvironment on neutrophil phenotype and investigated changes in neutrophil phenotype in cancer patients from when neutrophils are located in the bone marrow, to the circulating blood, to the healthy esophagus and esophageal tumor. It is not yet well understood how the tumor microenvironment in human influences neutrophil phenotype, and most studies in this area have been carried out in animal models using aggressive cancer cell lines.

Hypothesis: *How tumor microenvironment in humans influence neutrophil phenotypic characteristics*

The objectives of this aim are to determine:

- The changes in protein expression profile in neutrophils in four body compartments: bone marrow, peripheral blood, esophageal tumor, and non-diseased esophagus
- The influence of tumor cells on neutrophil protein expression profile using an *in vitro* cancer cell-conditioned medium assay

Chapter 1 Background

1.1 Gastro-esophageal cancer

Gastro-esophageal cancer (GEC), which includes esophageal squamous cell carcinoma, proximal esophagogastric junction adenocarcinomas (esophageal and gastric cardia adenocarcinomas), and distal gastric adenocarcinoma, is an important worldwide public health concern [20]. GEC is the fastest rising malignancy in the western world and has a poor prognosis with an overall 5-year survival of about 20% [21, 22]. While tremendous efforts have been made in locoregional and systemic treatment strategies, cancer recurrence remains the limiting factor to the survival of patients with GEC [22, 23]. This bleak statistic highlights the need to develop novel therapeutic approaches to address poor oncologic outcomes and systemic recurrence in this cancer. Employing our immune cells in various forms of immunotherapies to fight cancer and understanding the mechanisms underlying cancer treatment and prevention using immune cells have gained tremendous attention in the last two decades [24]. Neutrophils are the most abundant circulating leukocyte in our immune system. An increasing amount of evidence highlights the significance and involvement of neutrophils, as part of our immune system, in cancer progression, invasion, and metastasis [6, 10-12]. Therefore, it is compelling and logical to understand better the role of neutrophils in cancer progressions and the mechanisms underlying neutrophils/tumor microenvironment interactions. We can design novel cancer therapy by targeting neutrophils and using neutrophils to predict oncologic outcomes in cancer patients.

1.2 Role of Neutrophil in innate and adaptive immunity

Neutrophils are the predominant granulocyte and the most abandon leukocyte, accounting for 50 to 70% of circulating blood leukocytes [25]. They are the first line of innate immune system defense to fight invading pathogens and migrate from blood to inflamed sites caused by microorganism invasion, injuries, or diseases such as cancer [25, 26]. Neutrophils are powerful effector cells that destroy microorganisms through various mechanisms: i) phagocytosis and intercellular degradation, including production of cytoplasmic granules containing antimicrobial

proteins such as neutrophil elastase (NE) and arginase and release of these granules (exocytosis) upon the activation of neutrophil; ii) generation and release of reactive oxygen species (oxidative burst) such as H_2O_2; and iii) release of neutrophil extracellular traps (NETs) [27]. NETs are made up of extracellular DNA fibers decorated with antimicrobial proteins that can capture and destroy invader pathogens, preventing their spreading from the initial infection site [28].

Neutrophils can also interfere with the adaptive immune system indirectly or directly through crosstalk with T cells, B cells, and dendritic cells (DCs) [10, 11]. Neutrophils play both promoting and suppressing roles in adaptive immunity. Neutrophils promote T cell activation by secreting chemokines, such as CXCL1/5/6/8 which attract T cells to the site of inflammation also cytokines such as IL-4/6/10/12 that influence various T cell functions, activation, and differentiation [11]. Furthermore, neutrophils can directly activate T cells by acquiring the feature of antigen-presenting cells (APCs) to display antigens to T cells. Acquiring APC-like ability is an essential mechanism in the regulatory roles of neutrophils in adaptive immune cells. Neutrophils gain the APC-like function upon activation by cytokines (e.g., G-CSF, IFN-γ, IL-3, IL-4, and TNF-α) via autocrine, paracrine, and exocrine manners [29]. Activated neutrophils can synthesize the molecules required for antigen presentation on their cell surface, such as major histocompatibility complex (MHC) class II, and translocate these molecules from cytoplasm to cell surface upon neutrophil activation [30]. Neutrophils also gain APC-like function via cell-to-cell contact with T cells [10]. Neutrophils indirectly activate T cells by activation of DCs to present antigens to T cells. Neutrophils interact with DCs via cell contact and/or release of soluble factors to upregulate DCs activation [10, 31].

Neutrophils produce neutrophil extracellular traps (NETs) that are made up of extracellular DNA fibers and histones and decorated with antimicrobial proteins, such as NE and myeloperoxidase (MPO) [28]. Microbe-associated, inflammatory cytokines, or endogenous (“sterile”) stimuli can induce NET production (NETosis) in neutrophils by binding of these stimuli to cytokines receptors such as Toll-like receptors (TLRs) family that signal and induce NETosis cascade. The function of NETs in our body is capturing and killing invader microorganisms and preventing their spreading from the initial infection site. However, if dysregulated, NETs can contribute to the pathogenesis

of diseases, such as cystic fibrosis and asthma [32]. NETs can regulate inflammatory cytokines directly or indirectly via modulating other immune cells [10]. The NET structure is immunogenic and capable of triggering adaptive immune responses by activating plasmacytoid DCs, and prime T cells to reduce their activation threshold [33]. The involvement of NETs in cancer progression is described more in detail later.

Despite neutrophil abilities to promote adaptive immunity, neutrophils can also acquire immunosuppressive function through different mechanisms. These mechanisms include the production and release of arginase 1 that depletes L-arginine, essential for T cell activation and proliferation, as well as production and release of reactive oxygen species (ROS) and nitric oxide (NO) that suppress T cell activation [11]. Mechanisms through which neutrophils suppress adaptive immunity are described in detail in the below sections.

1.3 Neutrophil life cycle

1.3.1 Granulopoiesis

Neutrophils are produced in great numbers in the bone marrow through the differentiation of hematopoietic stem cells (HSC) to granulocyte–monocyte progenitors (GMPs) [34]. Mediated by granulocyte colony-stimulating factor (G-CSF), GMPs are then transformed to myeloblasts, followed by an irreversible maturation process to form band cells (immature neutrophils), and subsequently, the formation of mature or segmented neutrophils. About 10^{11} mature neutrophils are produced each day, creating a reservoir of 6×10^{11} neutrophils in the bone marrow [35, 36].

1.3.2 Neutrophil release from the bone marrow

Under normal hemostasis, only 1 to 2% of neutrophils are released from the bone marrow into the blood circulation [37]. Upon urgent need of the body to mobilize neutrophils to the inflamed site, neutrophils are rapidly produced and released from the neutrophil storage in the bone marrow under actions of G-CSF and CXC cytokines [37, 38]. Osteoblasts and other stromal cells

in the bone marrow produce CXCL12, a ligand of chemokine receptor CXCR4 expressed on neutrophils. The expression of CXCR4 on neutrophils is increased by the aging of neutrophils. Upon ligation of CXCL12 to CXCR4, CXCL12 employs a negative signal on neutrophils that leads to retaining neutrophils in the bone marrow. On the other hand, CXC chemokine receptor 2 (CXCR2) is highly expressed on mature neutrophils in blood and bone marrow. When the body needs neutrophils to be mobilized, ligands for CXCR2, such as CXCL1, CXCL2, CXCL5, and CXCL8, are expressed by endothelial cells at the proximity of the inflamed site. These ligands interfere with the CXCR4-CXCL12 ligation on neutrophils in the bone marrow and thereby induce neutrophil release to the bloodstream. G-CSF has a multifunctional role in neutrophil production and release: it regulates both neutrophil production and release to the bloodstream. G-CSF upregulates the expression of ligands for CXCR2, reduces the production of CXCL12 in osteoblasts, and downregulates the expression of CXCR4 on neutrophils [38]. The retention of neutrophils within the bone marrow and the homing of neutrophils back to bone marrow are therefore regulated through the action of two chemokine receptors, CXCR2 and CXCR4, and their ligands. Ligands for CXCR2, such as CXCL1 and CXCL2, guide neutrophil's chemotaxis toward the vasculature, enabling neutrophil release into the blood, while CXCR4 ligand (CXCL12) promotes neutrophil retention in the bone marrow. Increased expression of CXCR4, therefore, specifies the homing of neutrophils to and their retention in the bone marrow, whereas increased expression of CXCR2 on neutrophils points to neutrophil release into blood [38, 39]. Under normal hemostasis condition, the balance of chemokine production favors neutrophil retention in the bone marrow, whereas under stress conditions, such as infection and cancer, the expression of inflammatory cytokines, most notably G-CSF, is increased, favoriting neutrophil release into blood [39].

1.3.3 Neutrophil transmigration and clearance

Once circulating in the bloodstream, neutrophils stay in circulation for few hours before starting extravasation from the vasculature into tissue. Inflammation produces a wide range of pro-inflammatory cytokines, which deliver cues to neutrophils to migrate towards the infected or

damaged sites. This includes, CXC and CC chemokine ligands subfamily (CXCL1/2/6/8 and CCL/3/5), TNF-α, IFN-γ, and G-CSF, which increase the movement of neutrophils from the bone marrow to blood and towards inflammation [40]. Neutrophil expresses more than 30 different receptors on its surface, including Toll-like receptors (TLRs), G protein-coupled receptors (GPCR), and cognate immune receptors that can sense these pro-inflammatory mediators, modulating neutrophil migration, function, and behavior.

Extravasation of neutrophils from vasculature involves several steps [41]: i) neutrophils slow rolling on endothelial cells on the lumen side of the blood vessel wall, which is mediated by the interaction between P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin on neutrophils and P-selectin/E-selectin on endothelial cells; ii) firm attachment (arrest) of neutrophils to endothelial cells by binding between β2 integrins (e.g., Mac-1) on neutrophils and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells; iii) intravascular crawling that is allowed by shedding of L-selectin from neutrophil's surface; and finally IV) migration of neutrophils out of the blood vasculature. Extravasation of neutrophils allows the migration of neutrophils towards inflamed sites and instructing tissue-infiltrated neutrophils to deliver an effective immune response to invading pathogens. Tissue-infiltrated neutrophils migrate towards inflamed sites under the governance of several cellular and molecular mechanisms proposed in the literature [42].

Once neutrophils are released from the bone marrow, macrophages and DCs are responsible for clearing aged and apoptotic neutrophils via phagocytosis in the liver, spleen, and bone marrow [43]. Extravasated neutrophils mostly die in tissue, but some of them can go back to vasculature through reverse neutrophil migration [44]. The replacing of cleared neutrophils is coordinated by two interleukins, IL-23 and IL17. Phagocytosis of neutrophils results in the reduction of IL-23 production in macrophages and DCs and increases the expression of IL-17 by T lymphocytes [43]. An increase in expression of IL-17 promotes the release of neutrophils from the bone marrow by upregulation of G-CSF, leading to the steady replacement of cleared neutrophils in the bloodstream [43].

Circadian rhythm helps body to anticipate and adapt to the regular environmental changes during day. Through CXCR4/CXCL12 signaling axis, circadian oscillation causes the production of cues at

daytime that promotes the release of neutrophils from the bone marrow into the blood stream [45]. After several hours, circulating neutrophils infiltrate into tissues following circadian patterns, with a peak of infiltration at nighttime, except for intestine, liver, and white adipose tissue (WAT) in which circadian neutrophil infiltration was not detected [45]. It has been suggested that the circadian neutrophil infiltration across different tissues has important pathophysiological consequences. For example, high number of infiltrated neutrophils in kidneys at night can protect kidneys from fungal infection or increased recruitment of neutrophils in heart is suggested to be responsible for exacerbated myocardial ischemia-derived cardiac damage at night [45].

1.4 Cancer-associated inflammation

Studies have emphasized the pivotal role of cancer-associated inflammation in tumor progression, invasion, and cancer patient survival [1, 2]. Inflammation is a crucial response to invading pathogens and physical damage and the first step towards tissue regeneration in damaged areas. However, in the case of cancer, because the body cannot remove the actual cause of inflammation, the persistent inflammatory response to tumor cells becomes chronic, and inflammation indeed facilitates cancer progression and fosters tumor cell proliferation, survival, and migration [2, 46].

1.4.1 Neutrophil in cancer

Current research into immunotherapy against cancer mainly focuses on utilizing the adaptive immune response, notably T cells, to destroy tumor cells and neglected innate immune response, the first line of defense in our body. Furthermore, a significant number of tumor-associated immune cells are myeloid-derived innate immune cells, including neutrophils, macrophages, and myeloid-derived suppressor cells (MDSCs), and many recent studies reported the crosstalk between tumor-associated myeloid-derived cells and tumor cells [7-9]. Increasing evidence demonstrated that neutrophils actively influence tumor biology and thereby tumor behavior through producing pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF)-α, interleukin

(IL)-1, interferons (IFNs), angiogenic factors, and matrix-degrading enzymes that facilitate tumor initiation, progression, and invasion by inducing DNA damage, stimulating cancer cell proliferation, prolonging cancer cell survival, and promoting metastasis [15, 19, 47]. Neutrophils can also indirectly help tumor progression by suppressing adaptive anti-tumor immunity, notably suppressing cytotoxic CD8+ T-cells, which are part of our adaptive immune response to kill tumor cells. On the other hand, tumor cells initiate the migration of neutrophils from blood and bone marrow to tumor sites via specific cytokines (e.g., chemokines) including, ligands for CXCR2, such as CXCL1, CXCL2, CXCL5, CXCL8 (IL-8), VEGF, TNF-α, IFN-γ, TGF-β, MIF, and G-CSF. These signaling molecules can be directly secreted by tumor cells or indirectly by the influence of tumor cells on stromal and other immune cells [15, 16, 48].

1.4.2 Neutrophil subpopulations in cancer

Accumulating data suggest that neutrophils can switch phenotypes and display distinctive subpopulations [16, 49, 50]. Neutrophils have been initially considered a homogenous population of leukocytes, however recent studies, primarily based on *in vitro* and *in vivo* works, have illustrated the existence of different neutrophil phenotypes and their associations with cancer prognosis [16, 49, 50]. It has been reported that TANs are a distinct population of neutrophils, significantly differing in their phenotypic characterizations from naive neutrophils [51]. Influenced by the tumor microenvironment, TANs can play a spectrum of roles in cancer progression: they can possess anti-tumor (N1) to pro-tumor (N2) activities [15-19]. Respiratory burst (rapid release of reactive oxygen species) and release of antimicrobial proteins from neutrophils are two main mechanisms of killing cancer cells by neutrophils. TANs from early tumors are more cytotoxic toward tumor cells and produce higher NO and H_2O_2 (type N1). In established tumors, these functions are downregulated, and TANs acquire a more pro-tumor phenotype (type N2). Specific tumor-mediated signals, such as TGF-β (transforming growth factor-β), induce the formation of pro-tumor (N2) phenotype, while IFN-β (Interferon-β) alters neutrophil phenotype to anti-tumor type (N1) [17, 18, 52, 53]. Below we describe the spectrum of neutrophil phenotypes in more detail.

1.4.2.1 Myeloid-derived suppressor cells (MDSCs)

MDSCs are a heterogeneous population of immature myeloid cells that can inhibit T- and NK-cells activities and thereby contribute to tumor-associated immune suppression [54]. Based on the expression of granulocytic or monocytic markers, MDSCs are divided into granulocytic MDSCs (G-MDSCs) or monocytic MDSCs (M-MDSCs) subsets [6]. G-MDSCs have an increased level of reactive oxygen species and an undetectable level of NO, whereas M-MDSCs have an increased level of NO but undetectable levels of reactive oxygen species [55]. Human MDSCs are identified based on myeloid cell markers such as $CD11b^{+}$, $CD33^{+}$, HLA-$DR^{low/-}$, and two MDSC subsets can be further differentiated by $CD11b^{+}CD33^{+}$HLA-$DR^{-}CD14^{+}/CD15^{-}$ in M-MDSC and $CD11b^{+}CD33^{+}$HLA-$DR^{-}CD14^{-}/CD15^{+}$ in G-MDSC [54]. However, MDSCs are heterogenous, both biochemically and functionally, and the expression of markers in MDSCs may vary in different types of cancer [54]. More importantly, MDSCs have similar expression of markers to tumor-associated macrophages or tumor-associated neutrophils (depending on being M-MDSCs or G-MDSCs subset), and there is therefore still no defined consensus on the markers that should be used for determining MDSCs presence in tumors [56].

It has been reported that MDSCs have pro-tumorigenic activities, and their number increased in tumor and peripheral blood of several types of cancer, including non-small-cell lung carcinoma (NSCLC), pancreatic ductal adenocarcinoma (PDAC), and glioma [54, 57]. Increased G-MDSCs was associated with promoting tumor growth and development of the pre-metastatic niche through an array of mechanisms to create an immunosuppressive environment in tumors [6, 54]. These mechanisms include depleting amino acids that are essential for T-cell activity and proliferation, expression of specific factors by MDSCs involved in immunosuppression (e.g., PD-L1), expression of immunosuppressive cytokines (e.g., IL-10), and recruitment of immunosuppressive Tregs via expression of CD40 by MDSCs [54].

1.4.2.2 Low- and high-density neutrophils (LDNs and HDNs)

Low density (LDNs) and high density (HDNs) are two neutrophil subsets that can be separated through gradient centrifugation. It has been reported that LDNs exhibit pro-tumor activities, and increased percentage of LDN fraction in blood and tumor was associated with increased tumor growth and progression and promoted metastatic cancer [58]. Compared to HDNs, LDNs exhibited a decreased phagocytic activity, impaired reactive oxygen species (ROS) production, and increased immunosuppressive function [58, 59]. LDNs are bigger than HDNs, and they consist of both immature (banded nuclei) and mature (segmented nuclei) neutrophils. The difference between immature LDNs and G-MDSC is controversial, as they both possess the same nuclear morphology and express similar markers. Some researchers believe that the immature proportion of LDNs is indeed G-MDSC originated from the spleen [26].

1.4.2.3 Tumor-associated neutrophils (TANs)

Functional and phenotypical diversity and plasticity of TANs: Tumor cells initiate the migration of neutrophils from blood and bone marrow to tumor sites via the influence of specific cytokines (e.g., chemokines). These signaling molecules can be directly secreted by cancer cells, or indirectly by the influence of cancer cells on stromal and immune cells, and vice versa [15, 16, 48]. Interestingly TANs also produce chemokines (e.g., CCL2 and CXCL8) that increase further neutrophil migration to tumor site [12]. TANs release diverse active molecules from their granules and cytoplasm, including enzymes, cytokines, and chemokines, that affect the tumor microenvironment by acting on both tumor and stromal cells [6-14]. The release of these neutrophil-derived active molecules was attributed to the functional and phenotypical diversity and plasticity of TANs. Recent studies, primarily *in vitro* and animals, have shown that TANs can possess a spectrum of roles in cancer progression from anti-tumorigenic (N1) to pro-tumorigenic (N2) activities, depending on tumor type, tumor microenvironment, and tumor immunology [6, 16, 19, 60, 61]. Crosstalk and interactions of TANs with cancer cells and with other tumor-associated immune cells, such as macrophages, dendritic cells, NK cells, and lymphocytes, are complicated and still not fully understood. It has been suggested that TANs can mediate immune

response to the tumor, and various TAN subsets can function as immunosuppressive or immunostimulant in the context of cancer progression [15, 16].

Anti-tumor mechanisms of TANs (N1-Like TANs): Different signaling pathways and molecules are attributed to the anti-tumor activities of TANs. Production of reactive oxygen species (ROS) by TANs, such as hydrogen peroxide (H_2O_2), hypochlorite (HOCl), and nitric oxide (NO), has been considered the primary killing mechanism of tumor cells of neutrophils by lysing tumor cells [12]. Through antibody-dependent cell-mediated cytotoxicity (ADCC) mechanisms, TANs can recognize optimized (antibody-coated) tumor cells and destroy them [6, 62]. In ADCC, antibody molecules bind to tumor antigens, and upon recognition of these antibodies by Fc receptors on TAN, TAN is activated and destroys tumor cells through both ROS-dependent and ROS-independent mechanisms [6, 62]. In ROS-dependent mechanism, TANs contact tumor cells via integrins and directly deliver ROS to the tumor cell membrane, causing cell lysis [12]. In ROS-independent mechanism, TANs kill tumor cells via degranulation (release) of perforin and granzyme, which synergistically destroy tumor cells [6, 62]. Perforin binds to the tumor cell's plasma membrane to form pores, whereas granzyme induces programmed death cell (apoptosis) in tumor cells [63, 64]. Although TANs kill tumor cells via ROS production, it should be noted that neutrophil-derived ROS may also lead to carcinogenesis. ROS can result in further cell DNA base damages and mutations, essential for cancer initiation, proliferation, and immune suppression [14]. Moreover, neutrophil-derived ROS can also contribute to pro-tumor activities of TANs, and the level of ROS is usually elevated in the tumor microenvironment. Pro-tumor actions of neutrophil-derived ROS are explained in more detail in the next section.

In addition to the ROS-dependent cytotoxicity, TANs can inhibit tumor cell growth through the Fas ligand/Fas axis by direct physical contact to arrest the cell cycle [65]. Fas ligands (CD95L) expressed on neutrophils bind to tumor cells Fas receptors, hindering protein synthesis in tumor cells, and thereby halting their progression from G1 to S phase during the cell cycle.

TANs can also indirectly destroy tumor cells by promoting and supporting the anti-tumoral functions of other immune cells. TANs produce T-cell attracting chemokines (e.g., CCL-3, CXCL9, and CXCL10) and pro-inflammatory cytokines (e.g., IL-12, TNF-α, and GM-CSF), which promote

CD8+ T cells (cytotoxic T lymphocytes) recruitment and activation [12, 66]. TANs can also activate DCs via cell-cell contact and through the secretion of TNF-α [67]. Active DCs will thereafter promote anti-tumor immunity. As mentioned above, cytokines produced by cancer and immune cells induce the expression of MHC class II on TANs, and thereby TANs acquire the function of antigen-presenting cells to activate CD8+ T cells [15, 16, 50]. Moreover, matrix metalloproteinase-8 (MMP-8) produced by TANs contributes to anti-tumor activities of TANs by reducing metastasis formation through the modulation of tumor cell adhesion and invasion [68].

Pro-tumor mechanisms of TANs (N2-Like TANs): Exocytosis of granules from TANs results in the release of proteinase with potent tumorigenic activities, such as NE and arginase, into the tumor microenvironment. NE further increases the activation of phosphatidylinositol 3-kinase (PI3 K) proliferation pathway in tumor cells through degrading insulin receptor substrate-1 (IRS-1). Upregulation of PI3 K pathway by NE promotes tumor cell proliferation and metastasis by increasing cancer cell extravasation through endothelium [69]. TANs express high levels of arginase 1 (Arg1), which is known to suppress the proliferation of $CD8^+$ T-cells by conversing L-arginase to urea [70]. L-arginine modulates $CD8^+$ T cell metabolism and enhances their survival/proliferation and anti-tumor activity. Therefore, through L-arginine depletion in the environment, degranulation of neutrophils and exocytosis of Arg1 leads to suppression of anti-tumor immunity.

Although neutrophil-derived ROS species, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO), are cytotoxic towards tumor cells and higher expression of ROS can be considered as anti-tumor function of neutrophils. However, ROS species can also inhibit T cell activation and suppress adaptive immunity. Mechanisms through which neutrophil-derived ROS species suppress T cell activation include changing the surface thiol expression of T cells, decreasing NF-κB activation in T cells, and oxidation of the actin remodeling protein cofilin in T cells, resulting in the impaired formation of the immune synapse and T cell activation [71, 72]. As described above, Arg1 and ROS species can independently suppress T cell activation and proliferation, however, there is also evidence showing their cooperative effects. In activated TANs, co-expression and co-release of Arg1 and nitric oxide synthase (NOS) from TANs generates NO and superoxide (O_2^-).

O_2^- then combines with NO to form the highly reactive peroxynitrite ($ONOO^-$) product. Therefore, TAN-derived Arg1 suppresses T cells via both reduction of L-arginine and de novo production of ROS species ($ONOO^-$) [11].

It has also been reported few percentages of TANs express PD-L1 (programmed death-ligand 1) on their surface and this expression is attributed to pro-tumor activities of TANs [73]. PD-L1 binds to the PD-1 receptor on T cells and activates PD-1 signaling, which dampens T cell responses in several ways, including reduced T cell activation, proliferation, and survival [74]. The PD-1/PD-L1 axis is suggested to be a mechanism for tumors to escape T cell immunologic response. PD-L1 expression in TANs leads to the development of CD8+ T-cells incapable of generating anti-tumor response [73]. Secretion of CCL17 by TANs promotes the recruitment of immunosuppressive regulatory T cells (Tregs) with defective cytotoxic functions and thereby suppresses anti-tumor immunity of CD8+ T-cells [75].

TANs produce CXCL8 (and other ELR^+ CXC chemokines) and growth factors, such as vascular endothelial growth factor (VEGF), that further increase neutrophil migration to tumor, angiogenesis, and tumor growth [6]. Moreover, TAN-derived matrix metalloproteinase-9 (MMP-9) degrades the vascular basement membrane and remodels the extracellular matrix (ECM). This promotes tumor angiogenesis and tumor cell intravasation and prevents tumor cell apoptosis, all facilitating cancer progression and metastasis [76, 77]. MMP-9 is produced by many other cells, however, human neutrophil-derived MMP-9 is TIMP-free (TIMP: tissue inhibitor of metalloproteinases), which provides a potent catalytic stimulator of angiogenesis [78].

Cancer stem cells (CSCs) are a minor subpopulation of malignant cells within tumors, with the ability of unlimited self-renewal and differentiation, suggesting that these cells are responsible for tumor aggressiveness, tumor heterogeneity, metastasis, and resistance to antitumor treatments [79]. It has been shown that TANs can secret a large amount of growth factors, such as bone morphogenic protein (BMP)-2 and transforming growth factor beta 2 (TGF-β2) that can confer cancer stem-like phenotypes on hepatocellular carcinoma (HCC) cells [80]. These TAN-induced HCC stem-like cells are hyperactive in nuclear factor kappa B (NF-κB) signaling, secrete

higher levels of CXCL5 chemokine, which in turn recruit more neutrophils to the tumor site, providing a positive feedback loop.

1.5 Neutrophil extracellular trap (NET) and its role in cancer progression

As mentioned above, microbe-associated stimuli and inflammatory cytokines induce NETosis in neutrophils to protect the body against infection by capturing and trapping invasive pathogens [28]. NETs have been shown to be implicated in the progression of several diseases, and studies have recently highlighted the association between NETs and malignancy [32]. It has been reported that NET formation, in response to cancer-associated inflammatory cytokines, can indeed capture cancer cells, support their growth, and promote metastasis [81-86]. In patients undergoing curative liver resection for metastatic colorectal cancer, increased postoperative NET formation was associated with an above 4-fold reduction in disease-free survival [85]. The adhesion of tumor cells to NETs is mediated by β1- integrin, expressed on both cancer cells and NETs [84]. It has been suggested that NETs trigger HMGB1 (High mobility group box 1) protein release and activate TLR9-dependent pathways in cancer cells to promote their adhesion, proliferation, migration, and invasion [85]. In a surgical stress mice model, the authors reported that treatment of animals with DNAse or peptidyl arginine deiminase 4 (PAD4) inhibitors (or using a PAD4 deficient mice model) improved oncological outcome and reduced the growth of metastatic tumors [85]. PAD is essential for NET formation. Moreover, DNAse disintegrates the NET structure, and it has been shown in animal models that DNAse treatment of tumor-bearing animals reduced disease burden and metastasis [82, 87].

It should be noted that NET formation in tissues and blood can lead to pathophysiological consequences, causes collateral damages to tissues, and facilitates the progression of certain diseases [88, 89]. Persistent NET release during respiratory chronic diseases, such as cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease (COPD) contribute to the reduction of pulmonary function by blocking airways [89]. NETs can also exacerbate tissue damage during inappropriate inflammation and delay wound healing in diabetic patients [89]. In

addition, NETs interact with endothelium, platelets, red blood cells, and coagulation factors and can thereby stimulate deep vein thrombosis (DVT) [90]. More importantly, in relation to the current COVID-19 pandemic, researchers have identified NETs as the potential culprits of pulmonary dysfunction and death in COVID-19 patients [91]. It has been shown that the presence of NET byproducts in blood, such as extracellular DNA fibers, were correlated with parameters of lung damage in these patients [91].

1.6 Neutrophil-to-lymphocyte ratio (NLR) and prognostic evaluation

In the last decade, neutrophil-to-lymphocyte ratio (NLR) has been proposed as an independent and reliable marker of cancer-associated inflammation [3, 4]. The high number of blood and tumor NLR and TANs have been reported to be associated with poorer patient prognosis and survival in several types of cancer, including hepatocellular carcinoma [92], colorectal cancer [93], gastric cancer [94], and renal carcinoma [95]. A prevalence of neutrophils over lymphocytes could be a sign of failure of immune surveillance to recognize and destroy neoplastically transformed cells in the body [5]. Contrarily, fewer studies did not show any correlation between NLR and unfavorable patient outcomes and the relationship between NLR and pathological features of tumors remains inconsistent [3, 96, 97]. This specifies the complex and not fully understood roles of neutrophils in tumor development and progression and emphasizes neutrophil's plasticity to switch between pro-tumorigenic and anti-tumorigenic phenotypes [98].

Chapter 2 Determine the association of neutrophil-to-lymphocyte ratio (NLR) with neutrophil phenotype in cancer patients

2.1 Introduction

The role of inflammation in tumor progression has long been known and considered as one of the hallmarks of cancer progression [1, 2]. At the clinical level, cancer-associated inflammation can be recognized by an increased ratio of circulating neutrophils to lymphocytes (NLR) [3], and this clinical metric standardizes neutrophil counts between patients [4]. An elevated circulating NLR has been repeatedly shown to represent a poor prognostic marker in several types of cancer, such as many solid organ malignancies, including gastro-esophageal adenocarcinoma [5-9]. For example, Lawati et al reported that dynamic alterations in NLR during the course of treatment of patients with esophageal adenocarcinoma predicts oncological outcomes in which the patients with reduced post-treatment NLR have significantly better survival outcomes compared to patients who demonstrate a persistently high or rising NLR [9]. The study also showed that patients with a low NLR are more likely to achieve a pathologic complete response to standard of care neoadjuvant chemotherapy [9]. These clinical studies, linking NLR to patient outcome across a broad range of solid tumors, hint at a fundamental involvement of neutrophils in tumor development and progression.

Data in the literature have highlighted distinct subsets of neutrophils as pro-tumorigenic N2 cells based on their morphology and protein expression profiles [10-14]. It has been reported that low-density neutrophils (LDNs) exhibit pro-tumor activities, and increased percentage of LDNs fraction in blood and tumor was associated with increased tumor growth and progression and promoted metastatic cancer [15]. Expansion of protumor neutrophil subsets has been documented in both animal and human studies. However, their relative contribution to a rising NLR remains poorly elucidated. Furthermore, the downstream implications of an elevated NLR with respect to the tumor inflammatory milieu are similarly poorly described. Thus, given the

degree to which neutrophils impact the course of tumor development in animal models, and the degree to which elevated NLR is observed in human cancer patients, the possibility that NLR represents more than a simple biomarker of poor prognosis logically arises. Accordingly, in this study, we sought to characterize the changes in neutrophil phenotype associated with an elevated circulating and primary tumor NLR. In addition, we sought to determine the effect of an elevated circulating NLR at the level of the primary tumor with respect to neutrophil infiltration in patients with gastro-esophageal adenocarcinoma. In addition, metastasis to regional lymph nodes remains a hallmark of tumor progression in adenocarcinoma. This raises the question as to whether neutrophils and NETosis in this context are implicated in the development of nodal metastasis. Accordingly, in this study, we investigated the presence of nodal neutrophils and NETs as a promoter of premetastatic niche in lymph nodes and aimed to establish the correlation of nodal neutrophils and NETs with survival rate in cancer patients.

2.2 Methods

2.2.1 Patients

Patients with histologically confirmed gastro-esophageal adenocarcinoma who underwent curative-intent resection between January 2011 and May 2018 were included in this study. Ethical approval for this project was granted by McGill University Health Centre Research Ethics Board, and appropriate informed consent was obtained from each participant. Patient demographics, histopathologic characteristics, neutrophil and lymphocyte counts, and survival outcomes were recorded and retrieved from a prospectively entered institutional database. The demographic and clinico-pathologic data of these patients are outlined in table 1.

Table 1 Demographic and clinico-pathologic data of patients

Patients with gastric and esophageal cancer (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 & 4	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

2.2.2 Quantification of neutrophil and lymphocyte counts in peripheral blood

Circulating NLR of patients was calculated from laboratory blood tests performed between the date of diagnosis and a maximum of 7 days prior to surgery. The cut-off NLR value of 4 was used

to assign patients into low or high NLR groups. The cut-off was chosen based on previous data in the literature [3]. Tissue (tumor) NLR was determined via immunohistochemistry dual staining of tissue microarrays (TMAs) as described below. Patients with tissue NLR above or equal to the median value were considered in the high NLR group and those with tissue NLR below the median were considered in the low NLR group.

2.2.3 Tissue microarray (TMA) construction

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. TMA blocks had 6 cores for each patient: 2 cores punched from tumor core areas of 2 separate representative tumor blocks, 1 core punched from the tumor periphery (the same block as tumor core), 1 core from the non-diseased stomach, 1 core from the positive local lymph node, and 1 core from the negative local lymph node. After sectioning TMA blocks and IHC staining, a pathologist verified that each core contained the intended tissue of interest.

2.2.4 Immunohistochemistry (IHC) staining and image analysis

Dual IHC was performed on TMA sections to identify neutrophils and lymphocytes. Neutrophil Elastase (NE) and CD3 positive cells were considered neutrophils and lymphocytes, respectively. Sequential 4 µm sections from TMA blocks were obtained using microtomy. Automated dual IHC was performed using The Discovery Ultra platform (Ventana). Briefly, the sections were first deparaffinized and rehydrated. Antigen retrieval was carried out in Tris-EDTA buffer (pH 9) at 96 °C for 24 mins. To block the endogenous peroxide and proteins, sections were immersed in Inhibitor CM (Ventana) for 4 mins at 37 °C. The sections were then incubated for 60 mins at room temperature with NE-antibody, R&D Systems #MAB91671100, 1:2900 dilution. After washing, the secondary antibody was added (anti-mouse AP), incubated for 60 mins, washed, and Ventana yellow kit was used to detect the signal of NE+ cells. As per the manufacturer's guidelines,

antibodies were then denatured. After washing, the slides were incubated with anti CD3-antibody, Dako #A0452, 1:70 dilution for 60 mins at room temperature. After washing, the secondary antibody (anti-rabbit HRP) was added, incubated for 60 mins, washed, and Ventana purple kit was used to detect the signal of CD3+ cells. Subsequently, slides were counterstained with hematoxylin to stain the cell nucleus, dehydrated in a series of gradient ethanol, and covered with coverslips. The optimal titrations of antibodies were chosen using IHC staining of human gastric/esophageal tumor and non-diseased stomach slides, selected by a pathologist, containing neutrophils and lymphocytes. All slides were scanned using Aperio AT Turbo at 20× objective (0.05 µm/pixel).

The number of CD3+ (lymphocytes) and NE+ (neutrophils) cells in each core was quantified using Aperio Technologies, Inc. system (IHC Nuclear Image Analysis algorithm, version 10). The area of each core (area of analysis) was marked manually using the annotation tools. To tune the algorithm to be able to detect the nuclear staining (hematoxylin), parameters such as the color vector (RBG OD: optical density for the Red, Green, Blue components of hematoxylin staining), intensity threshold, cell segmentation, cell size and shape were optimized. The algorithm detected cytoplasmic/membrane staining and corrected for it in the staining intensities and in the segmentation of the nuclei. Cells positive for cytoplasmic/membrane staining, anti-NE and anti-CD3, were detected by tuning RBG OD of yellow and purple colours and their intensity thresholds. The number of neutrophils and lymphocytes per area of analysis was calculated in each core. Areas of necrosis and cells located in blood vessels were excluded from the area of analysis. The mean data obtained from 2 cores was used for statistical analysis. All IHC staining and optimization steps were validated and performed under the supervision of a pathologist.

2.2.5 Immunofluorescence (IF) staining and image analysis

Additional TMA slides were deparaffinized and subjected to antigen retrieval as described above for IF staining. IF was performed using following antibodies: anti-NE (R&D Systems, #MAB91671100, 1:2900 dilution), anti-arginase 1 (Cell Signaling Technology, #93668T, 1:100

dilution), anti-CD66b (BioLegend, #392902, 1:80 dilution), and Histone H3 Citrulline, H3Cit (Abcam #ab5103, 1:400). Similar to IHC, positive and negative controls were used for the optimization of IF staining antibodies. To visualize these antibodies, OPAL kit (PerkinElmer) fluorophores were used and prepared according to the manufacturer's protocols. The slides were counterstained with DAPI to stain the cell nucleus and coverslipped (#1.5). TMA slides were then digitalized at 20× magnification using Axio Scan Z.1. whole slide scanner (Zeiss).

HighPlex FL v3.1.0 and Area Quantification FL v1.0 algorithms of HALO image analysis software (Indica Labs) were used to quantify the number of positive cells, the average intensity of the markers, and the area covered by the markers. Nuclei were detected using the following optimized parameters: nuclear detection minimum intensity (DAPI) = 0.052; nuclear contrast = 0.503; nuclear segmentation aggressiveness = 0.099; nuclear size setting = 6.8 – 107 μm^2; and nuclear roundness = 0.105. Positive cells/areas were detected by adjusting Arg1, NE, CD66b, H3Cit dye thresholds. The area of analysis was marked manually using the annotation tools and the percentages of positive cells per area were calculated from the total cell count. Arg1 and NE markers were chosen as they have been suggested to be associated with the tumor-promoting phenotype of neutrophils. For measuring the level of Arg1 and NE expressions in TANs, Arg+/CD66+ and NE+/CD66b+ cells were respectively considered as TANs (tumor-associated neutrophils). To determine the level of expression of Arg1 and NE in TANs, the QuickScore method was used and calculated by multiplying the percentage of positive cells (0% to 100%) by the mean intensity of the staining, scaled 0 to 3 (0 = negative, 1 = weak staining, 2 = moderate staining, and 3 = strong staining) [16-18]. Anti-H3Cit was used to identify areas covers with NETs, and NE/H3Cit colocalized positive area was considered as NETs positive areas.

2.2.6 Isolating and immunophenotyping of low density (LDNs) and high density (HDNs) neutrophils from blood

EDTA anti-coagulated peripheral blood samples were collected from 19 treatment naïve gastro-esophageal adenocarcinoma patients. LDNs and HDNs were isolated from blood samples using

gradient centrifugation. Briefly, 450 µl of 6% dextran (Fisher Scientific) in phosphate-buffered saline (PBS) was mixed with 3 ml blood and incubated for 1 hour at room temperature to sediment red blood cells (RBCs). The leukocyte-rich supernatant was removed, centrifuged at 450 x g for 5 mins (4°C), and then resuspended in 2 ml PBS. Using a pipette, leukocyte-rich supernatant was slowly layered on top of 3 ml Ficoll-Paque (GE Healthcare) separation medium, and then centrifuged for 25 mins at 450 x g at room temperature without breaks. LDNs were carefully collected from the mononuclear cell layer at the interface between plasma and Ficoll. HDNs were collected from the granulocyte/RBCs sediment followed by hypotonic lysing (Invitrogen) of the remaining RBCs. Both cell fractions were washed twice in PBS and resuspended in 1 ml PBS and counted. To identify and quantify LDNs and HDNs in each cell fraction using flow cytometry (BD LSRFortessa - 20×), 10^6 cells were suspended in 1 ml PBS and stained with APC-CD66b and PE-CD16 antibodies, purchased from Miltenyi Biotec. Cells were incubated with antibodies for 30 mins at 4 °C, washed with PBS, fixed using Miltenyi cell fixation kit, and resuspended in 500 µl flow cytometry media containing 0.5% BSA and 2 mM EDTA in PBS. Using flow cytometry, LDNs and HDNs were gated as CD66+/CD16+ population and their percentage from the total number of events was obtained and used to calculate the total number of LDNs and HDNs in blood samples. The percentages of LDN fraction (%LDN) and HDN fraction (%HDN) from the total number of neutrophils (LDN + HDN) were calculated for each patient. For the same patients, NLR was obtained from the laboratory blood test (neutrophil and lymphocyte counts) using the same blood sample.

We performed immunophenotyping of LDNs and HDNs using a panel of 12 neutrophil biomarkers and a viability dye (Viobility Fixable Dye-V500, Miltenyi). Table 1 in the Appendixes section summarizes the flow cytometry biomarker panel used in this study with a description for each biomarker. The surface (membrane) staining and fixing procedures were described above. The antibodies for flow cytometry staining of surface-associated proteins used in this study were: anti-CD45-BV421 (130-110-637 Miltenyi); anti-CD16-PE (130-113-393 Miltenyi); anti-CD66b-APC (130-117-692 Miltenyi); anti-CD54-BV711 (64078 BD Biosciences); anti-CD62L-BV650 (563808 BD Biosciences); anti-CD182-PE-Cy7 (130-100-930 Miltenyi); anti-CD184-FITC (130-117-370 Miltenyi); anti-HLA-DR-PE-CF594 (30-111-797 Miltenyi); anti-CD11b-BV605 (742639 BD

Biosciences); anti-CD274-BV785 (563739 BD Biosciences). The concentrations of these antibodies were determined based on the manufacturer's recommendation and our preliminary optimization experiments. After surface staining, cells were washed in flow cytometry media, fixed, and then resuspended in 100 μl of permeabilization solution containing antibodies for intracellular protein staining and incubated for 10 mins at RT. Anti-NE-APC Cy7 (bs-6982R-A750 Biossusa) and anti-arg1-PerCP-eFluor 710 (6-3697-82 eBioscience) were used for intercellular staining. After permeabilization and intracellular staining, cells were washed in 2 ml of permeabilization solution and then resuspended in flow cytometry media. Samples in flow cytometry tubes were then acquired within 24 hours using multi-colour flow cytometry - BD LSRFortessa 20 (FACSDiva Software). To ensure the consistent performance of the cytometer on different days, cytometer Setup and Tracking beads (CS&T beads, BD) was run before sample acquiring according to the manufacturer's instruction and the same application was applied for all data acquisition. Single color compensation controls using compensation beads were prepared to determine the compensation settings, and the same compensation matrix was applied to all samples in FlowJo software after data collection. Fluorescence minus one (FMO) control substituting with the respective isotype control antibody were used as negative controls for each experiment to be able to gate positive cells. To select neutrophils, we followed the subsequent flow cytometry gating strategy. First, debris was excluded, and the neutrophil population was roughly gated on the SSC-A/FSC-A plot. Single cells (singlets) were identified on the SSC-H/SSC-A plot, and then live cells were selected by gating on cells negative to the viability dye. Leukocytes were selected by gating on CD45+ cells, and finally, neutrophils were selected as CD66b+ and CD16+ cells. CD16 biomarker was used to eliminate eosinophils because, like neutrophils, eosinophils also express granulocyte markers such as CD66b. Once the neutrophil population was identified on the flow cytometry plots, positive neutrophils for each of the above biomarker were gated on the neutrophil population. Figure 1 in the Appendix shows the gating strategy used in this project.

2.2.7 Statistical analysis

Overall survival (OS) and disease-free survival (DSF) were evaluated using the Kaplan-Meier method, and log-rank statistical test was used to compare survival curves between groups. Univariate Cox regression and multivariable Cox-proportional hazard model were employed to estimate the magnitude of the association between variables and survival and to determine whether these variables were independently associated with survival. Mann–Whitney test was used for unpaired data to compare the mean values of two continuous variables. Wilcoxon test was used for paired data to compare the mean values of two continuous variables. Kruskal–Wallis test was used for unpaired data to compare the mean values of more than two groups. Welch test was used for unpaired data to compare the mean values of two continuous variables without assuming equal population variances due to unequal size of groups. Fisher's test was used for the association between two categorical variables. Pearson correlation test was used to establish the correlation between two continuous variables. For all statistical tests, a *p*-value below 0.05 was considered statistically significant. Statistical analyses were performed using Prism-Graphpad and R software programs.

2.3 Results

2.3.1 An elevated circulating NLR was associated with the expansion of the low-density neutrophil fraction in patients with gastro-esophageal cancer

The percentage of circulating LDN fraction substantially varied among cancer patients (0.2% - 40%). Figure 1 shows that the average percentage of circulating LDN was 16% in patients with an elevated baseline circulating NLR (NLR $\geq$ 4), which was significantly higher than those with a low circulating NLR (2%). Furthermore, circulating NLR was positively correlated (r = 0.6674 value, *P* = 0.0018) with the percentage of circulating LDNs (Figure 1). We also observed a positive correlation between neutrophil counts in blood and the percentage of circulating LDN fraction.

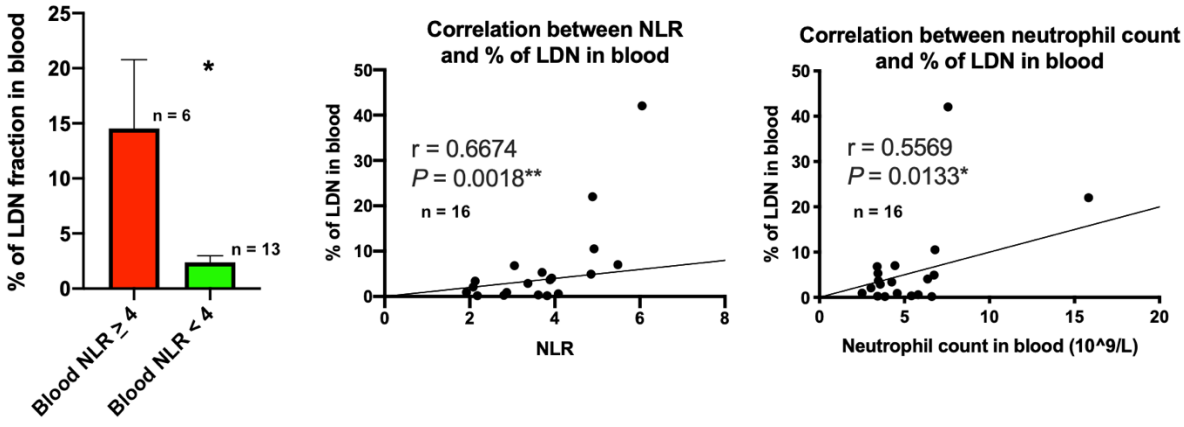

Figure 1 An elevated NLR is associated with the expansion of the low-density neutrophil (LDN) fraction: Expansion of the LDN fraction in the circulation of patients with elevated blood NLR (≥ 4). Positive Pearson correlation of both circulating NLR and neutrophil counts with the percentage of LDN fraction. Mann-Whitney test for unpaired data, mean ± SEM, **P* < 0.05, ***P* < 0.01, ****P* < 0.001

To phenotypically characterize LDNs, we then determined the protein expression profile of LDNs in 9 patients using flow cytometry. LDNs and HDNs exhibited distinct protein expression profiles and formed separated clusters in the t-SNE dimensionality reduction visualization of cells (Figure 2). Based on the shift of FSC to higher values, flow cytometry results revealed that LDNs were bigger in size than HDNs. Moreover, LDNs exhibited higher expression of pro-tumorigenic markers arginase 1, CXCR2, and CD66b (CEACM-8) compared to HDNs (Figure 2B and C).

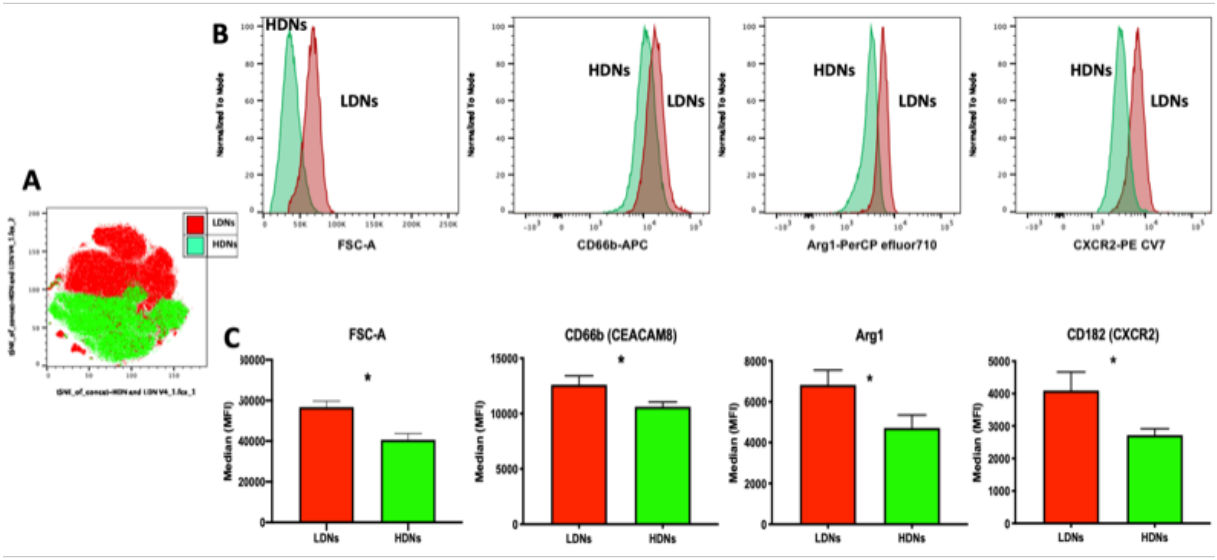

Figure 2 Circulating HDNs and LDNs exhibit distinct phenotypes: (A) LDNs and HDNs exhibited distinct protein expression profiles and formed separate clusters in a t-SNE dimensionality reduction graph. (B and C) LDNs are bigger in size, as shown by the shift of FSC to higher values. Significantly higher expression of CD66b, Arg1, and CXCR2 was demonstrated by flow cytometry in circulating LDNs compared to HDNs. n = 7, Wilcoxon test for paired data, mean ± SEM, * $P < 0.05$

LDNs are heterogenous and composed of both mature and immature neutrophils (Figure 3). The percentage of immature LDNs (defined by low CD16 expression) showed a large variation between patients with an average percentage of 12.9% $\pm$ 11.9% (mean $\pm$ SD). A significant positive correlation between the percentage of circulating LDN and the proportion of the immature LDN fraction was observed (r = 0.9279). Thus, the higher the percentage of circulating LDN in cancer patients, the higher the percentage of immature LDNs within the LDN population. We subsequently determined the protein expression profile of immature LDNs and identified an immature LDN phenotype in cancer patients characterized by $CD16^{low}/CD66b^{high}/CXCR2^{low}/CD62L^{low}$ expression (Figure 3). The average percentage of this LDN phenotype was 5.5% $\pm$ 9.2% (mean $\pm$ SD) of the total LDN population.

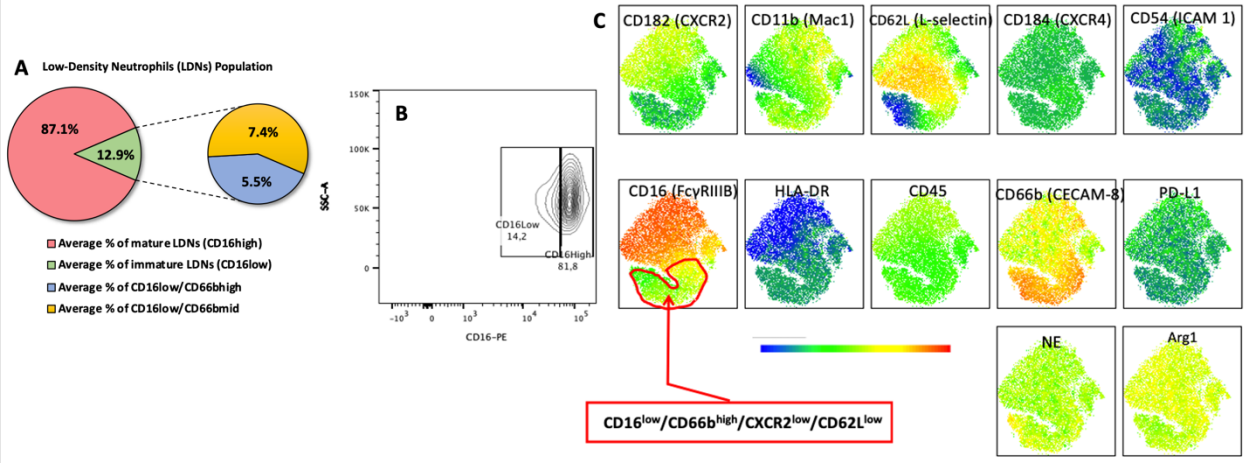

Figure 3 Circulating LDN are heterogenous and composed of phenotypically distinct immature and mature fractions: (A) average % of different neutrophil subsets in LDN population. (B) Gating strategy to identify immature LDNs (CD16 low) and mature LDNs (CD16 high). (C) t-SNE representation of LDNs demonstrates the presence of a distinct immature LDN phenotype in cancer patients characterized by $CD16^{low}/CD66b^{high}/CXCR2^{low}/CD62L^{low}$ expression.

2.3.2 An elevated circulating NLR was associated with an elevated NLR within primary gastro-esophageal tumors

The prognostic implications of an elevated circulating NLR are clear, however, the question remains as to whether the NLR represents a simple biomarker of poor prognosis or whether it plays a causative role in driving tumor progression in these NLR high patients. Accordingly, we sought to determine the implications of an elevated circulating NLR on the pattern of inflammatory infiltration within gastro-esophageal adenocarcinoma and non-diseased stomach tissue derived from surgical specimens. Figure 4 shows the IHC staining image of TMA cores, representing neutrophils (NE+ cells, yellow) and lymphocytes (CD3+ cells, purple). The average NLR within tumor core, tumor periphery, and non-diseased stomach tissue was respectively 0.97, 0.37, and 0.18, where the NLR in tumor core was significantly higher than that in tumor periphery and non-diseased stomach (Figure 5). The average NLR was also significantly higher in the tumor periphery compared to the non-diseased stomach (Figure 5). Differences in NLR were

accentuated at the level of the tumor core when patients were dichotomized according to blood NLR. As figure 6 shows, patients with a circulating NLR ≥ 4 exhibited an NLR of 1.59 within tumor cores compared to 0.49 in patients with an NLR < 4 ($P < 0.01$). Finally, no significant association between circulating NLR and neutrophil infiltration at the periphery or within non-diseased stomach tissue was observed (Figure 6).

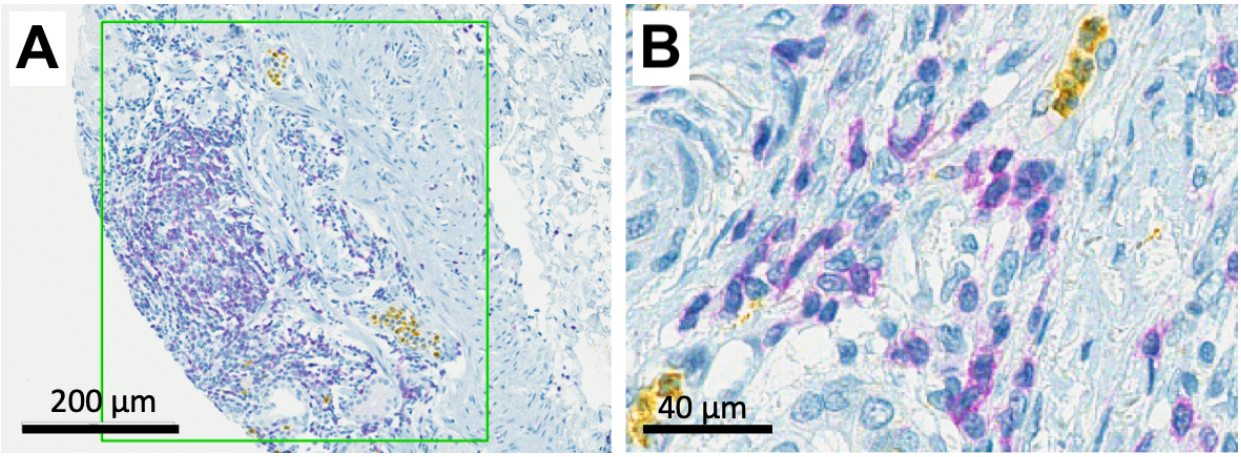

Figure 4 Immunohistochemistry of gastro-esophageal adenocarcinoma primary tumors: (A) IHC staining image of TMA cores, neutrophils (NE+ cells, yellow), and lymphocytes (CD3+ cells, purple). (B) IHC staining at higher magnification, neutrophils stained in yellow, and lymphocytes stained in purple.

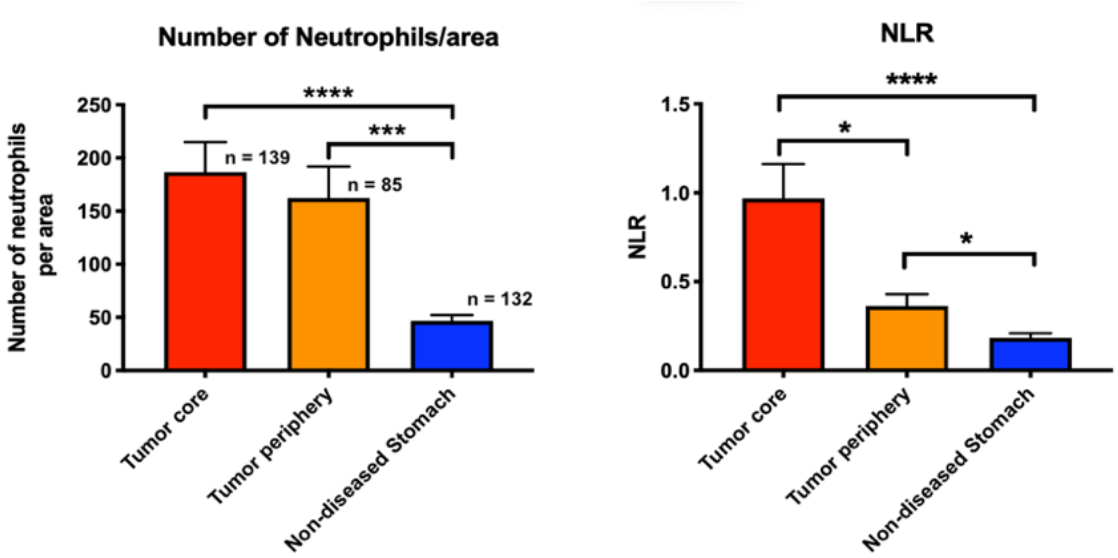

Figure 5 Relative neutrophil infiltration is restricted to the primary tumor: Higher number of neutrophils per area in tumor core and in tumor periphery compared to non-diseased stomach. NLR was higher in the tumor core than in the tumor periphery and in the non-diseased stomach. Kruskal–Wallis test for unpaired data, mean ± SEM, *$P < 0.05$, *** $P < 0.001$, ****$P < 0.0001$.

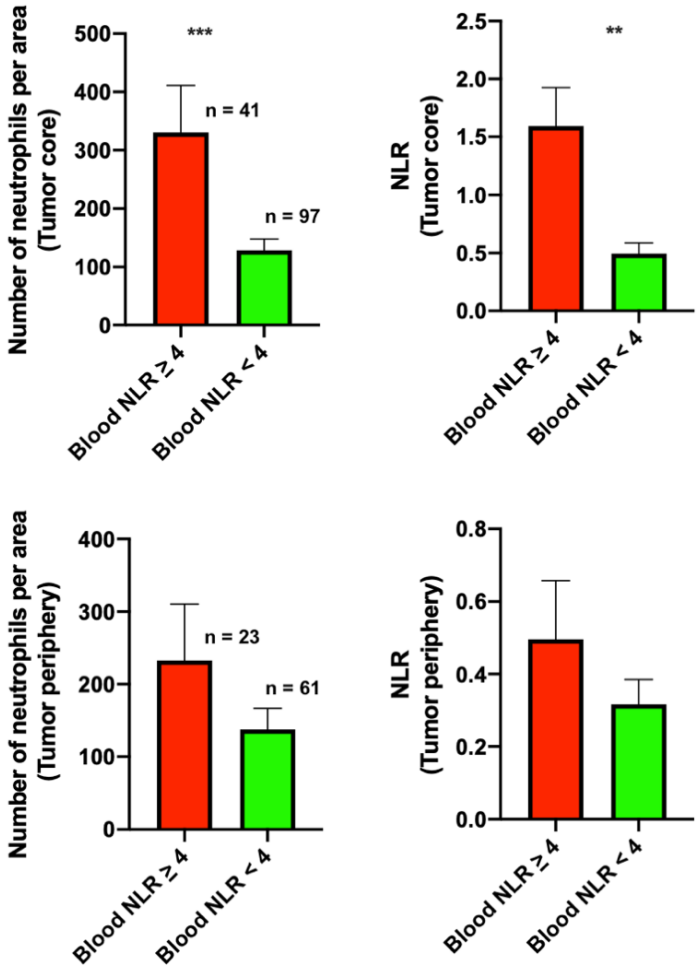

Figure 6 Increased neutrophil infiltration to the tumor core when the circulating NLR is elevated: Significantly higher neutrophil accumulation and NLR in the intratumoral area in patients with high blood NLR (≥ 4). These differences were not significant in the tumor peripheral area. Mann-Whitney test for unpaired data, mean ± SEM, **$P < 0.01$, ***$P < 0.001$

2.3.3 The NLR in the tumor core (intratumoral NLR), but not in the tumor periphery, is an independent predictor of survival

Given the observation that the circulating NLR portends poor survival and that an elevated NLR is associated with neutrophil infiltration within the tumor core, we sought to establish the association between tumor NLR and survival. Univariate Cox regression analysis (Table 2) shows that high intratumoral NLR (≥ median) and prognostic factors such as high pathologic stage, high tumor grade, and LVI (lymphovascular invasion) are all significantly associated with a higher risk of death. The risk of death in patients with a high intratumoral NLR was significantly higher than those with low intratumoral NLR. Multivariable Cox regression analysis confirmed that intratumoral NLR is an independent predictor of patient survival (Table 2). We did not observe a significant association between NLR in tumor periphery or non-diseased stomach with survival.

Kaplan-Meier (K-M) survival analysis confirmed the Cox regression results. OS (overall survival) in patients with high (≥ median) intratumoral NLR was significantly lower than those with low intratumoral NLR (Figure 7A). Median OS in NLR high group was 708 days versus 1316 days in NLR low group, P = 0.0053. An analogous trend was observed with respect to DFS, disease-free survival (DFS NLR high 520 days versus NLR low 1087 days P = 0.0047). In contrast to intratumoral NLR, NLR in tumor periphery and NLR in non-diseased tissues were not significantly associated with patient survival outcomes.

Table 2 Univariate and multivariable Cox regression analyses of prognostic parameters for overall survival in cancer patients. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$.

		Univariate			Multivariable		
Variable	**N**	**HR**	**95% CI**	***P*-Value**	**HR**	**95% CI**	***P*-Value**
Tumor core NLR							
< median	71	1.00	Referent	-	1.00	Referent	-
≥ median	71	1.95	1.26-3.00	**0.0025****	2.621	1.67 -4.1	**< 0.001*****
Tumor periphery NLR							
< median	42	1.00	Referent	-	-	-	-
≥ median	43	1.21	0.70-2.10	0.49	-	-	-
Pathologic Stage							
1	26	1.00	Referent	-	1.00	Referent	
2	31	0.88	0.39-1.95	0.75	0.81	0.36-1.81	0.6
3	79	2.05	1.12-3.75	**0.02***	2.37	1.29-4.37	**0.0056****
4	11	5.40	2.13-13.67	**< 0.001*****	7.02	2.71-18.20	**< 0.001*****
Pathologic T							
1	20	1.00	Referent	-	-	-	-
2	28	1.04	0.43-2.52	0.93	-	-	-
3	73	2.13	1.04-4.36	**0.039***	-	-	-
4	26	4.53	2.09-9.83	**< 0.001*****	-	-	-
Pathologic N							
0	38	1.00	Referent	-	-	-	-
1	26	0.95	0.46-1.98	0.89	-	-	-
2	39	1.35	0.70-2.58	0.37	-	-	-
3	44	3.03	1.67-5.50	**< 0.001*****	-	-	-
Pathologic M							
0	132	1.00	Referent	-	-	-	-
1	12	3.40	1.547-7.473	**0.002****	-	-	-
Recurrence							
No	85	1.00	Referent	-	-	-	-
Yes	58	2.46	1.591-3.813	**< 0.001*****	-	-	-
Grade							
Poor	75	1.00	Referent	-	-	-	-
Moderate	60	0.47	0.294- 0.74	**0.0011****	-	-	-
Well	8	0.88	0.35-2.21	0.79	-	-	-
LVI							
No	40	1.00	Referent	-	-	-	-
Yes	104	3.32	1.80-6.12	**< 0.001*****	-	-	-
Neoadjuvant							
No	58	1.00	Referent	-	-	-	-
Yes	82	0.82	0.54-1.25	0.36	-	-	-

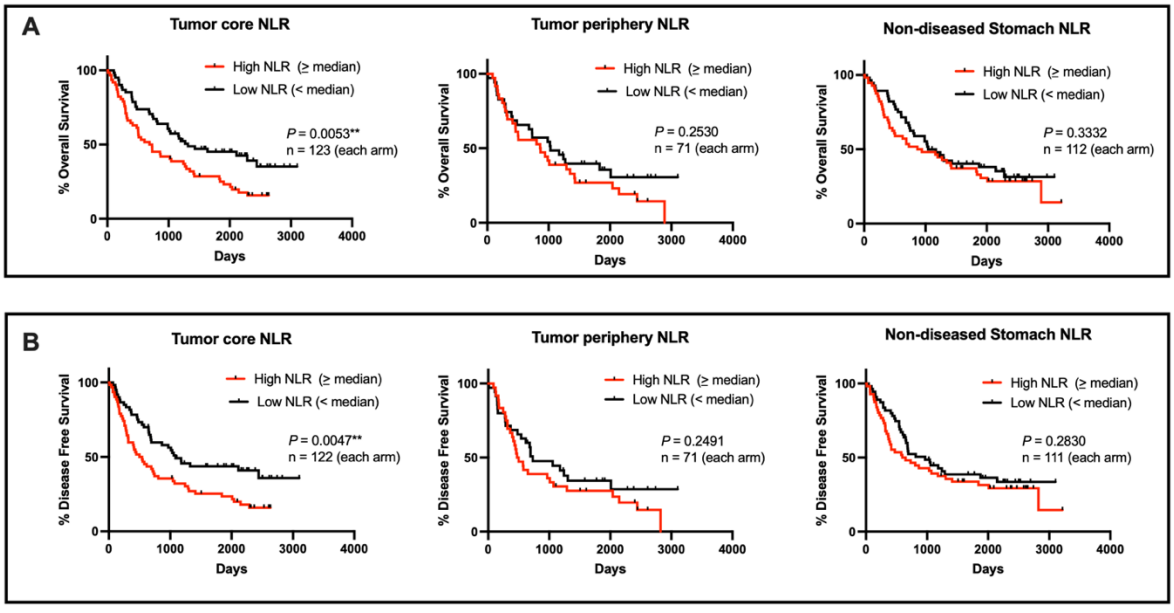

Figure 7 Survival outcomes in gastro-esophageal adenocarcinoma patients according to NLR: (A) Kaplan-Meier (K-M) overall survival (OS) and (B) disease-free survival (DSF) curves between patients with high NLR and low NLR in intratumoral, peritumoral, and non-diseased stomach TMA cores. Significantly lower OS and DSF in the high intratumoral NLR (≥ median) group than patients with low intratumoral NLR. There was no significant difference in terms of OS and DSF between high NLR and low NLR groups in the tumor periphery, and the same for the non-diseased stomach. n is the total number of patients in both arms together. Log-rank test, $**P < 0.01$.

2.3.4 An elevated tumor and blood NLR in cancer patients was associated with increased expression of Arg1 and NE in tumor-associated neutrophils (TANs)

At the next step, using IF staining (Figure 8) and image analysis, we looked at the association of Arg1 and NE expressions in TANs with circulating and intratumoral NLRs. The level of expression of Arg1 and NE was calculated using a scoring method described in the Method section. We found that a high level of Arg1 and NE expressions in TANs were associated with high blood and intratumoral NLR in gastro-esophageal cancer patients (Figure 9). As shown in figure 9, the level of expression of Arg1 and NE in TANs were significantly higher in patients with high intratumoral NLR (≥ median) as well as in those with high baseline blood NLR (≥ 4). In addition, overall survival (OS) was also significantly lower in patients with an elevated level of expression of Arg1 in TANs (Figure 10). Similar results were likewise obtained for NE markers where high expression of NE in TANs was associated with poorer survival in gastro-esophageal cancer patients (Figure 10).

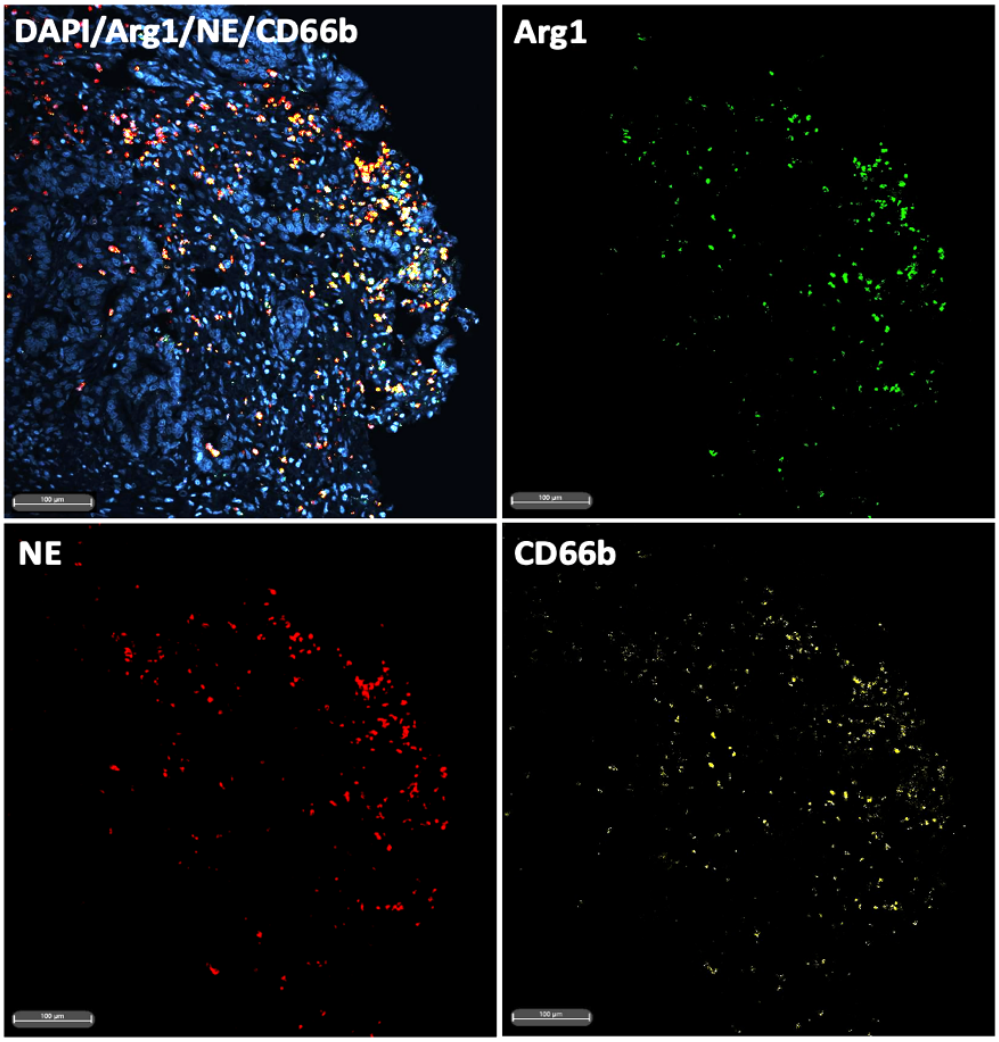

Figure 8 Immunofluorescence staining of tumor core for DAPI, Arg1, NE, and CD66b: Colocalization of Arg1, NE, CD66b positive cells was observed. For measuring the level of Arg1 and NE expressions in TANs, Arg+/CD66+ and NE+/CD66b+ cells were respectively considered as TANs. The number of TANs and the average florescence intensity of Arg1 and NE were measured using HALO image analysis.

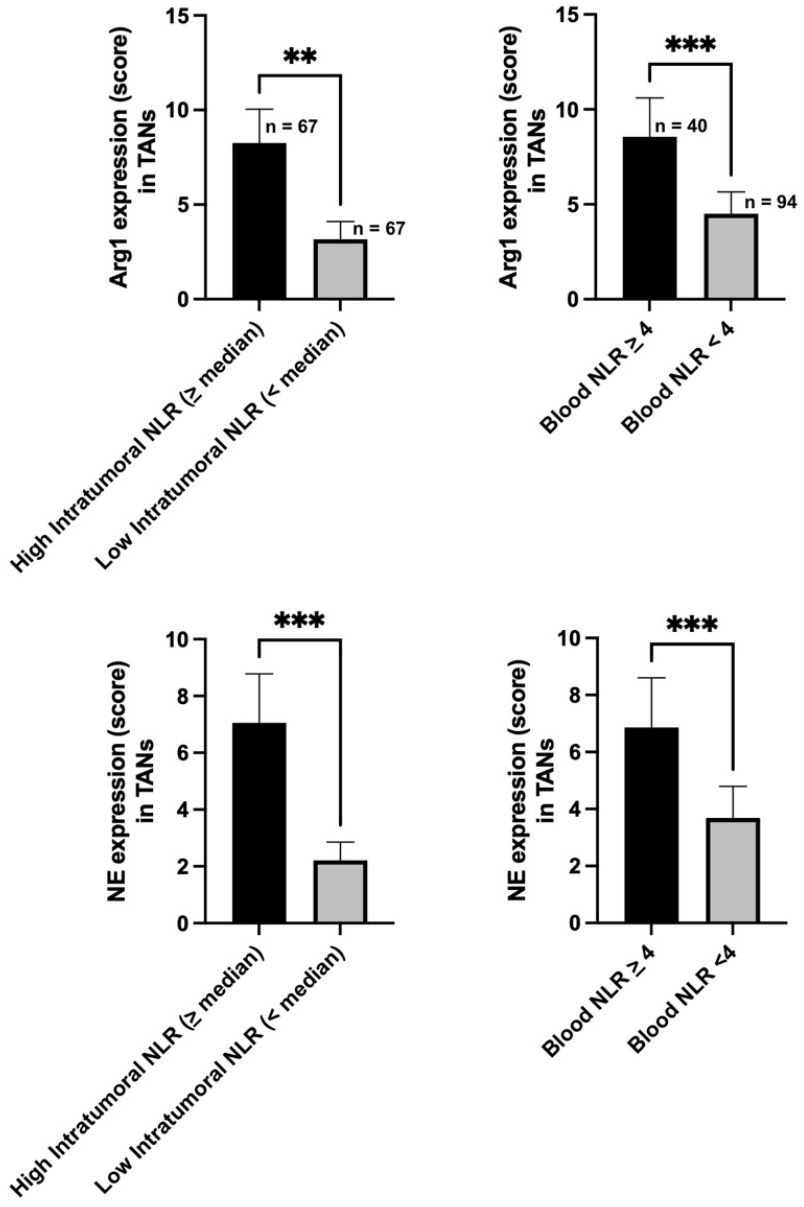

Figure 9 Association between NLR and the expression of Arg1 and NE in TANs: Elevated blood and tumor NLRs were both associated with increased expression of Arg1 and NE in TANs. Mann-Whitney test for unpaired data, mean ± SEM, ** $P < 0.01$, *** $P < 0.001$

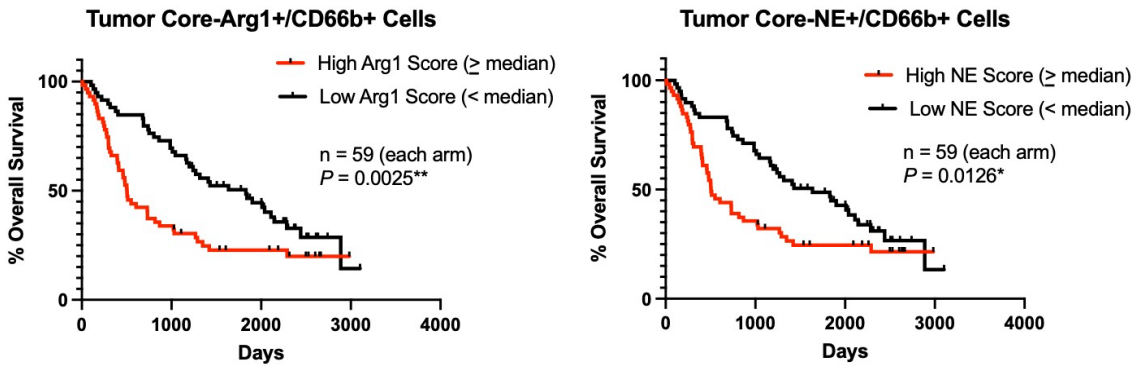

Figure 10 Association of overall survival (OS) rate in gastro-esophageal adenocarcinoma patients with the level of expression of Arg1 and NE in TANs: Kaplan-Meier overall survival (OS) showed that patients with high Arg1 and NE expressions in TANs (above median) had significantly poorer survival compared to those with low expression of Arg1 and NE in TANs. Log-rank test, $*P < 0.05$, $**P < 0.01$

2.3.5 Nodal neutrophil and NETs increased in gastro-esophageal adenocarcinoma patients, harbouring lymph node metastasis

We further looked at the number of neutrophils and NET formation in involved local lymph nodes (LN+) and uninvolved local lymph nodes (LN-) from patients with lymph node metastasis as well as local lymph nodes from patients without lymph node metastasis (LN0). NET formation was identified in IF images by colocalization of positive NE and H3Cit areas (Figure 11). Our results showed that in patients with lymph node metastasis, lymph nodes (LN+ & LN-) had a higher number of neutrophils (neutrophils/area) compared to lymph nodes from node-negative (LN0) patients (Figure 12A). We also observed an increased number of neutrophils in involved nodes (LN+) compared to uninvolved lymph nodes (LN-) in patients with nodal metastasis (Figure 12A). Interestingly, compared to lymph nodes from patients without nodal metastasis (LN0), uninvolved lymph nodes (LN-) in node-positive patients also had a significantly higher number of neutrophils (Figure 12A). NET formation was significantly elevated in lymph nodes (LN+ & LN-) of patients with nodal metastasis compared to those without nodal metastasis (LN0) (Figure 12B). NET formation was also significantly higher in LN- of patients with nodal metastasis compared to

LN0 in patients without nodal metastasis. While a trend towards greater NET formation in LN+ of patients with lymph node metastasis was observed compared to LN-, it did not reach statistical significance (Figure 12B).

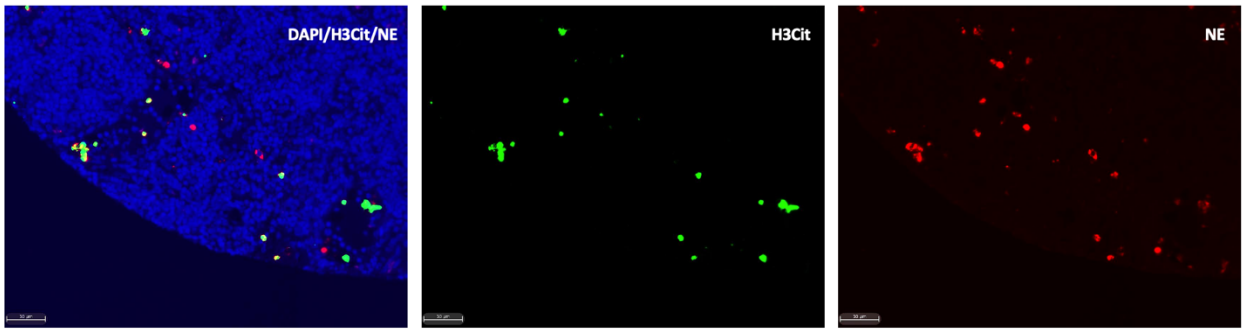

Figure 11 Immunofluorescence staining of lymph nodes for DAPI, NE, H3Cit: Colocalized NE and H3Cit positive areas were considered as NETs.

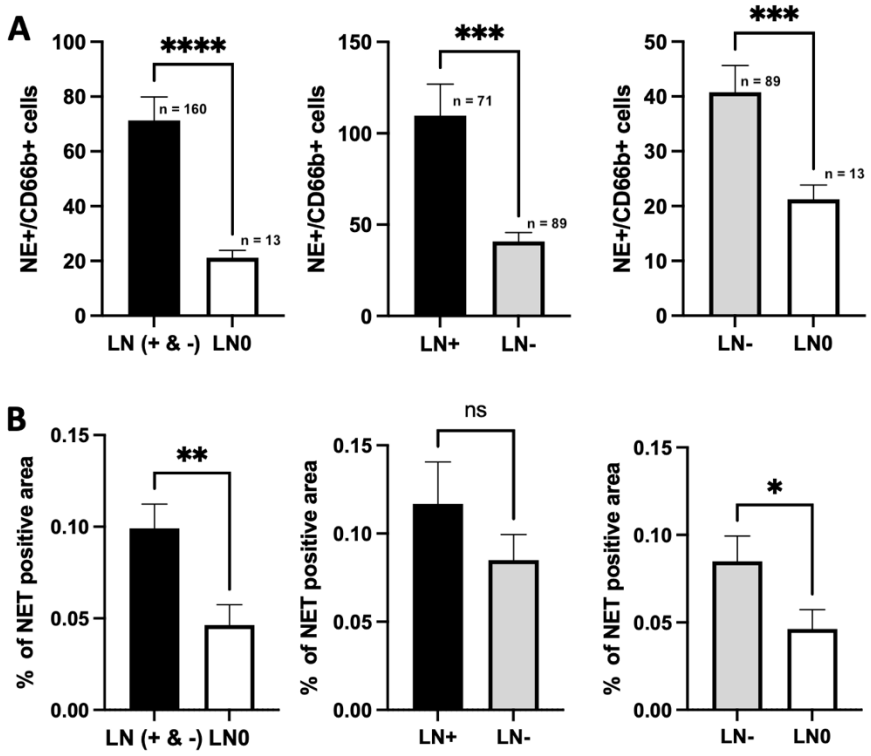

Figure 12 (A) Number of neutrophils/area and (B) % of the NET positive area in positive lymph nodes (LN+) and negative lymph nodes (LN-) of patients with lymph node metastasis and in

lymph nodes (LN0) of patients without lymph node metastasis: Both neutrophil count and NETs were higher in the lymph nodes of patients with nodal metastasis compared to those without nodal metastasis. Both neutrophils count and NETs were higher in the negative lymph nodes of patients with nodal metastasis compared to those without lymph node metastasis. Welch test for unpaired data, mean ± SEM, ns (nonsignificant), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Furthermore, we looked at the correlation of NET formation in lymph nodes with survival, demonstrated by Kaplan-Meir curves (Figure 13). Patients with high NET formation (above median) in their involved lymph nodes (LN+) had significantly lower overall survival rate (OS), $P = 0.0014$, compared to those in the low NET group. Interestingly, in patients with nodal metastasis, we also observed a weaker but positive association between NET formation in their uninvolved lymph nodes (LN-) and survival (Figure 13): patients with higher NETs in their LN- had significantly lower overall survival ($P = 0.03$) than patients with lower NETs in their LN-. Multivariable Cox regression analysis confirmed that regardless of the stage of cancer, NET formation in lymph nodes was an independent predictor of patient survival (Table 3). Based on the pathologic pN data (number of local involved lymph nodes), Fisher's test showed that patients with higher NETs (above median) in their involved lymph nodes (LN+) were significantly more likely to have a higher number of involved lymph nodes than those with lower NETs in their LN+, (Table 4).

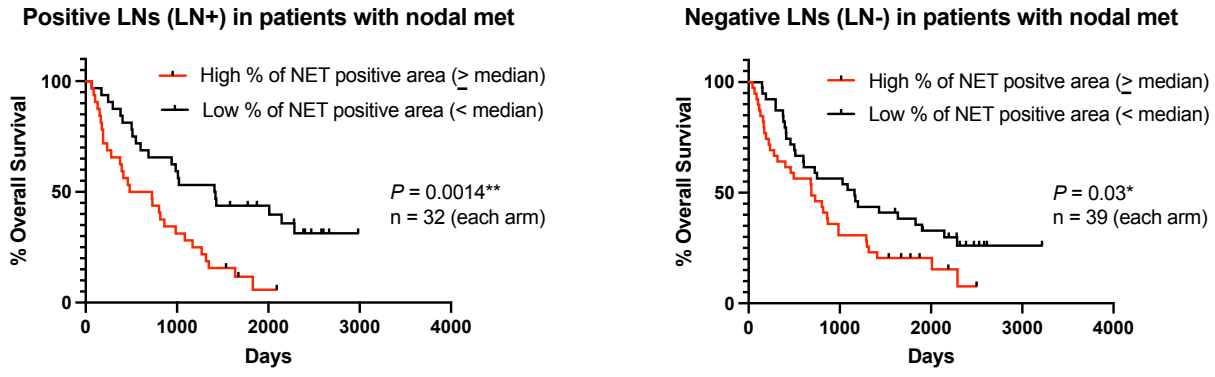

Figure 13 Kaplan Meir survival curves of lymph node-positive patients with a high and low NET formation in their positive lymph nodes (LN+) and in their negative lymph nodes (LN-):

Patients with high NETs (above median) in their LNs+ had lower overall survival (OS). In patients with LN met, high NETs in LN- was also associated with lower survival. Log-rank test, *$P < 0.05$, *$P < 0.01$

Following the above results, we then looked at the correlation of NET formation between positive (LN+) and negative (LN-) lymph nodes in patients with lymph node metastasis. As shown in figure 14, Pearson correlation demonstrated that NET formation was indeed significantly positively correlated between involved (LN+) and uninvolved (LN-) lymph nodes (r = 0.562, $P < 0.0001$).

Table 3 Univariate and multivariable Cox regression analyses of NET formation (H3Cit/NE positive area) for overall survival in cancer patients. *$P < 0.05$; **$P < 0.01$

		Univariate			Multivariable		
Variable	**N**	**HR**	**95% CI**	***P*-Value**	**HR**	**95% CI**	***P*-Value**
Positive LN - % of H3Cit/NE positive area							
< median	32	1.00	Referent	-	1.00	Referent	-
≥ median	32	2.55	1.41-4.61	**0.0019****	2.74	1.49 -5.05	**0.0011****
Negative LN (W LN met) - % of H3Cit/NE positive area							
< median	39	1.00	Referent	-	1.00	Referent	-
≥ median	39	1.70	1.02-2.83	**0.041***	1.58	0.94-2.65	0.08
Pathologic Stage							
2	12	1.00	Referent	-	1.00	Referent	
3 & 4	51	3.22	1.36-7.64	**0.0078****	3.55	1.48-8.49	**0.0043****

Table 4 Patients with higher a NET formation (≥ median) in their LNs+ had a significantly greater frequency of pN3 than those with a lower NET formation in their LNs+ (60.61% v.s. 31.43%). Fisher's test, * $P < 0.05$

	Positive Lymph Nodes-H3Cit/NE - % of positive area		
Variable	***High ≥ median*** ***n (%)***	***Low < median*** ***n (%)***	***P-value*** ***(Fisher's test)***
Pat. Stage, 3 & 4	28 (82.35%)	27 (77.14%)	0.766
pN, 3	**20 (60.61%)**	**11 (31.43%)**	**0.0276***
Recurrence, yes	22 (66.67%)	15 (41.67%)	0.0535

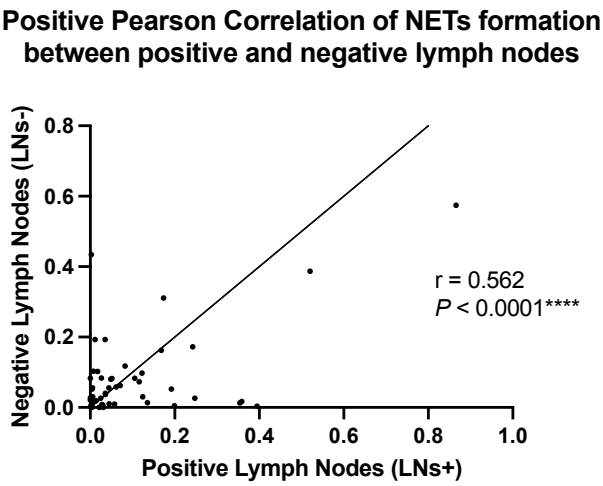

Figure 14 Positive Pearson correlation of NET formation between LN+ and LN-: percentage of the NET positive area in positive (LN+) and negative (LN-) lymph nodes was significantly positively correlated (Pearson correlation) in patients with nodal metastasis.

When we looked at the correlation of the number of nodal neutrophils, in LN+, LN-, LN0, with survival in cancer patients, our results did not show a significant difference of survival between high nodal neutrophil and low nodal neutrophil groups (data was not shown). We also investigated the association of NET formation in tumor with survival, and our results did not demonstrate a significant difference in survival between high intratumoral NETs and low intratumoral NETs groups (data was not shown).

2.4 Discussion

2.4.1 High blood and tumor NLRs in cancer patients are associated with the tumor-promoting neutrophil phenotype

With respect to circulating neutrophils, we demonstrated an expansion of the LDN fraction in patients with an elevated circulating NLR. Through the mediation of G-CSF, neutrophils are differentiated and maturated from hematopoietic stem cells (HSC) and stored in great numbers in the bone marrow [19-21]. Under normal hemostasis, only 1 to 2% of neutrophils are released from the bone marrow into circulation [22]. However, under stress conditions such as cancer, the

expression of cancer-associated inflammatory cytokines, most notably G-CSF, is increased that forces a rapid production and release of neutrophils into the bloodstream [23]. It was shown that cancer-cell-derived G-CSF is necessary but not sufficient to mobilize immature LDNs into the circulation, and tumor-derived factors blocking neutrophil differentiation also need to be involved in this process [15]. The continuous and rapid release of neutrophils from the bone marrow can lead to the circulation of many immature neutrophils in the bloodstream, increasing both NLR and the percentage of immature LDNs in the bloodstream. LDNs are heterogenous and consist of both mature and immature neutrophils [15, 24, 25]. Immature LDNs are postulated to comprise granulocytic myeloid-derived suppressor cells (G-MDSC) owing to their ability to suppress T cell proliferation and to skew the immune response towards tumor-promoting T helper type 2 (Th2) phenotype [24, 26]. One of the main mechanisms by which G-MDSC exert their immunosuppressive activity is through the depletion of arginine by expression of Arg1 [26]. Our results also demonstrated higher expression of Arg1 in the LDN population compared to HDNs. We also demonstrated that as the LDN fraction expands, so too does the relative proportion of immature LDNs, characterized in this study by $CD16^{low}/CD66b^{high}/CXCR2^{low}/CD62L^{low}$ expression. It is important to note that LDNs are ultimately defined by their functional capabilities, particularly with respect to T cell suppression. A consistent protein expression profile characteristic has yet to be clearly defined for LDNs. The observation that expansion of the LDN fraction carries adverse oncologic outcomes has been previously described. For example, Shaul et al demonstrated the expansion of circulating LDN fraction, characterized by $CD66b+/CD10^{low}/CXCR4+/PDL1^{inter}$, in patients with advanced NSCLC [27]. Kumagai et al demonstrate that LDNs induced by surgical stress after abdominal surgery can *in vitro* suppress T cell proliferation [28]. Furthermore, these cells were primed to elaborate NETs, which themselves were able to trap circulating gastric cancer cells and induce their proliferation *in vitro*. When taken collectively, these observations suggest that the expansion of the LDN fraction observed in our study in patients with elevated blood NLR may represent a skewed innate response which favours tumor progression and metastasis.

We showed that patients with high tumor NLR exhibited higher mortality, evidenced by lower OS and DSF. Other studies also reported that both circulating and tumor NLR exhibited a superior

prognostic significance respectively than blood neutrophil counts and the number of TANs [29]. More specifically, we showed that NLR in the tumor core (intratumoral NLR), but not NLR in the tumor periphery, predicts unfavorable survival in cancer patients. Too many neutrophils in tumor periphery may encapsulate tumor and fill space into which new tumor cells can diffuse and invade surrounding tissues, inhibiting tumor growth [30]. Although most studies demonstrated the association of high circulating and tumor NLR with lower survival rate, the relationship between NLR and oncological outcomes is inconsistent in some studies [3, 31, 32]. For example, in colorectal cancer, tumor-infiltrating neutrophils in patients with early-stage cancer were found to be a marker of good prognosis. Similarly, Zhang et al demonstrated a positive correlation between the degree of tumor infiltration by neutrophils in gastric cancer and improved survival when patients were treated with 5-FU based chemotherapy [33]. Finally, Rakaee et al demonstrated divergent prognostic implications to TANs in patients with NSCLC according to histologic subtype [34]. In patients with adenocarcinoma, TANs were associated with adverse survival outcomes [35]. Conversely, in patients with squamous cell carcinoma, TANs were positively associated with survival [36]. When taken collectively, the bulk of data, including our study, demonstrates the implications of TANs in poor oncological outcomes. TANs promote both lymphangiogenesis and angiogenesis, facilitating the spreading of cancer cells to distant tissues/organs [37, 38]. More importantly, it has been shown that TANs in the primary tumor might spread through lymphatic vessels, travel to lymph nodes, and become involved in cancer-related lymphangiogenesis, increasing the risk of lymph node metastasis [39]. It should also be noted that increased NLR could also be an indication of worsening cancer-related complications and patient's well-being, including obstruction, dysphasia, bleeding, and in general, deteriorating physiological conditions related to cancer. Such patients may have a greater mortality rate than patients without these severe complications.

In this study, we also demonstrated a positive association of both blood and tumor NLR with increased expression of Arg1 and NE in TANs. Exocytosis of granules from TANs results in the release of antimicrobial proteins such as Arg1 and NE into the tumor microenvironment. NE increases the activation of the AKT Kinase pathway, which promotes cancer cell proliferation and metastasis by increasing cancer cell extravasation through endothelium [40]. TANs express high

levels of Arg1, which is known to suppress the proliferation of $CD8^+$ T cells by conversing L-arginase to urea [41]. L-arginine modulates CD8+ T cell metabolism and enhances their survival/proliferation and anti-tumor activity. Therefore, through L-arginine depletion in the environment, degranulation of neutrophils followed by exocytosis of Arg 1 leads to suppression of anti-tumor immunity. Degranulation and exocytosis of NE and Arg1 from TANs in patients with high blood NLR and tumor NLR may explain the implication of NLR in poor oncologic outcomes. It should also be noted that in this study, we demonstrated that blood NLR and tumor NLR are positively correlated.

2.4.2 Nodal neutrophil and NETosis (NET formation) enhance premetastatic promotor niche in lymph nodes

Our data showed that the number of neutrophils and NET formation increased in the lymph nodes of patients with lymphatic metastasis, and increased NET formation in lymph nodes was associated with poorer survival. This observation was not restricted to involved nodes specifically; even negative nodes in node-positive patients showed elevated neutrophils and NETs compared to patients without nodal metastasis. The contribution of neutrophils and NETs to the formation of the pre-metastatic niche has been gaining growing attention [42, 43]. For example, Abbate et al showed that in patients with oral SCC (squamous cell carcinoma), the presence of neutrophils in lymph nodes was an independent predictor of lymph node metastasis [44]. Similarly, Hiramatsu et al demonstrated that an elevated tumor NLR was associated with increased NLR within tumor-draining lymph nodes in gastric cancer patients [39]. High NLR was associated with adverse oncologic outcomes and lymph node metastasis, but as we showed in this study, neutrophil infiltration was present in all tumor-draining lymph nodes regardless of involvement. Tohme et al demonstrated the implication of NETs in the development of liver metastasis in cancer patients and proposed elimination of NETs may reduce risks of cancer reoccurrence [45]. Park et al reported that NETs promoted tumor cell migration and invasion to lungs in a breast cancer mouse model and the treatment of mice with NET-digesting DNase I-coated nanoparticles inhibited lung metastasis [46]. NETs are networks of extracellular

neutrophil DNA fibers that are capable of binding to tumor cells arriving at the secondary organs and thereby promote tumor migration and invasion in secondary organs [42, 43, 45, 47-49]. Importantly in our study, we showed that even in uninvolved lymph nodes in patients with nodal metastasis, which means in pre-metastatic lymph nodes, neutrophil number and NET formation were both higher than in lymph nodes of patients without nodal metastasis. This postulates that neutrophil trafficking followed by NETosis in pre-metastatic lymph nodes makes lymph nodes more receptive towards incoming primary tumor cells, and therefore, NETosis promotes the pre-metastatic niche in lymph nodes. The concept of the pre-metastatic niche was introduced by Kaplan et al [50] and was suggested that factors derived from the primary tumor make tissues in the secondary organs to provide more favorable conditions for tumor growth and eventual metastasis.

When taken together, our results suggest that NET formation in lymph nodes may precede the development of metastasis and that nodal NET formation is at the very least a permissive, if not an overt facilitator, of metastasis. Tumor-draining lymph nodes have been shown to go through a process involving increased lymph-angiogenesis and architectural changes that support tumor implantation and growth in lymph nodes [51]. Accordingly, it is reasonable to postulate that our results point to the implication of NETs in this process in human gastro-esophageal adenocarcinoma patients.

One limitation of this study arises from the fact that most patients were treated with neoadjuvant chemotherapy. Any preoperative treatment may influence the number and phenotype of immune cells in the tumor microenvironment. However, most patients with gastro-esophageal adenocarcinoma require neoadjuvant treatment as the current standard of care, and thus fewer untreated surgical specimens exist. Furthermore, it has been shown that circulating NLR remains of prognostic significance both prior to and after the administration of neoadjuvant therapy, suggesting that markers of cancer-associated inflammation remain relevant regardless of therapeutic approach [52, 53].

2.5 Conclusion

We demonstrated the expansion of the pro-tumor phenotype of neutrophils (LDNs) in the circulation of patients with an elevated blood NLR. High NLR in the tumor core and not in the periphery was specifically and independently associated with poorer survival outcomes. More importantly, elevated blood and intratumoral NLRs were positively correlated, and both were associated with the tumor-promoting phenotype of TANs, characterized by increased expression of Arg1 and NE in the tumor core. This study highlights the contextual importance of high NLR within the tumor core as potential facilitators of tumor progression. This study also demonstrated that NET formation in lymph nodes is potentially involved in lymph node metastasis and acts as a pre-metastatic promoter niche in lymph nodes.

2.6 References

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In chapter 2, we looked at the correlation between NLR, as a predictor of poorer oncological outcomes, and neutrophil phenotype. We demonstrated that high peripheral blood and tumor NLRs were associated with the tumor-promoting phenotype of neutrophils. Chapter 2 suggests that the link between high NLR and poorer patient outcomes is not only due to the higher prevalence of neutrophils over lymphocytes, and elevated NLR is indeed associated with neutrophils that promote tumor growth and metastasis. In chapter 3, we went one step further and looked at how the tumor microenvironment affects neutrophil phenotype in cancer patients. To do so, we compared phenotypic characteristics of TANs with those of neutrophils in bone marrow, peripheral blood, and more importantly, neutrophils in non-diseased tissue. In chapter 3, the phenotypic characteristics of neutrophils were determined by immunophenotyping of neutrophils using 12 different biomarkers.

Chapter 3 Determine the influence of tumor microenvironment on neutrophil phenotype

3.1 Introduction

A large proportion of immune cells infiltrated into the tumor microenvironment are neutrophils and TANs play important roles in tumor development and influence the adaptive immune response to tumors [1-7]. There is a growing number of evidence suggesting that TANs possess a wide functional diversity and plasticity in phenotypical characteristics [8-12]. TANs can be part of tumor-promoting inflammation by increasing angiogenesis in the tumor site, promoting extracellular matrix remodeling in the tumor, elevating tumor cell proliferation, providing pre-metastatic niche via NETosis in secondary organs, and suppressing anti-tumor adaptive immunity. Conversely, there are also tumor-inhibiting subsets of TANs that can mediate anti-tumor responses by direct recognizing and killing of tumor cells and by participating in and orchestrating anti-tumor immunity response.

TANs exhibit anti-tumor activity by releasing reactive oxygen species (ROS) to kill tumor cells and/or by stimulating T cells response to tumor cells [7, 13]. In fact, TANs can directly promote the recruitment and activity of CD8+ T cytotoxic cells, which are responsible for destroying cancer cells, by producing T-cell attracting chemokines (e.g., CCL-3, CXCL9, and CXCL10) and pro-inflammatory cytokines (e.g., IL-12, TNF-α, and GM-CSF) [7, 13]. TANs can indirectly stimulate CD8+ T activities by activating dendritic cells (antigen-presenting cells), via cell-cell contact and through secretion of TNF-α [14]. It has also been shown that TANs can themselves acquire the function of antigen-presenting cells to directly activate CD8+ T cells [14, 15]. On the other hand, the pro-tumor activities of TANs are supported by the degranulation of TANs and the release of antimicrobial enzymes such as NE and Arg1 into the tumor microenvironment. Extracellular NE promotes cancer cell proliferation and metastasis by increasing cancer cell extravasation through

endothelium [16]. Pro-tumor TANs express high levels of arginase, and exocytosis of arginase is known to suppress the proliferation of CD8+ T-cells by conversing L-arginase to urea [17].

The diversity and plasticity of TANs underline their dual roles from pro-tumor to anti-tumor activities. Factors influencing the plasticity of TANs to switch between pro-tumor and anti-tumor phenotypes are complex and not yet fully understood. It is not yet clear how the tumor microenvironment in humans influences neutrophils to acquire certain phenotypes and functions. To address this question, in this study, we investigated how phenotypic characteristics of neutrophils change in patients with esophageal adenocarcinoma. We looked at neutrophil phenotype when cells migrate from the bone marrow to the peripheral blood and then to tumor site and how neutrophil phenotype differs between TANs and those infiltrated in non-diseased tissues. In this study, the phenotypic characteristic of neutrophils was established based on neutrophil's surface and intracellular protein expression profile. We selected a panel of 12 neutrophil biomarkers that were reported in the literature to be involved in cancer progression, and we determined their expression in neutrophils using the multi-colour flow cytometry method. In each patient, the protein expression profile of neutrophils is compared between neutrophils isolated from tumor, healthy (non-diseased) tissue, blood, and bone marrow. Neutrophil protein expression profile alters when cells transmigrate from the bone marrow to bloodstream and then infiltrate into tissues. To account for the changes in protein expression of neutrophils due to the transmigration and determine the effects of tumor microenvironment on neutrophil phenotypes, we compared the protein expression profile of TANs with that of neutrophils from healthy tissues. We furthermore investigated the influence of tumor cells on neutrophil protein expression profile in an *in vitro* study using cancer cell-conditioned medium (CM). Our study is advantageous compared to past works in this field as we studied neutrophils in human tissues obtained from cancer patients. To date, data on functions and phenotypes of TANs have mostly been obtained in mice cancer models using aggressive cancer cell lines. However, in terms of many aspects of neutrophil biology, from its migration steps to its effector functions, there are several differences between mouse and human neutrophils [15].

3.2 Methods

3.2.1 Patients

In total 16 patients with clinically confirmed esophageal adenosarcoma who underwent resection surgery at the Montreal General Hospital between March 2019 and January 2020 were selected in this study. Surgical samples, including bone marrow, peripheral blood, esophagus tumor, and distant non-diseased esophagus, at least 5 cm away from the tumor, were collected from patients. The demographic and clinico-pathologic data of these patients are outlined in Figure 1.

3.2.2 Preparation of neutrophil suspensions from four body compartments: bone marrow, peripheral blood, esophageal tumor, non-diseased esophagus

Freshly operated human esophageal tumor and non-diseased esophagus tissues (distant tissue), circulating blood, and bone marrow samples were collected from the same patient on the surgery day (Figure 1). To prepare cell suspension from tissue samples, tumor and non-diseased tissues were sliced into small pieces (1-2 mm^2) using dissecting scissors and then mechanically and enzymatically dissociated using gentleMACS^{TM} Octo Dissociator (Miltenyi Biotec) following the manufacturer protocol. Cell suspensions were then washed in RPMI medium and passed through a 70 µm nylon cell strainer to remove large remaining pieces. For blood samples, red blood cells were first lysed by hypotonic lysing (Invitrogen), washed in PBS, and then cell suspension went through the same dissociation process as tissues to account for the effects of dissociation enzymes on neutrophil surface epitopes. To obtain the patient's bone marrow, a small piece of bone rib sample was collected from the operation. The rib bone was placed in a 15 ml tube and centrifuged at 450 x g for 2 minutes to spin down the bone marrow. The bone was then removed, and the sample was flushed few times in lysing solution using an 18G needle to break down the bone marrow chunks. After lysing, the cells were washed in PBS, pass through a 70 µm strainer

to remove any small pieces of bone, and then the cells went through the same dissociation process as explained above.

3.2.3 Neutrophil immunophenotyping using multicolor flow cytometry

Sample preparation, flow cytometry staining/method, gating neutrophils, and biomarkers used for neutrophil immunophenotyping were described in the previous chapter. Using FlowJo software, tSNE (t-distributed Stochastic Neighbor Embedding) and UMAP (Uniform Manifold Approximation and Projection) dimensionality reduction and clustering techniques were employed on gated the neutrophil population to visualize clusters of neutrophils with close biomarker expression. t-SNE heatmaps showing fluorescence intensity of each biomarker at the single-cell level for the neutrophil population were prepared using the FlowJo program. At least 10,000 events (neutrophils) were randomly selected to prepare these heatmaps.

Patients with esophageal adenocarcenoma (n = 16)	
Mean age, years	64.18
Sex, male	81.25%
Neoadjuvant Treatment, yes	93.75%
Clinical Stage 1	0.00%
Clinical Stage 2	6.25%
Clinical Stage 3	68.75%
Clinical Stage 4	25.00%
Clinical T1	0.00%
Clinical T2	6.25%
Clinical T3 & 4	93.75%
Clinical N, yes	81.25%
Pathologic Stage 1	12.50%
Pathologic Stage 2	6.25%
Pathologic Stage 3	31.25%
Pathologic Stage 4	50.00%
Pathologic T1	12.50%
Pathologic T2	12.50%
Pathologic T3 & 4	75.00%
Pathologic N0	18.75%
Pathologic N1	31.25%
Pathologic N2	25.00%
Pathologic N3	25.00%
Grade, poor	43.75%
Grade, moderate	50.00%
Grade, well	6.25%
Lymphovascular Invasion, yes	81.25%

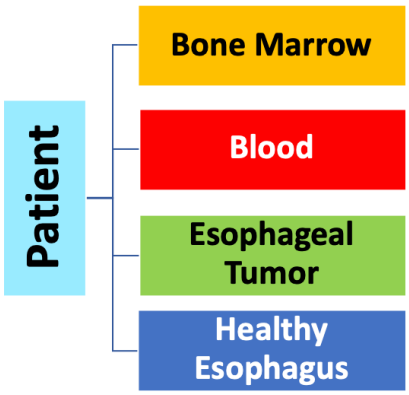

Figure 1 Demographic and clinico-pathologic data of patients: from each patient, neutrophils were isolated from bone marrow, peripheral blood, esophageal tumor, and healthy distant esophagus.

3.2.4 Neutrophils treated in cancer cell-conditioned media (CM): an *in vitro assay*

To investigate the *in vitro* effects of tumor cells on the neutrophil phenotypic characteriscs, peripheral blood neutrophils (PBNs) isolated from healthy volunteers were treated with different cancer cell-conditioned media (CM) obtained from four different esophageal cancer cell lines.

PBNs isolation: We collected 10 ml peripheral blood from volunteers in two heparin-coated tubes (each contained 5 ml of blood). 5 ml of PBS was added to each tube of blood and using an 18 G needle mounted on a syringe the diluted blood was layered on top of 10 ml Lymphocyte Separation Media (LSM) in a 50 ml tube at room temperature. The tube was then centrifuged at 800 x g for 30 min at room temperature without break. The top two layers containing leukocytes and LSM were then carefully removed, and the RBCs and neutrophils at the bottom were diluted in 20 ml of PBS and 20 ml of 6% Dextran solution, gently mixed, and kept at room temperature for 30 min to allow RBCs to sediment. We then transferred the neutrophil-rich supernatant into a fresh tube and centrifuged at 450 x g at 4 °C for 5 min to collect the pellet containing mostly neutrophils and few RBCs. The remaining RBCs were then lysed (Invitrogen) followed by washing neutrophils in PBS and resuspending cells in fresh PBS.

Preparation of cancer cell-conditioned media (CM): Four esophageal cancer cell lines, OE19, OE33, ESO26, FLO-1, purchased from Sigma Canada, used in this project. OE19, OE33, OE26 cells were cultured in T75 flasks (37°C, 5% CO_2) using RPMI medium, supplemented with 1% glutamine, 10% FBS, and 1% penicillin-streptomycin (100 u/ml) antibiotic solution. FLO-1 cells were cultured in a DMEM medium instead of RPMI. At 70% confluency, the medium was removed, cells were washed with warmed PBS twice and incubated again in a serum-free medium. Following a 24-hour incubation, CM was collected, centrifuged at 750 x g for 10 mins to remove debris and unattached cells and then stored at -80 °C for further use.

CM-PBN treatment: 1 million freshly isolated PBNs from healthy volunteers were incubated in 1 ml of each above CM (OE19, OE33, ESO26, FLO-1) and incubated for 2 hours at 37°C. CM-treaded PBNs were then washed in PBS and stained for flow cytometry as described before. PBN treated in RPMI medium alone was used as control.

3.2.5 Statistical analysis

Mann–Whitney test was used for unpaired data to compare the mean values of two continuous variables. Wilcoxon test was used for paired data to compare the mean values of two continuous variables. ANOVA with Tukey post-hoc test was used for paired data to compare the mean values of more than two groups. Friedman t-test was used for paired data to compare multiple variables to a control group. For all statistical tests, a *p*-value below 0.05 was considered statistically significant. Statistical analyses were performed using the Prism-Graphpad program.

3.3 Results

3.3.1 Tumor-associated neutrophils (TANs) exhibited distinguished expression of biomarkers compared to neutrophils isolated from other body compartments

Figure 2 shows UMAP dimensionality reduction visualization of neutrophils isolated from different body compartments (bone marrow, peripheral blood, healthy (non-diseased) esophagus, and esophageal tumor) in 3 different patients. Neutrophils isolated from the same body compartment tended to cluster together in UMAP visualization, which means neutrophils isolated from the same body compartment had more similar expressions of biomarkers. The cluster of neutrophils from healthy esophagus was located closely next to or superposed on the cluster of neutrophils from esophageal tumor (TANs), indicating that the expression of biomarkers in neutrophils isolated from healthy tissue and that in TANs were more alike than those isolated from the bone marrow (BM) and blood. The cluster of BMNs was further apart

from the clusters of neutrophils isolated from other body compartments, demonstrating that neutrophils from BM had the most distinct expression of biomarkers.

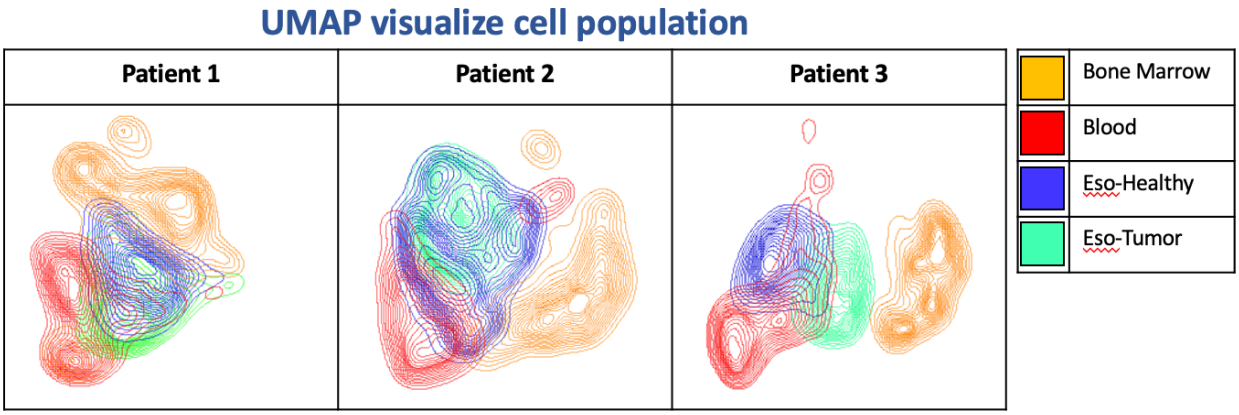

Figure 2 UMAP dimensionality reduction visualization of neutrophils isolated from body compartments (sources) of three different patients: neutrophils isolated from the same source had more similar expressions of biomarkers. The expression of biomarkers in TANs was more similar to that of neutrophils in the healthy esophagus.

We then generated the t-SNE heatmaps showing the fluorescence intensity of each biomarker at the single-cell level. Figure 3 shows the fluorescence intensity heatmaps of all biomarkers, comparing neutrophils isolated from all four body compartments in a cancer patient, and figure 4 compares those in TANs and in healthy tissue-infiltrated neutrophils for another patient. As figure 3 shows, neutrophil isolated from the same body compartment clustered together in both t-SNE (figure 3A) and UMAP (figure 3B) visualizations. Figure 4A and 4B also show that TANs and neutrophils from healthy esophagus formed separated populations in t-SNE and UMAP graphs, indicating that the expression of biomarkers in TANs differs from that in tissue-infiltrated neutrophils. The heatmap presented in figure 3C shows that in comparison between TANs and PBNs, TANs exhibited $CD66b^{high}$ and $CD11b^{high}$, indicating higher activation of TANs than PBNs. Moreover, CD16, a marker for neutrophil's maturity and activation, was lower and more heterogenous in TANs, confirming that TANs were activated compared to PBNs. Interestingly, the expression of CXCR2 decreased in TANs, which could be due to the ligation of this receptor in the

tumor site. Expression of CD62-L was also decreased in TANs, CD62L shed during neutrophil transmigration.

Given, some of the changes in the expression of these biomarkers occur due to the transmigration of neutrophils from circulation to tissue, and it is not indeed related to the presence of tumor, we also compared the expressions of biomarkers in TANs with those of neutrophils in the healthy esophagus (Figure 4). As these heatmaps show, the expression of these biomarkers was different between TANs and tissue-infiltrated neutrophils, where TANs exhibited $CD66b^{high}$, $CD54^{high}$, $CD62L^{low}$, $CXCR2^{low}$, and $Arg1^{low}$ (Figure 4C). It should be noted that we did internal (cytoplasmic) flow cytometry staining for Arg1 and NE; therefore, lower expression of Arg1 and NE could indicate the degranulation of neutrophils and exocytosis of Arg1 and NE.

Although the t-SNE heatmaps give us valuable information about the expression of biomarkers in neutrophils, these heatmaps are generated only for the individual patient, and to obtain statistical analysis; we need to combine data from all patients. We therefore gated on the positive neutrophil population for each biomarker and presented the level expression of each biomarker for all patients in the form of graphs (Figure 5 to 9). In these figures, we also presented the expression of biomarkers in cancer cell-conditioned medium (CM)-treated PBNs, isolated from healthy volunteers, and compared them to untreated PBNs.

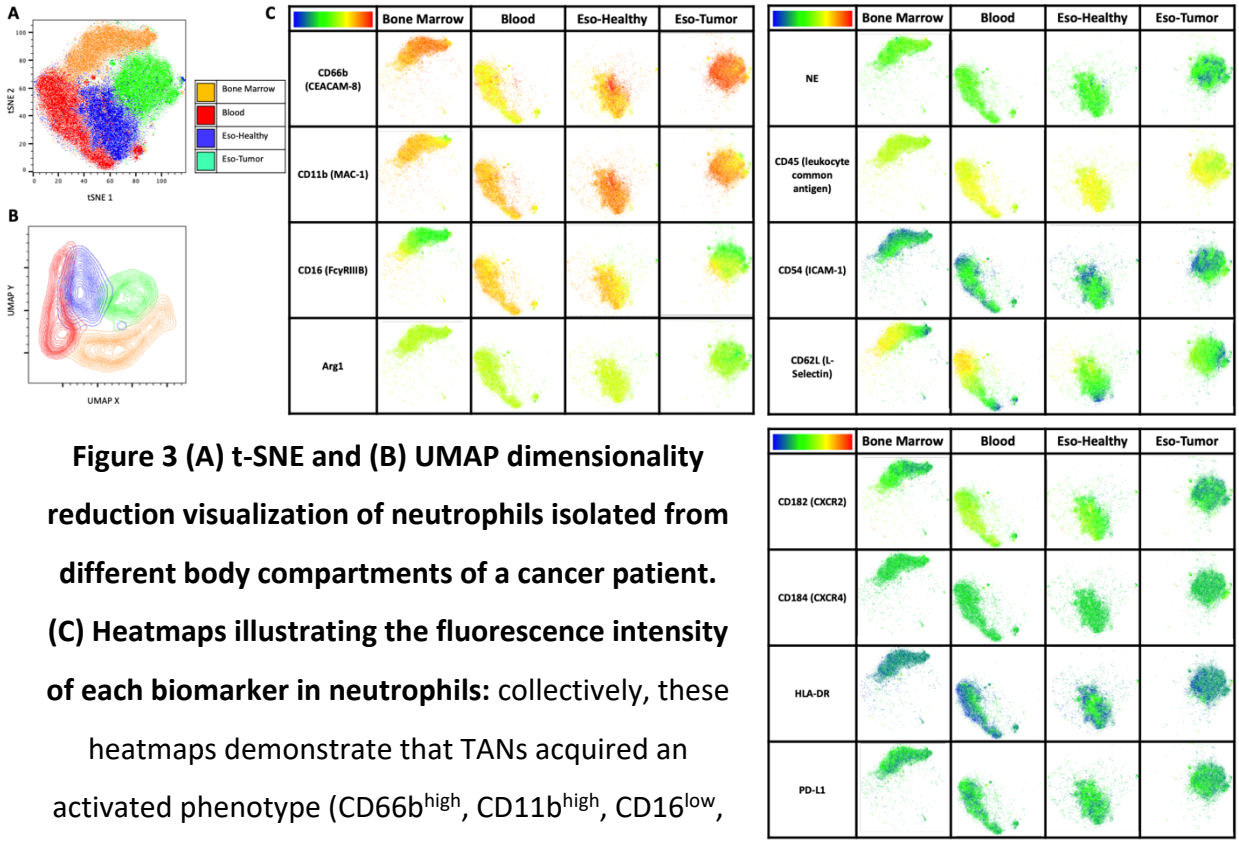

Figure 3 (A) t-SNE and (B) UMAP dimensionality reduction visualization of neutrophils isolated from different body compartments of a cancer patient. (C) Heatmaps illustrating the fluorescence intensity of each biomarker in neutrophils: collectively, these heatmaps demonstrate that TANs acquired an activated phenotype (CD66b^{high}, CD11b^{high}, CD16^{low}, CD54^{high}, CD62L^{low}, CXCR2^{low}). We also observed Arg1^{low} and NE^{low} in TANs, which could be due to the degranulation of TANs.

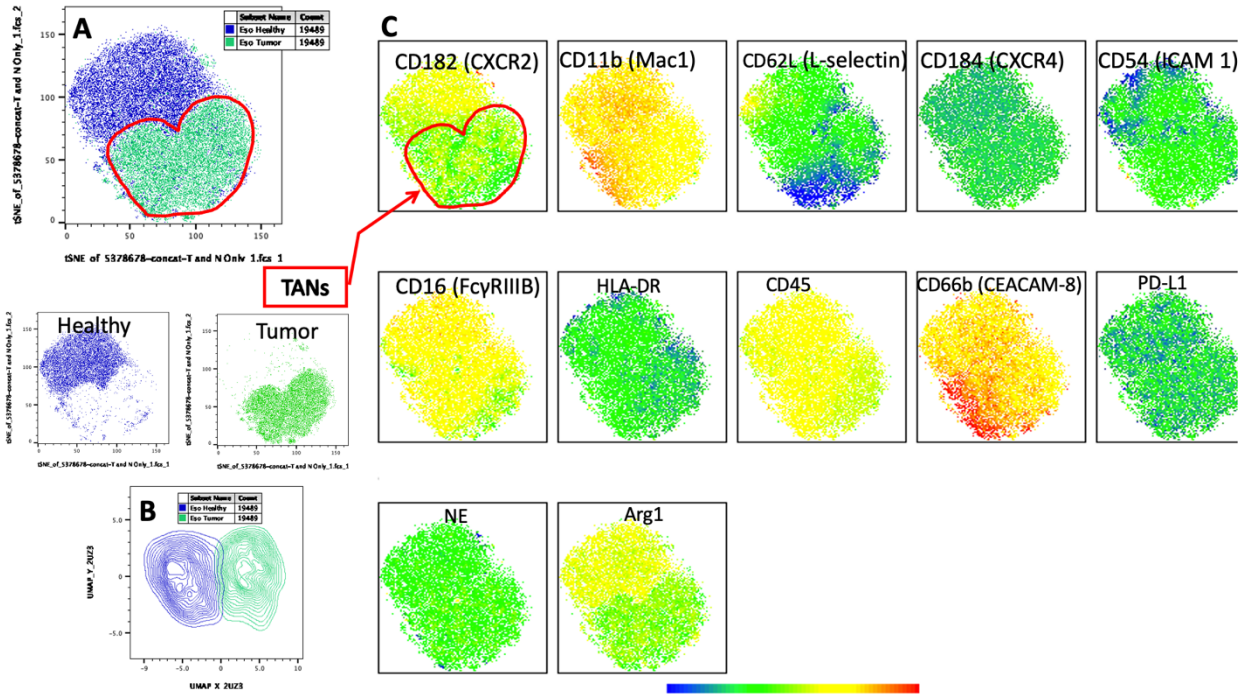

Figure 4 (A) t-SNE and (B) UMAP (B) dimensionality reduction visualization of neutrophils isolated from esophageal tumor and healthy (non-diseased) esophagus. (C) Heatmaps illustrating the fluorescence intensity of each biomarker in neutrophils: these heatmaps demonstrate that compared to the tissue-infiltrated neutrophils, TANs exhibited $CD66b^{high}$, $CD54^{high}$, $CD62L^{low}$, $CXCR2^{low}$, and $Arg1^{low}$

3.3.2 TANs acquired an activated phenotype

Compared to PBNs, TANs acquired an activated phenotype evidenced by increased CD66b and CD11b expression, a higher number of CD54+ neutrophils, and a lower number of CD62L+ neutrophils (Figure 5A). Increased expression of adhesion molecules, CD66b (CEACAM-8), CD11b (MAC-1), CD54 (ICAM-1), and shedding of CD62L (L-selectin) are the hallmarks of neutrophil activation [16-18]. Compared to tissue-infiltrated neutrophils, TANs were also more activated as the expression of CD66 increased, the number of CD54+ cells increased, and the number of CD62L+ cells decreased (Figure 5B). Higher activation of TANs than healthy tissue-infiltrated neutrophils promote neutrophil mobilization to and accumulation in the tumor site. We also

observed increased neutrophil activation by incubation of PBNs from healthy volunteers in different cancer cell-conditioned media (CM), evidenced by increased expression of CD66b, CD11b, and CD54 (Figure 5C). Expression of CD62L did not change in CM-treated PBNs; it should be noted that CD62L is mostly shed during neutrophil extravasation in the body [19]. The surface receptor CD16 (FCγRIIIB) is a marker for neutrophil maturation and its expression increases by neutrophil maturation and sheds by aging, activation, and apoptosis [20, 21]. As expected, the expression of CD16 substantially increased from BM to blood (Figure 5A), as PBNs were more mature compared to immature neutrophils stored in BM. CD16 expression was decreased in TANs, which could be due to the higher activation of TANs as well as delayed apoptosis of TANs compared to PBNs and healthy tissue-infiltered neutrophils (Figure 5A and B). Interestingly CM-treated PBNs showed higher CD16 expression compared to untreated PBNs. Cytokines released from cancer cells in CM can activate neutrophils and delay apoptosis, maintaining CD16 expression in treated PBNs than untreated PBNs [20, 21].

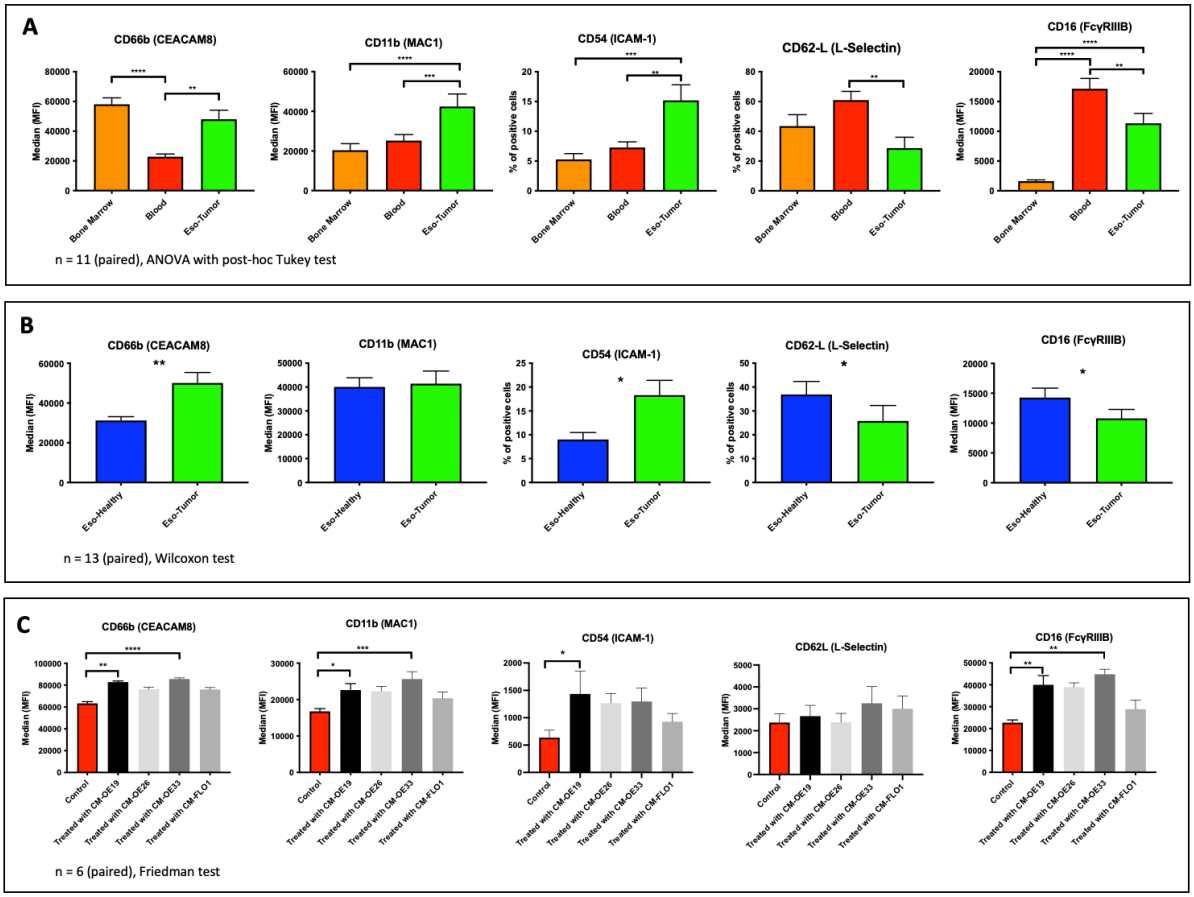

Figure 5 (A) Expression of CD66b, CD11b, CD54, CD62L, and CD16 in BMNs, PBNs, TANs, (B) in TANs and tissue infiltrated neutrophils, and (C) in *in vitro* CM-treated PBNs and untreated (control) PBNs: TANs acquired activated phenotype evidenced by increased CD66b and CD11b expression, higher number of CD54+ and lower number of CD62L+ neutrophils. Data presented mean ± SEM, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

3.3.3 Degranulation in TANs

Figures 6A and B show that the expression of NE decreased in TANs compared to PBNs and the expression of Arg1 was also lower (*P* = 0.056, not significant) in TANs compared to healthy tissue-infiltrated neutrophils. NE and Arg1 granule enzymes are stored in granules of neutrophils, and upon neutrophil activation, the degranulation of neutrophils leads to the fusion of these enzymes to the cell membrane and their release into extracellular space (exocytosis) [22]. Reduced expression of these enzymes, measured by flow cytometry using internal (cytoplasmic) staining, could indeed indicate the degranulation in TANs. We observed an increase, not decrease, in Arg1 expression in CM-treated neutrophils (Figure 6C), but more importantly, in IF images of the tumor and healthy esophagus tissues, which also consider extracellular and membrane Arg1 and NE, we obtained higher Arg1 and NE positive areas in tumor tissue compared to healthy tissue (Figure 6D). Collectively these data support the assumption of increased degranulation in TANs.

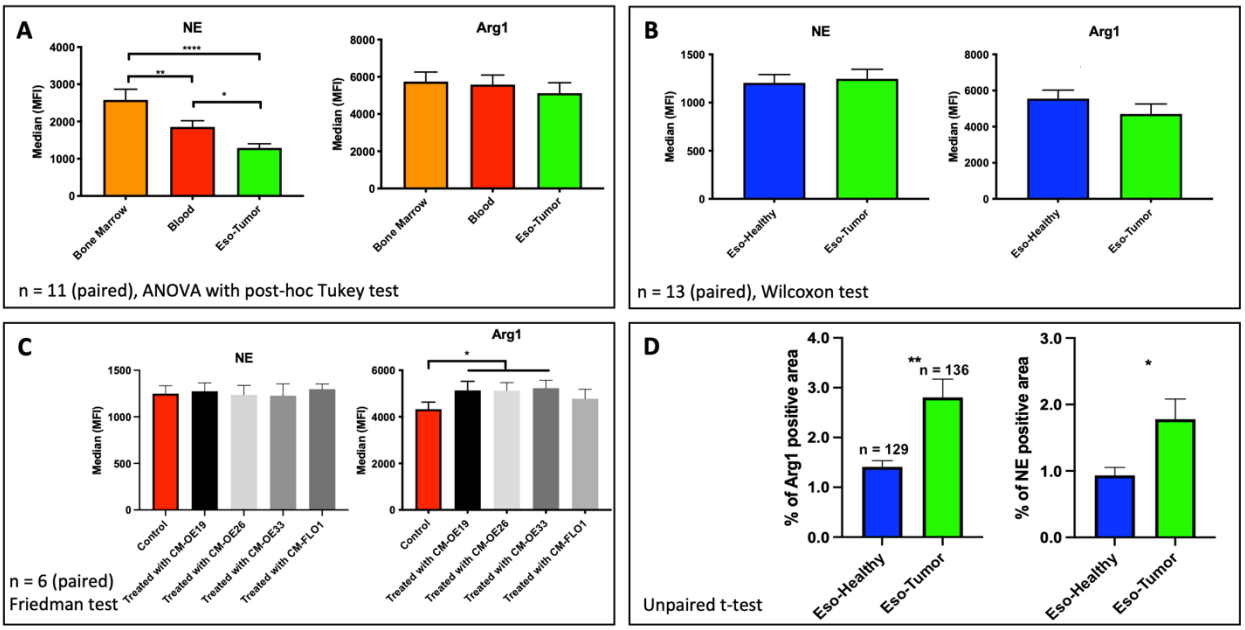

Figure 6 (A) Expression of NE and Arg1 in BMNs, PBNs, TANs, (B) in TANs and tissue infiltrated neutrophils, (C) in *in vitro* CM-treated PBNs and untreated (control) PBNs, and (D) IF staining of NE and Arg1 in tumor and non-diseased esophagus: degranulation of TANs evidenced by deceased the expression of Arg1 and NE in granules measured by flow cytometry (A and B) and increased Arg1 and NE positive areas in tumor compared to healthy tissue (D) measured by IF imaging. Data presented mean ± SEM, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

3.3.4 TANs are aged (delayed apoptosis)

The number of CXCR4+ cells increased, and the number of CD62L+ cells decreased in TANs compared to both PBNs and tissue-infiltrated neutrophils (Figure 7). Increased CXCR4 expression impedes neutrophil homing to the bone marrow, and the expression of CXCR4 increases, and that of CD62L decreases on neutrophils as they age [23]. Collectively this suggests that compared to tissue-infiltrated neutrophils, TANs live longer and are aged (delayed apoptosis) in cancer patients.

3.3.5 Ligation of chemokine receptor CXCR2 in TANs

CXCR2 attracts neutrophils into tumors, and G-CSF produced by cancer cells increases the expression of CXCR2 on neutrophils [24]. However, we observed in cancer patients, the number of CXCR2+ cells decreased in TANs compared to both PBNs and tissue-infiltrated neutrophils (Figure 8). Contrarily, the number of CXCR2+ cells elevated in CM-treated PBNs. Due to the tumor-associated inflammation, the tumor microenvironment contains a large amount of CXCR2 ligands, such as IL-8, and the ligation/internalization of CXCR2 on neutrophils results in lower fluorescence intensity of this biomarker measured by flow cytometry [25].

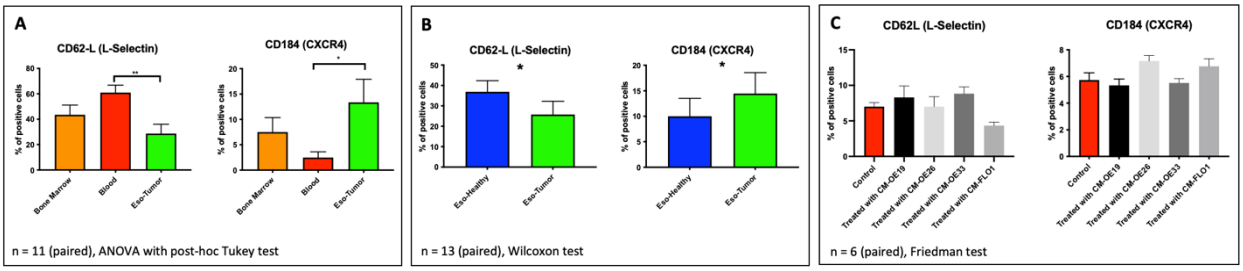

Figure 7 (A) Expression of CD62L and CXCR4 in BMNs, PBNs, TANs, (B) in TANs and tissue infiltrated neutrophils, and (C) in *in vitro* CM-treated PBNs and untreated (control) PBNs: increased number of CXCR4+ and decreased number of CD62L+ cells in TANs demonstrate that TANs are aged compared to PBNs and tissue-infiltrated neutrophils.

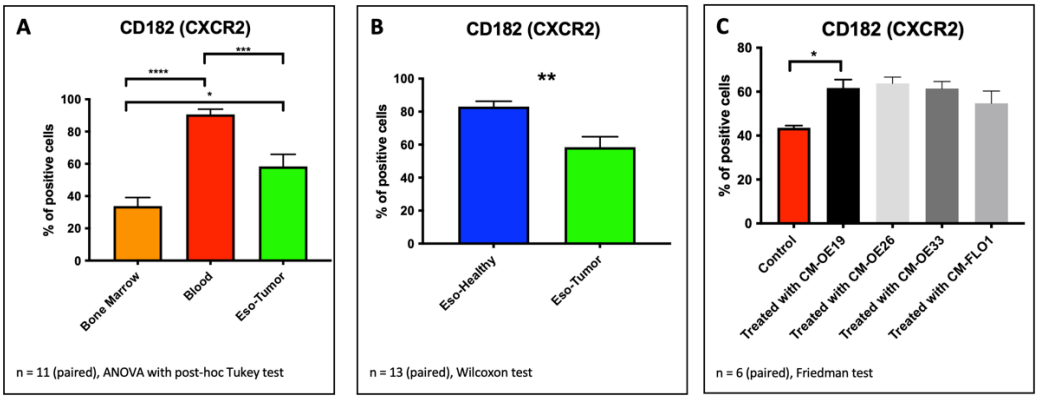

Figure 8 (A) Expression of CXCR2 in BMNs, PBNs, TANs, (B) in TANs and tissue infiltrated neutrophils, and (C) in *in vitro* CM-treated PBNs and untreated (control) PBNs: increased number of CXCR2+ cells in TANs compared to PBNs and tissue-infiltrated neutrophils. CM-

treated PBNs demonstrated an elevated number of CXCR2+ cells compared to control. Decreased number of CXCR2+ cells in TANs could be due to the CXCR2 ligation in the tumor site.

3.3.6 Expression of both HLA-DR and PDL-1 increased in CM-treated neutrophils

HLA-DR is expressed on the surface of human antigen-presenting cells (APC) and the expression of HLA-DR on neutrophils is a marker for tumor-inhibiting activities of neutrophils. Neutrophils can acquire the function of antigen-presenting and HLA-DR expressing neutrophils are able to activate T cells and thereby promote adaptive immunity against tumor cells [8, 9, 26]. On the other hand, high PD-L1 is suggested to be a marker for tumor-promoting activities of neutrophils. PD-L1 binds to the PD-1 receptor on T cells and dampens T cell responses to cancer [27, 28]. Interestingly we observed an increase in both tumor-inhibiting HLA-DR+ neutrophils and tumor-promoting PD-L1+ neutrophils in CM-treated PBNs (Figure 9C). In cancer patients, the percentage of HLA-DR+ TANs was higher than PBNs (Figure 9). It should be noted that the numbers of positive neutrophils for these two biomarkers were very low, and on average, the percentage of PD-L1+ and HLA-DR+ TANs was respectively 2.6% and 8.7%. It has been suggested that HLA-DR and PD-L1 have opposing roles in T cell activation.

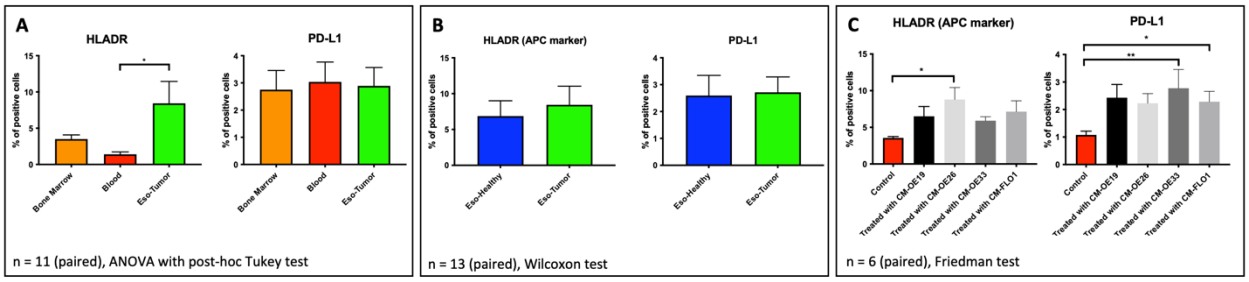

Figure 9 (A) Expression of HLA-DR and PD-L1 in BMNs, PBNs, TANs, (B) in TANs and tissue infiltrated neutrophils, and (C) in *in vitro* CM-treated PBNs and untreated (control) PBNs: The number of HLA-DR+ was higher in TANs than PBNs. An increase in both tumor-inhibiting HLA-DR+ neutrophils and tumor-promoting PD-L1+ neutrophils in CM-treated PBNs.

3.4 Discussion

3.4.1 Tumor microenvironment activates tumor-associated neutrophils

In this study, we used a combination of surface and internal biomarkers to determine the effects of tumor microenvironment on neutrophil phenotypes, which gives us some indications about TANs functional aspect in human cancer development. Using multiple color flow cytometry, we determined the expression of biomarkers for neutrophil activation, maturity, degranulation, and aging. Our results showed that in esophageal cancer patients, TANs demonstrated to possess an activated phenotype compared to PBNs, evidenced by higher expression of CD66b/CEACAM-8, CD11b/MAC-1, CD54/ICAM-1, and shedding of CD16/FcγRIIIB, CD62L/L-selectin. The relationship between the activation status of neutrophils and the upregulation/downregulation of these biomarkers has been previously reported in the literature [29, 30]. We also investigated the phenotype of neutrophils from the non-diseased esophagus to show which characteristics of TANs are specifically due to the tumor microenvironment and which are simply due to the transmigration of neutrophils from blood to tissue. Neutrophils from healthy tissue versus TANs were more alike to each other than to PBNs (blood neutrophils). We found that TANs exhibit an even more activated phenotype compared with neutrophils isolated from non-diseased tissue. Neutrophil activation pattern was also observed in PBNs from healthy volunteers treated *in vitro* with different cancer cell-conditioned media.

Consistent with our results, Rodriguez et al reported that activated PBNs using PMA stimulation expressed high levels of CD66b, CD11b, and VEGFR1 and low levels of CD62L and CD16 [17]. Interestingly, they demonstrated that activation of PBNs induces pro-tumor phenotypic characterizations and functional changes in PBNs similar to suppressor cells (MDSC) and neutrophil activation promotes the release of Arg1 from intracellular granules. Eruslanov et al also reported TANs acquired an activated phenotype compared with PBNs and neutrophils from non-diseased tissue in patients with early-stage of lung cancer [29]. Tumor-associated inflammation increases the production of inflammatory cytokines, including G-CSF and IL-6, which then induce an activated phenotype of neutrophils as well as promote further mobilization

of both mature and immature neutrophils through emergency granulopoiesis to be released from the bone marrow to the bloodstream and subsequently migrate to the tumor sites [31]. These cytokines are directly secreted by tumor cells or indirectly by the influence of tumor cells on stromal cells, including tumor infiltrated immune cells, such as neutrophils, macrophages, and lymphocytes. Although activated neutrophils can directly recognize anti-body coated tumor cells and kill them through phagocytosis and ROS production, activated TANs has been suggested to favor tumor growth, progression, and metastasis through various mechanisms [32-35]. This includes: activated TANs have higher production of chemokines (e.g., CXCL8) and growth factors (e.g., VEGF) that further increase neutrophil migration to the tumor site, angiogenesis and tumor growth; activated TANs exhibit higher production of CCL17 that recruits immunosuppressive regulatory T cells (Tregs); activated TANs possess higher degranulation (exocytosis) of arginase and NE, promoting cancer cell proliferation, metastasis, and the suppression of CD8+ T cells; and finally activated TANs produce a higher amount of matrix metalloproteinase-9 (MMP-9) that degrades extracellular matrix, increasing angiogenesis, and preventing apoptosis of tumor cells.

In our study, we did not observe increased expression of PD-L1 in TANs, a protumor biomarker in neutrophils, compared to PBNs and neutrophils in non-diseased tissues. Consistent with our study, Eruslanov et al also did not observe elevation in PD-L1 expression in TANs. However, our results showed that the number of PD-L1 expressing PBNs was significantly higher in CM-treated PBNs than in the control group. A higher number of PD-L1 expressing PBNs has been previously observed in cancer patients and other inflammatory-related diseases compared to healthy subjects [27, 36, 37]. It has also been reported that the number of PD-L1 expressing neutrophils increases in TANs and they are considered pro-tumor and associated with poor patient prognosis because of the ability of these neutrophils to suppress CD8+ T cells with reduced cytotoxicity towards cancer cells [27, 38-41]. Some studies suggested that PD-L1 expressing neutrophils are predominantly located in the peritumoral area of the tumor rather than in the core of the tumor (intratumoral neutrophils) [27]. However, this observation is inconsistent between different papers as some studies reported no difference or lower number of PD-L1 expressing neutrophils in the peritumoral area [41]. It should be noted that in our study, the tissues from the core of

tumors were dissociated, and thereby, TANs in our work were isolated from the core of tumors and not from the periphery of tumors.

HLA-DR is an MHC (major histocompatibility complex) class II cell surface. It has been suggested that tumor cell-derived cytokines, such as GM-CSF, IFN-γ, IL-3, and TNF, induce the expression of HLA-DR on TANs and antigen-presenting TANs are be able to activate T cells and increase adaptive anti-tumor immunity [5, 42, 43]. Our results revealed that PBNs express no or little HLA-DR and the percentage of HLA-DR expressing neutrophils increased in tumor compared to blood. However, we did not see any difference in the percentage of HLA-DR expressing cells between TANs and neutrophils in healthy tissue. In early-stage lung cancer patients, Singhal et al identified a subset of TANs that exhibited characteristics of both neutrophils and antigen-presenting cells (APCs) [44]. These APC-like neutrophils were able to trigger and augment anti-tumor T cell responses.

3.4.2 Tumor microenvironment causes degranulation of TANs

Our results suggested that TANs had an increased level of degranulation compared to PBNs and tissue-infiltrated neutrophils. Exocytosis of enzymes from neutrophil granules (degranulation) contributes to the pro-tumor activities of TANs. Degranulation of activated neutrophils has been previously reported and shown to increase the accumulation of Arg1 in plasma and extracellular matrix due to neutrophil activation [17, 45, 46]. As mentioned above, Arg1 release leads to suppression of anti-tumor immunity via converting arginine to urea. Similar to Arg1, elevated extracellular NE has been detected in several cancers and was suggested to contribute to the pro-tumor activity of neutrophils via several mechanisms and pathways [47-49]. For example, neutrophil-derived NE activates membrane receptors on the surface of tumor cells, such as epidermal growth factor receptor (EGFR) and toll-like receptor 4 (TLR4), which upregulate mitogen activated protein kinase (MAPK) signaling pathway, promoting tumor cell proliferation [49]. NE can also enzymatically degrade the insulin receptor substrate-1 (IRS-1), preventing the formation of IRS-l and phosphoinositide 3-kinase (PI3K) complex, leading to augmentation of free

PI3K in the cytoplasm. The free form of PI3K can associate with platelet-derived growth factor receptor (PDGFR) and induce the tumor cell proliferative signaling pathway [50-52].

3.4.3 Tumor microenvironment delays apoptosis of neutrophils and increases the accumulation of neutrophils in the tumor site

CXCR2 and CXCR4 regulate neutrophil migration and control the release of these cells from the bone marrow and their recruitment to inflammation sites, including tumors. Increased expression of CXCR4 on neutrophils retains them in the bone marrow, whereas increased expression of CXCR2 on neutrophils raises their release into the bloodstream [53, 54]. We showed *in vitro* that tumor cells increased the expression of CXCR2 on neutrophils, which can then further promote neutrophil migration to the tumor site. Interestingly our results showed that in cancer patients, CXCR2 expression decreased in TANs compared to both PBNs and neutrophils from healthy tissues. Tumor cells directly or indirectly by influence on stromal and immune cells produce CXCR2 ligands, such as CXCL1, CXCL2, CXCL6 (IL-6), and CXCL-8 (IL-8), and their ligation to CXCR2 triggers the extravasation and subsequently the infiltration of PBNs to tumor site [24, 55-57]. However, once in the tumor, increased concentration of CXCR2 ligands in the tumor microenvironment leads to binding of these ligands to CXCR2 receptors and thereby causes downregulation of CXCR2 expression on TANs due to internalization of the ligand-receptor complex [25]. Increased CXCR2 expression on TANs has been shown to be associated with pro-tumor activities of neutrophils [35]. CXCR2 signaling in neutrophils promotes tumorigenesis and tumor metastasis, and the inhibition of CXCR2 improved T cell entry into tumor core and survival [58].

Aged neutrophils exhibit increased expression of CXCR4 and decreased expression of CD62-L and CXCR2 [23, 59], where CXCR2 expression induces neutrophil aging, whereas CXCR4 expression antagonizes it [59]. Our results suggested that TANs are aged compared to tissue-infiltrated neutrophils, evidenced by increased CXCR4 and decreased CD62-L and CXCR2 expressions. Increased CXCR4 expression on neutrophils is associated with elevated neutrophil transmigration

and accumulation in tissue [60, 61]. It has also been shown that increased expression of CXCR4 in neutrophils protects neutrophils against cell death (apoptosis). Therefore, higher expression of CXCR4 in TANs than neutrophils in the healthy tissues in our study suggests that the tumor microenvironment promotes the accumulation of neutrophils in the tumor site and prolongs their survival, which is in agreement with previous studies [60, 61]. In a mouse model, Fridlender et al demonstrated that most genes related to apoptosis were indeed expressed at similar levels in TANs and in PBNs, however, several anti-apoptotic members of the NF-kB family were upregulated in TANs [62].

In this study, we were unable to obtain tumor samples from patients with small tumor size. For patients with small tumor size, the pathologist needs the entire intact tumor for histopathology and was not able to give us tumor samples from early-stage cancer patients. This means inevitably, the post-operative samples for in this study were obtained mostly from patients with advanced disease and aggressive tumors. For future works, Cytek Aurora flow cytometer can be employed for analysis of patient samples, as up to 48 antibodies can be applied simultaneously in this system.

3.5 Conclusion

We demonstrated that tumor-associated neutrophils (TANs) acquired an activated phenotype compared to neutrophils isolated from other body compartments. Tumor-associated inflammation increases the production of inflammatory cytokines (e.g., G-CSF, IL-6) that can activate neutrophils and promote their mobilization to the tumor site. Immunophenotypic characterization suggested that TANs were aged and retained longer in tumors than neutrophils infiltrated into healthy tissues. Higher degranulation of TANs compared to healthy tissue-infiltrated neutrophils could contribute to the pro-tumor activities of TANs. Our findings suggest that TAN phenotypes were skewed towards pro-tumor neutrophils.

3.6 References

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General Discussion

In chapter 2, we characterized the composition of the circulating neutrophils in patients with an elevated NLR. We demonstrated that elevated blood NLR is associated with the expansion of circulating LDNs fraction, a protumor phenotype of neutrophils, expressing a higher level of arginase compared to the HDNs fraction. Subsequently, we demonstrated that an elevated circulating NLR is associated with increased NLR in the primary tumor of patients with gastro-esophageal adenocarcinoma. Furthermore, we showed that NLR into the tumor core was significantly associated with poor survival outcomes. We also revealed that high intratumoral NLR and blood NLR in cancer patients were associated with increased expression of arginase and neutrophil elastase in TANs. Finally, we demonstrated that neutrophil trafficking to and NET formation in lymph nodes promote premetastatic niche in lymph nodes of cancer patients. Thus, when taken collectively, the results of chapter 2 highlight the implication and importance of tumor and nodal neutrophils in cancer progression and the association of high NLR with tumor-promoting TANs in cancer patients. In chapter 3, we went one step further and studied the phenotypic characteristic of TANs using 12 different neutrophil biomarkers and compared this with neutrophils from other body compartments to determine the effects of tumor microenvironment on TAN phenotypes. Based on the expression of these biomarkers, we demonstrated that in our patient group, TANs acquired an activated phenotype with phenotypic characteristics skewed towards tumor-promoting phenotype. The results of this chapter underline the pro-tumor activities of TANs compared to the neutrophils isolated from other body compartments. In the current chapter, we will discuss the potential clinical applications of our findings in chapters 2 and 3, and in general, the potential of using TANs as a target in human cancer therapy and prognosis.

3.7 Neutrophil as a prognostic tool in oncology

It has been suggested that circulating neutrophils and/or TANs can be used as prognostic tools to predict oncological outcomes in cancer patients [4, 14]. Neutrophils are considered as the least favorable immune cell population, linking to poor survival in cancer patients, which highlights the

significance of neutrophils in cancer prognosis [99]. As it has been shown in this project and other studies, the prevalence of neutrophils over lymphocytes (tumor NLR) in blood and in tumor has been considered as a stronger predictor for poorer oncological outcomes in cancer patients than the neutrophil count alone [3, 4]. Aside from neutrophils, neutrophil-releasing factors in circulating blood could also be employed in cancer prognosis. For example, detecting NETs in peripheral blood could be used as a prognostic marker since the high level of circulating NETs is strongly correlated with poorer oncological outcomes in patients [100]. Detection of neutrophils and/or neutrophil-releasing factors in blood is easy, inexpensive, and applicable in cancer prognosis purposes [101]. To utilize neutrophil count or NLR as prognostic tools, further clinical studies are required. First, we need to ensure these are in fact independent markers for cancer prognosis [4, 102]. Second, the reliability of neutrophil count to predict oncological outcomes should be established for different cancer stages and tumor types. In other words, does neutrophil count (or NLR) possess similar and adequate reliability to predict cancer outcomes in patients with different cancer stages and tumor types? Or, neutrophil, as a prognostic tool, is only applicable in early-stage of tumors and only in certain types of cancer.

3.8 Neutrophil as a target for human cancer therapy

In this project, we demonstrated the implications of TANs in tumor progression and metastasis. TANs can be therefore utilized as a target for cancer therapy through different approaches [13, 14]: i) depletion of neutrophils; ii) inhibition of TANs accumulation in tumor site; iii) modulating TAN phenotypes by inhibiting pro-tumor and promoting anti-tumor TANs; iv) disintegrating NETs or preventing NETosis.

Depletion of neutrophils: TANs usually play a pro-tumor role, and therefore depletion of neutrophils can be utilized as a strategy to reduce tumor growth. It has been shown in preclinical animal models that the depletion of circulating neutrophils resulted in fewer TANs and thereby reduced tumor growth and metastasis [103, 104]. However, depletion of neutrophils cannot be considered as a viable anti-cancer therapy in humans since significantly reducing circulating

neutrophils can severely compromise the ability of the immune system to fight bacterial infections.

Inhibition of TANs accumulation in tumor site: Rather than depletion of circulating neutrophils, maybe a better approach to target TANs in cancer therapy is inhibiting their accumulation in the tumor. This can be achieved by blocking chemokine receptors on neutrophils, particularly CXCR2, responsible for neutrophil recruitment to the tumor site [47, 105-107]. Animal studies demonstrated that blocking these chemokine receptors by antibodies resulted in reduced tumor burden and metastasis [108-110]. Based on these promising preclinical results, a phase II clinical trial with the CXCR2 inhibitor, AZD5069, is ongoing in pancreatic cancer patients [108]. CXCL8 is the major CXCR2 ligand in humans, and the elevated level of CXCL8 in blood and tumor was associated with increased accumulation of TANs and unfavorable survival in cancer patients [111]. Targeting CXCR2 ligands (CXCL8) has therefore emerged as a novel approach in cancer therapy. The clinical benefit of CXCL8 antibody treatment is currently under evaluation in patients with advanced solid tumors [13]. One thing that needs to be taken into consideration in targeting CXCR2 is that this chemokine receptor is not specifically expressed on neutrophils, and in fact, it is also expressed on cancer cells and other immune cells such macrophages and lymphocytes [6]. It is therefore imperative to assess whether CXCR2 blocker treatment in humans specifically decreases TAN accumulation and/or modulates TAN phenotype and activation rather than affecting other immune cells and/or tumor cells [13]. Aside from chemokine receptors, tumor cell-derived G-CSF has been shown in animal experiments to promote tumor development and metastasis by recruiting TANs and by shifting their phenotypes towards pro-tumor TANs [112, 113]. However, human trials failed to demonstrate the association of increased G–CSF expression with poorer oncological outcomes in cancer patients [114].

Modulating TAN phenotypes by inhibiting pro-tumor and promoting anti-tumor TANs: Maybe the more desirable approach to target TANs in human cancer therapy is by modulating TAN phenotype to promote type N1 (anti-tumor) and/or to demote type N2 (pro-tumor) TANs. Using a mathematical model, Kim et al demonstrated that phenotypic shifts between anti-tumor and pro-tumor TANs affected tumor growth in a lung cancer model [115]. In their study, the N2-to-

N1 ratio was positively correlated with aggressive tumor growth, and anti-tumor activities increased when cell death of N1 inhibited, and that of N2 promoted. Based on *in vitro* and *in vivo* experiments, TGF-β induces pro-tumor (N2) phenotype, while IFN-β alters neutrophil phenotype to anti-tumor type (N1) [17, 18, 52, 53]. Downregulating TGFβ and/or upregulating IFN-β has been therefore proposed to alter TAN phenotype. In a mouse study, TGF-β blockade resulted in the recruitment and activation of TANs with an anti-tumor phenotype [19]. Andzinski et al showed that interferon therapy in mice altered TAN polarization towards anti-tumor phenotype (N1) [18]. They then investigated characteristics of PBNs isolated from the blood of melanoma patients who were under interferon therapy and demonstrated N1 biased PBN polarization in these patients compared to the untreated patients. Hypoxia was also reported to augment the N1-to-N2 ratio in animal studies and led to a reduction in tumor burden [116]. Although promising results in preclinical studies demonstrated the strategies to shift TAN phenotype from pro-tumor towards anti-tumor, this research is still in the preclinical stage, and alteration of TAN phenotype in cancer therapy has not yet been trialed and proved in humans [13, 14]. Due to the complexity of neutrophil plasticity and diversity in the tumor microenvironment, many more preclinical experiments in different tumor types/stages are needed to establish the efficacy of altering TAN phenotype on tumor growth and the safety of this therapeutic strategy in humans.

Disintegrating NETs or preventing NETosis: In this project, we demonstrated the implication of nodal NETs in lymph node metastasis. Other works also showed the contribution of NETs in the promotion of metastatic cancer, in which NETs capture and support the growth of tumor cells that are trafficking in a secondary organ [81-86]. NETs can also capture circulating tumor cells and promote metastasis. This encouraged researchers to investigate whether disintegration of NETs or blocking NET formation would result in inhibition of metastatic tumors and if we can use NETs as a potential target in cancer therapy [83, 86]. DNase can disintegrate the DNA structure of NETs and it has been shown in animal models that DNase treatment decreased tumor burden and metastasis [82, 87]. DNase treatment is not toxic in humans and FDA has approved DNase therapy in patients suffering cystic fibrosis [117]. Another way to target NETs is using the inhibitors of the molecules that are required in NET formation (NETosis). In a mouse tumor model, treatment with inhibitors of PAD4, an essential molecule for NETosis, improved

oncological outcome and reduced growth of metastatic tumors [85]. Although encouraging results were obtained in animal models to reduce tumor burden and metastasis through using NETs as a target, so far, clinical studies did not provide conclusive evidence of the efficacy of this approach in cancer therapy [13, 14, 86]. More preclinical and clinical studies are therefore required to obtain a better understanding of the complex role of neutrophils in cancer progression and the therapeutic effects of targeting neutrophils in cancer patients [13, 14]. These pieces of knowledge will provide a solid basis to design optimal targeting therapeutic approaches in cancer by employing neutrophil as a target.

Appendix

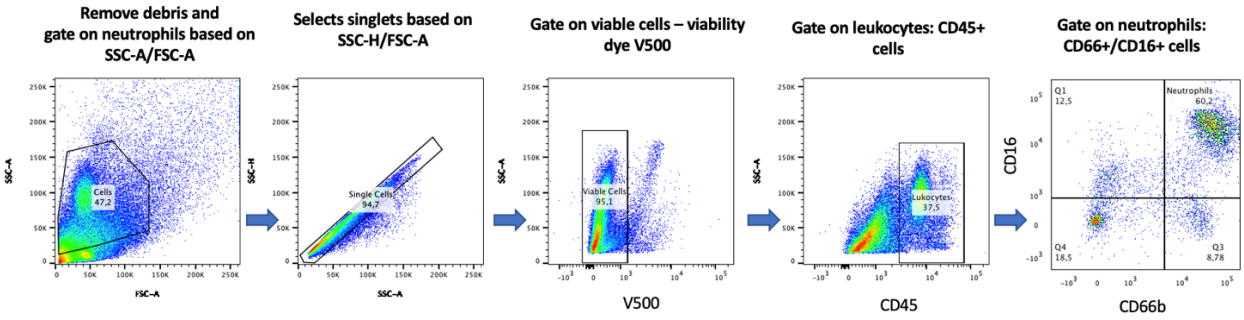

Figure 1 Gating strategy used in this project to identify neutrophil population: First, debris was excluded, and the neutrophil population was roughly gated on the SSC-A/FSC-A plot. Single cells (singlets) were identified on the SSC-H/SSC-A plot, and then live cells were selected by gating on cells negative to the viability dye. Leukocytes were selected by gating on CD45+ cells, and finally, neutrophils were selected as CD66b+/CD16+ cells.

Table1: Biomarkers of neutrophils used in immunophenotyping

Marker	Description	Reference
	Chemokine Receptor	
CD182 (CXCR2)	Decreased expression of CXCR2 may enhance neutrophil adhesion and impede neutrophil migration from blood to tumor site. CXCR2 was downregulated on TANs (lung cancer) and distant neutrophils compared to PBNs. CXCR2 positively regulates neutrophil release from the bone marrow. Increased CXCR2 expression on TANs has been shown to be associated with neutrophils pro-tumor activity.	[50, 53, 118-121]
CD184 (CXCR4)	A marker for neutrophil migration. CXCR4 negatively regulates neutrophil release from the bone marrow, however it is dispensable for neutrophil clearance from the circulation. CXCR2 expression increased by neutrophil aging. CXCR4 was upregulated on TANs (lung cancer).	[50, 120-122]
	Granulocytic Lineage	
CD66b (CEACAM-8)	A marker for neutrophil adhesion, migration, and activation. Activated neutrophils possess increased surface expression of CD11b, CD66b, and the loss of CD16. Increased expression of CD66b in TANs is associated with presence of metastasis and decreased survival. CD66b along with CD11c, CD32, CD35, CD45 were upregulated in the myeloid-derived suppressive cells (MDSCs) compared to neutrophils.	[123, 124]
CD11b (Mac1)	A marker for neutrophils activation, adhesion, and migration. Higher expression of CD11b on TANs indicated a degree of activation and migration. Neutrophils showed to capture circulating cancer cells by direct interactions through the cell surface molecule CD11b.	[72, 125, 126]

Arginase 1 (Arg1)	TANs express high levels of Arg1 which suppresses proliferation of CD8+ T-cells by conversing L-arginase to urea. L-arginine is essential for various T cell functions and proliferation. Arg1 was shown to be important in the suppression of immune responses by MDSCs in various murine models.	[70, 124, 127]
Neutrophil Elastase (NE)	NE contributes to the progression of cancers to enhance tumor invasion and metastasis. NE enzymatically degrade the insulin receptor substrate-1 (IRS-1) resulting in failure to form the association complex of IRS-l and phosphoinositide 3-kinase (PI3K), leading to free PI3K in cytoplasm. The free form of PI3K can associate with platelet-derived growth factor receptor (PDGFR) and induce the tumor proliferative signaling pathway.	[128-130]
Fc Receptor		
CD16 (FcγRIIIB)	A marker for neutrophils maturity, increased expression by mature neutrophils. PBN of cancer patients have lower expression of CD16 compared to those of healthy subjects. The expression of CD16 was downregulated in TANs (lung cancer) compared with that seen on PBNs. High expression of CD11c, CD16, and CD10 is characteristic of mature neutrophils and CD15 expression decreased during late stages of maturation.	[50, 72, 119, 121, 131-135]
Inhibitory Ligand		
CD274 (PD-L1)	Increased expression of PD-L1 in TANs at the periphery of colorectal cancer. PD-L1 supresses adaptive immune system. PD-L1 was expressed in several tumors, where it tended to be correlated with decreased survival. PD-L1 is highly expressed in tumor-infiltrating MDSCs.	[73, 136, 137]
Activation		
CD54 (ICAM-1)	Receptor for adhesion, facilitate the transmigration of neutrophils into tissues. A marker for neutrophil activation ($CD54^{high}$ $CD62L^{low}$) and its expression is increased in TAN (early-stage human lung cancer). ICAM-1 was proposed to be a marker of anti-tumor neutrophils in murine tumor models.	[50, 119, 121, 138-140]
CD62L (L-selectin)	Receptor for adhesion and transmigration of neutrophils into tissues is allowed by shedding of L-selectin from neutrophil surface. A marker for neutrophil activation ($CD54^{high}$ $CD62L^{low}$). Activated $CD16^{high}$ $CD62L^{dim}$ neutrophils inhibited migration, proliferation and induced apoptosis of FaDu cancer cell line.	[50, 72, 119, 135, 141]
Antigen Presenting Cell (APC) marker		
HLA-DR (Human Leukocyte Antigen – DR isotype)	HLA-DR is a major histocompatibility complex (MHC) II cell surface receptor. Expression of HLA-DR on neutrophils activate immune response to tumor (anti-tumor activity). Expressed in TANs in early stage of lung tumors and reduced expression as tumor develops.	[10, 121]
Leukocyte Common Antigen (LCA)		
CD45	Marker for leukocytes and is utilized to isolate leukocytes. CD45 is upregulated in the MDSC compared to neutrophils.	[124]

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