Dissertation

New insights into the role of neutrophils in non-small cell lung cancer: the case of low-density neutrophils and myeloperoxidase

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and I fully acknowledge the name all of individuals and organizations that contributed to the research constituting the thesis. Due acknowledgement has been made to all other material used for the thesis. Throughout this thesis and associated publication, I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

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ABREVIATIONS

Abbreviation	Explanation
ANOVA	Analysis of variance
ARG-1	Arginase
CCL2	C-C motif chemokine ligand 2
CCL5	C-C motif chemokine ligand 5
CPTAC	Clinical Proteomic Tumor Analysis Consortium
CXCL1	C-X-C motif chemokine ligand 1
CXCL2	C-X-C motif chemokine ligand 2
CXCL3	C-X-C motif chemokine ligand 3
CXCL5	C-X-C motif chemokine ligand 5
CXCL6	C-X-C motif chemokine ligand 6
CXCL7	C-X-C motif chemokine ligand 7
CXCL8	C-X-C motif chemokine ligand 8
CXCL12	C-X-C motif chemokine ligand 12
CXCR2	C-X-C motif chemokine receptor 2
CXCR4	C-X-C motif chemokine receptor 4
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FASL	FAS/FAS ligand
FBS	Fetal bovine serum
FMO	Fluorescence minus-one

FVD	Fixable viability dye
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDNs	High density neutrophils
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
HOI	Hypoiodous acid
HOSCN	Hypothiocyanous acid
i.p.	Intraperitoneal
ICAM-1	Intercellular adhesion molecule-1
ICI	Immune checkpoint inhibitor
IFN	Interferon
IFN-γ	Interferon-gamma
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-33	Interleukin-33
iNOS	Inducible nitric oxide synthase
KO	Knock out
KRAS	Kirsten rat sarcoma viral oncogene
LDNs	Low density neutrophils

LLC	Lewis lung carcinoma
LDCT	Low dose computer tomography
Lox1	Lectin-type oxidized LDL receptor 1
LUAD	Lung adenocarcinoma
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMP1	Matrix metalloproteinase 1
MMP3	Matrix metalloproteinase 3
MMP9	Matrix metalloproteinase 9
MPO	Myeloperoxidase
NF-κB	Nuclear factor-κB
NK	Natural killer
NKT	Natural killer T
NSCLC	Non-small cell lung cancer
P/S	Penicillin/ streptomycin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PD-1	Programmed death-1
pDC	Plasmacytoid dendritic cells
PD-L1	Programmed death-ligand 1
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase

PMA/Iono	Phorbol myristate acetate/Ionomycin
PMNL	Polymorphonuclear leukocytes
RBC	Red blood cell
RPMI	Roswell Park memorial Institute
ROS	Reactive oxygen species
s.c.	Subcutaneous
SB	Staining buffer
SCLC	Small-cell lung cancer
SD	Standard deviation
SEM	Standard error of mean
TAMs	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
Th1	T helper 1
Th2	T helper 2
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-alpha
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
Tregs	Regulatory T cells
TRPM2	Transient receptor potential cation channel, subfamily M, member 2
WHO	World Health Organization
WT	Wild type

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ZUSAMMENFASSUNG

Lungenkrebs ist die am häufigsten diagnostizierte Krebsart und führt die Liste der tödlichsten Krebsarten weltweit an. Nichtkleinzelliges Lungenkarzinom (NSCLC) steht bei den Diagnosen an erster Stelle und macht etwa 85 % aller Lungenkrebsfälle aus. Neutrophile sind heterogene und vielseitige Zellen, die zur Tumorentwicklung beitragen. Sie sind besonders häufig in der Tumormikroumgebung (TME) von NSCLC anzutreffen und es wurde gezeigt, dass sie die Entwicklung von NSCLC durch ihre tumorfördernde Wirkung begünstigen. Darüber hinaus wurden im Blut von Krebspatienten zirkulierende Neutrophilenpopulationen identifiziert, von denen man annimmt, dass sie eine immunsuppressive Wirkung haben. Trotzdem sind die Mechanismen, durch die Neutrophile ihre Wirkung im NSCLC entfalten, noch nicht vollständig geklärt, und die Klassifizierung der neutrophilen Subpopulationen im Blutkreislauf und im Tumor ist noch ein wichtiges Forschungsgebiet. Die vorliegende Arbeit gliedert sich in zwei unabhängige Projekte, die darauf abzielen, die Rolle der Neutrophilen im NSCLC aus zwei verschiedenen Perspektiven zu untersuchen:

- Im ersten Projekt verwendeten wir ein hochdimensionales Screening menschlicher Zelloberflächenmarker, um zelluläre Marker zu identifizieren, die es uns ermöglichen, zwischen zwei zirkulierenden Neutrophilenpopulationen zu unterscheiden: den sogenannten Neutrophilen niedriger Dichte (LDN) und den Neutrophilen hoher Dichte (HDN). Mit diesem Ansatz kategorisierten wir 12 Oberflächenmarker als gering exprimiert von LDNs gegenüber HDNs, während 41 Oberflächenmarker in der LDN-Untergruppe gegenüber HDNs hoch exprimiert waren. Wir entwickelten ein spezifisches Durchflusszytometrie-Panel, mit dem wir die Überexpression von CD36, CD41, CD61 und CD226 in der LDN-Fraktion nachweisen konnten. Darüber hinaus bestätigten wir die Existenz von LDNs ausschließlich im Blut von NSCLC-Patienten, während sie im Blut von gesunden Spendern fast nicht vorkamen.

- Im zweiten Projekt untersuchten wir die Rolle eines von Neutrophilen stammenden Enzyms namens Myeloperoxidase (MPO) in der Entwicklung von NSCLC. Mit Hilfe eines heterotopen NSCLC-Modells in MPO-defizienten Mäusen beobachteten wir eine Verringerung des Tumorwachstums, die mit einer Zunahme der Infiltration zytotoxischer T-Zellen im Vergleich zu MPO wildtyp-Mäusen einherging. Die CD8-Depletion hob die zuvor beobachtete Verringerung der Tumorgröße in MPO-defizienten-Mäusen auf, was darauf hindeutet, dass $CD8^+$ T-Zellen

eine wichtige Rolle spielen. *In vitro* führte die Zugabe von MPO zu T-Zellen zu einer verringerten Proliferation und Aktivierung von T-Zellen. Darüber hinaus wurde MPO in T-Zellen internalisiert, während eine Heparin-Vorbehandlung die MPO-Internalisierung blockierte und die proliferationshemmende Wirkung von MPO in T-Zellen aufhob. Interessanterweise wurden MPO^{+}-Lymphozyten in Tumorproben von Patienten mit nicht-kleinzelligem Lungenkrebs (NSCLC) gefunden, und eine In-silico-Analyse zeigte einen Überlebensvorteil für Patienten mit NSCLC und niedriger MPO-Expression.

Zusammenfassend unterstreichen unsere Ergebnisse die Bedeutung von Neutrophilen und deren Enzymen in der Biologie von Lungenkrebs und tragen zum Verständnis der komplexen Rolle von diesen Zellen in Krebs bei. Insgesamt bietet diese Dissertation Ideen, wie Neutrophile und deren Moleküle als potenzielle Kandidaten für Diagnostik und therapeutische Ziele eingesetzt werden können.

ABSTRACT

Lung cancer is the most frequently diagnosed type of cancer and tops the list of deadliest cancers around the world. Non-small cell lung cancer (NSCLC) leads the diagnoses and represents about 85% of all lung cancer cases. Neutrophils are heterogeneous and versatile cells that contribute to tumor development. They are especially abundant within the tumor microenvironment (TME) of NSCLC and have shown to exert pro-tumorigenic actions favoring NSCLC development. Moreover, specific circulating neutrophil populations have been identified in the blood of cancer patients and have been proposed to depict immune-suppressive actions. Despite this, the mechanisms by which neutrophils exert their actions in NSCLC are not completely elucidated yet and the classification of neutrophil subpopulations in circulation and in the tumor is still a major field to explore. The present thesis is divided in two independent projects that aimed to address the role of neutrophils in NSCLC from two different perspectives:

- In the first project we used a high-dimensional human cell surface marker screen to identify cellular markers that allow us to discriminate between two circulating neutrophil populations; the so-called low-density neutrophils (LDNs) and high-density neutrophils (HDNs). Using this approach, we categorized 12 surface markers as low expressed by LDNs vs HDNs, while 41 surface markers were highly expressed in the LDN subset vs the HDN. We designed a specific flow cytometry panel that allowed us to validate the overexpression of CD36, CD41, CD61 and CD226 in the LDN fraction. Moreover, we confirmed the exclusive existence of LDNs in the blood of NSCLC patients while they were almost absent in the blood of healthy donors.

- In the second project, we investigated the role of a neutrophil-derived enzyme called myeloperoxidase (MPO) in the development of NSCLC. Using a heterotopic NSCLC model in MPO-deficient mice, we observed a reduction of tumor growth that was accompanied by an increase in the infiltration of cytotoxic T cells as compared to WT mice. Moreover, the elimination of $CD8^+$ T cells reversed the previously observed decrease in tumor size in MPO KO mice. *In vitro*, MPO addition to T cells resulted in decreased proliferation and activation of T cells. Furthermore, MPO was internalized into T cells, while heparin pre-treatment blocked MPO internalization and reversed the anti-proliferative effect of MPO in T cells. Notably, MPO-positive lymphocytes were present in tumor samples obtained from NSCLC patients while an *in silico* analysis indicated that NSCLC patients with low MPO expression had a survival advantage.

In summary, our findings highlight the importance of neutrophils and neutrophil-derived enzymes in the biology of lung cancer and contribute to the understanding of the complex roles of these cells in cancer. Altogether, this dissertation offers ideas of how neutrophils and their molecules are potential candidates that can be used as diagnostic tools and therapeutic targets.

1 INTRODUCTION

1.1 Lung cancer

Based on the 2020 statistics from the World Health Organization (WHO), cancer is among the top causes of premature mortality (4). Lung cancer is the leading contributor to cancer-related deaths, responsible for 18% of all cancer-linked fatalities. It is also the second most prevalent cancer diagnosis, following breast cancer, constituting 11.4% of all cancer cases (4).

Based on its histological characteristics, lung cancer can be classified as small cell lung carcinoma, representing about 15 % of all lung cancers, and non-small cell lung cancer (NSCLC), comprising the remaining 85% (5,6). NSCLC is further subdivided into adenocarcinoma (most common subtype), squamous cell carcinoma and large-cell carcinoma (7).

Environmental and genetic factors have been associated with the development of lung cancer (5). Tobacco smoking as well as prolonged exposure to second-hand smoke lead the list of risk factors to develop lung cancer (5,8). Other important risk factors include long-term exposure to air pollution, occupational exposure to chemicals such asbestos, previous lung disease, inheritance of certain single-nucleotide polymorphisms and family history of lung cancer, among others (9). It is important to note that many cases of lung cancer are caused by a combination of these factors, rather than a single cause (9).

Although there have been significant developments in the diagnosis and treatment of cancer, lung cancer patients have not seen much improvement. Surgery can be a successful option for early-stage NSCLC, but conventional chemo- and radiotherapy have limited effectiveness (7). Additionally, new treatments like single-agent immune checkpoint inhibitors (ICI) have only partially succeeded, with only around 20% of NSCLC patients experiencing any benefits from this therapy (10). Late diagnosis of lung cancer, when the disease is advanced and treatment options are limited, results in a dismal 5-years survival rate below 18% (11).

Understanding the molecular and cellular mechanisms that drive lung cancer initiation and progression is crucial to improve early diagnosis and to generate new strategies to fight against this disease. In this regard, the complex and dynamic interactions between cancer cells and their surrounded niche, called tumor microenvironment (TME), not only play a critical role in cancer initiation, progression, and response to treatment, but also represents an opportunity to develop novel therapeutic approaches and better strategies for early diagnosis.

1.2 Tumor microenvironment (TME)

The TME is a heterogeneous and complex network of cellular and acellular components (12). Extracellular matrix (ECM), signaling molecules, mediators and blood vessels constitute the noncellular part of the TME (13). Besides, a wide variety of cells are also part of this unique niche including tumor cells, cancer stem cells, pericytes, adipocytes, fibroblasts, and immune cells (12).

The role of the TME in controlling carcinogenesis has been highlighted for decades (14,15). This intricate niche provides a favorable environment to proliferating cancer cells by generating new blood vessels to overcome hypoxia and supply nutrients. Moreover, the activation, differentiation, proliferation and apoptosis of tumor-infiltrating immune cells can be influence by the very rough conditions of the TME (e.g. acidic pH, hypoxic milieu and presence of immunosuppressive cytokines and chemokines) (16,17). An extra level of complexity is added being that the TME composition varies depending of the tumor entity and state of disease. The nature and extend of the infiltrating immune cells might determine the progress of the disease and the response to treatment (16). In this context, identifying and understanding the role of specific immune populations and leukocyte-derived molecules within the TME, will help us to develop new strategies to fight against cancer.

1.2.1 Immune cells of the TME

Immune cells can be classified as innate immune cells and adaptive immune cells. Adaptive immunity is triggered by exposure to specific antigens and utilizes immunological memory to better respond to threats (18). T cells and B cells are part of the adaptive immune response. Innate immunity, on the other hand, is a non-specific defense mechanism that is activated within minutes to hours of an external invader entering the body (18). Cells such as macrophages, neutrophils, natural killer (NK), eosinophils and dendritic cells (DC) comprise the innate immune response.

One of the mayor tasks of the cells of the immune system is to identify and eradicate aberrant cells in a process called immune surveillance (19). However, some cancer cells can escape from the immune system and later on facilitate the generation of tumors and development of an immunosuppressive TME (20).

Based on their role in tumor development, immune cells within the TME can be classified as tumor-antagonizing, and tumor-promoting cells (21). Infiltrating immune cells with anti-

tumorigenic capabilities include cells of the adaptive response such as $CD8^+$ cytotoxic T cells, effector $CD4^+$ T cells and DCs, as well as innate immune cells like NK cells (22). On the other hand, a broad diversity of immune cells that promote tumor development have been described including $CD4^+$ T helper 2 (Th2), regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAM). Moreover, cells such as B-cells, eosinophils and neutrophils seems to have more diverse roles acting in favor or against tumor development (22). It is important to highlight that this classification is to facilitate the organization of the information and with educational purposes. Almost all cells can change from pro- to anti-tumor and vice versa depending on the cellular context, type of tumor, comprehensive microenvironment composition, among many other factors. Therefore, the information in the following chapters needs to be approached keeping on mind that specific immune populations are versatile and have diverse and contrasting functions in cancer.

1.2.1.1 Tumor-antagonizing immune cells

$CD8^+$ T cells

T lymphocytes have been broadly studied in the setting of cancer due to their cytotoxic capabilities. In general, tumors infiltrated by cytotoxic T cells represent a positive outcome for patients with different kinds of cancer such as breast, ovarian, bladder, pancreatic and lung cancers (23–29). In fact, antigen specific cytotoxic $CD8^+$ T-cells can detect anomalous antigens found in cancer cells that are presented by major histocompatibility complex I (MHC-I) (18). Moreover, naïve $CD8^+$ T cells differentiate into effector and memory cells after antigen encounter by antigen presenting cells (30). In short, when naïve $CD8^+$ T cells ($CD44^{low}CD62L^{hi}$ in mice and $CD45RA^+CCR7^+$ in humans; cells that have not previously encountered foreign antigens) are exposed to antigens through the MHC-I complex, they become activated and rapidly proliferate within 1-2 weeks (30–32). They then transform into effector $CD8^+$ T cells ($CD44^{hi}CD62L^{low}$ in mice and $CD45RA^-CCR7^-$ in humans) that produce cytokines like interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and can eliminate target cells through either the granzyme/perforin-mediated cytotoxicity or the FAS/FAS ligand pathway (30-33). Once $CD8^+$ T cells have reached their peak in proliferation, roughly 90-95% of effector $CD8^+$ T cells undergo programmed cell death. The remaining cells, however, transform into memory $CD8^+$ T cells ($CD44^{hi}CD62L^{hi}$ in mice and $CD45RA^-$ $CCR7^+$ in humans) and stay dormant until they encounter the same or a similar antigen once more (30-32).

CD4+ Th1 cells

Activated CD4+ Th1 cells secret interleukine-2 (IL-2) which directly activates CD8+ T cells (34). Furthermore, CD4+ T cells can indirectly assist CD8+ T cells by sustaining pro-inflammatory dendritic cells involved in cross-presentation. An additional method through which CD4+ Th1 cells can directly combat tumor cells is by generating cytokines like IFN-γ and TNF-α (34).

NK and NKT cells

High infiltration of NK and NKT cells has been associated with enhanced overall survival in a diversity of solid tumors (35,36). NK cells are especially effective on killing circulating cancer cells and cells with aberrant or absent MHC-I expression through the activation of two major mechanisms; the release of lytic granules that contain perforin and granzymes or the activation of the death receptor-mediated apoptosis (37,38).

Dendritic cells (DC)

DCs are antigen-presenting cells that infiltrate tumors and process and present tumor-derived antigens to naïve T cells (39). Their role in priming the anti-tumor T cell response is fundamental. DCs are classified in different subtypes with specific roles in cancer (39). Conventional DC hold the highest potency among antigen-presenting cells and, consequently, are robust initiators of T cell-mediated immune reactions. (39). Plasmacytoid DC (pDC) are major producers of type I IFNs (type I IFNs comprise IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, IFN-ω and IFN-ζ; type II IFNs comprise IFN-γ; type III IFNs comprise IFN-λ1 ,IFN- λ2 ,IFN-λ3 and IFN-λ4) that can promote anti-tumor immunity (39).

1.2.1.2 Tumor-promoting immune cells

CD4+ T cells

Among the different CD4+ T-cell populations that infiltrate tumors, CD4+ Th2 cells and T regulatory cells (Tregs) are generally described as tumor promoting. In fact, high infiltration of Tregs into tumors has been linked to worse prognosis in breast (40), ovarian (41) and pancreatic (42) cancer, among others. Cytokines such as IL-4, IL-5 and IL-13 are produced by CD4+ Th2 cells and promote tumor growth (12,43). By their part, Tregs, characterized by the expression of FOXP3 and CD25 (44), exert an immunosuppressive function (45). Production of IL-10 and transforming growth factor beta (TGF-b) are some of the mechanisms by which Tregs constrain

the recognition and elimination of tumor cells (45). Cytolysis of effector T cells, metabolic disruption, and DC suppression are other mechanisms by which Tregs exert their suppressive function (46).

Tumor-associated macrophages (TAMs)

Macrophages play a central role in endorsing tumor growth and can represent up to 50% of tumor mass (47). Increased macrophage presence is associated with a worse prognosis for cancer patients with various types of tumors (48). The release of some hypoxia-induced chemoattractants such as vascular endothelial growth factor (VEGF) promote the recruitment of TAMs that usually accumulate in hypoxic or necrotic tumor areas (49). Moreover, hypoxia can also favor the secretion of cytokines such IL-4 therefore supporting the immunosuppressive phenotype of macrophages (M2) (50). The role of macrophages in supporting angiogenesis is well documented (51,52). Moreover, TAMs also promote malignant cell migration, invasion and metastasis (53–55).

Myeloid-derived suppressor cells (MDSC)

MDSCs are inhibitory cells that can have a polymorphonuclear (PMN-MDSC) or monocytic (M-MDSC) origin (56,57). Elevated numbers of MDSC are associated with an unfavorable prognosis in patients with various cancer types, including breast cancer and melanoma (58–60). MDSCs are involved in many pro-tumor functions including the inhibition of the activation of cytotoxic $CD8^+$ T cells (61), the development of Tregs (62,63), the polarization of macrophages to a M2 phenotype (64), the increase of angiogenesis (65) and the enhancement of tumor cell stemness (66).

1.2.1.3 Immune cells with conflicting roles in cancer

B cells

The role of B cells in the TME is complex (67). The existence of B cells within certain tumors has been linked to improved patient outcomes in cervical cancer (68) and NSCLC (25,69). Conversely, the presence of B cells is associated with a poorer overall survival in patients with bladder, breast, colorectal cancer, and other malignancies (70).

B cells can infiltrate tumors, where they often gather in specific structures called tertiary lymphoid structures (TLS) (70). B cells can help kill tumor cells directly by activating the

Fas/FasL or TRAIL/Apo2L pathways (71,72). Moreover, B cells produce IFN-γ, that recruits and activates NK cells and polarizes T cells towards Th1 (70,73). B cells can also produce anti-tumor antibodies that target specific proteins on the surface of cancer cells (70).
Conversely, certain antibodies can expedite tumor progression (70,74,75), and there exists an immunosuppressive category of B cells, called regulatory B cells, which can hinder the activity of $CD4^+$ and $CD8^+$ T while supporting tumor growth. (70,76).

Eosinophils

Eosinophils can exert both positive and negative influences on tumors. Their impact on TME can vary based on the specific type of tumor and its surroundings (77,78). Research indicates that elevated eosinophil levels in both the bloodstream and within tumors can be advantageous for several cancer types (77). In experimental tumor settings, eosinophils have the capacity to directly inhibit tumor growth by releasing their contents or by attracting other immune cells with anti-tumor properties(79–81). Nevertheless, eosinophils have also been linked to tumor-promoting functions such the secretion of pro-angiogenic and matrix-remodeling soluble mediators (77,78).

Neutrophils

Neutrophils have been more recently pointed out as an immune population that influence tumor growth, however their roles are rather complex and will be discussed with more detail in the following section.

1.3 Neutrophils in cancer

Neutrophils are innate immune cells that have a short lifespan and make up the majority (50-70%) of all immune cells in the bloodstream (82). They are rapidly recruited into site of inflammation where they quickly respond to trap and kill invading pathogens (83). Their physiological roles mainly involve the clearance of pathogens thought mechanisms such phagocytosis, degranulation, and the formation of extracellular neutrophil-derived networks of DNA, fibers and proteins called neutrophil extracellular traps (NETs) (84). However, current research has revealed that neutrophils are complex, transcriptionally active cells that respond to multiple signals and can release cytokines and inflammatory molecules that regulate inflammation. More recently, their role in cancer has come to the spotlight and more and more

investigations show that neutrophils are a heterogeneous cell population with phenotypic plasticity that possess anti- and protumor roles (82,85).

In terms of clinical implications, neutrophils are important components of the TME and increased neutrophil numbers have been reported in a variety of cancers. To notice, neutrophils constitute one of the dominant populations in the TME of NSCLC (86). Moreover, absolute neutrophil counts and the neutrophil to lymphocyte ratio (NLR) have been linked with poorer outcomes in several types of cancer including gastric, melanoma, breast and NSCLC (87–91). NLR has been proposed as a potential biomarker for identifying cancer patients at risk and response to treatment (92–95). Some studies have reported that NLR was able to differentiate between NSCLC patients who responded well to anti-PD-1 therapy and those who had stable or worsening conditions (87,96).

1.3.1 Neutrophil recruitment to the TME

Neutrophils are recruited into the TME through a complex process that happens in different stages and involves a series of cytokines, chemokines and receptors (97). Firstly, pre-mature neutrophils are expanded and matured in the bone marrow from hematopoietic stem cells, secondly, they attach to endothelial cells and enter circulation, and lastly, they move towards the tumor site in response to chemical signals (98). The process of neutrophil maturation is regulated by two factors: granulocyte colony-stimulating factor (G-CSF in mice, and its homolog in humans) and granulocyte macrophage colony-stimulating factor (GM-CSF in mice, and its homolog in humans) (82). As the neutrophils mature, their nucleus changes from a round shape to a segmented shape, and the surface antigen expression also changes (99).

The participation of two G-protein coupled receptors of the CXC chemokine receptor family, CXCR4 and CXCR2 in mice (and their homologs in humans), and their corresponding ligands, is crucial on the mobilization of neutrophils from the bone marrow to the bloodstream, tissues and tumors (100,101). Both receptors are expressed on the surface of neutrophils but exert contrasting functions. CXCR4 and its ligands, such as CXCL12, restrict neutrophil mobility; while CXCR2 (and its ligands) is mainly responsible of the release of neutrophils (100,101). Another important regulator of neutrophils recruitment is G-CSF, a cytokine that negatively regulates CXCR4, thus positively regulating neutrophil migration (102). The balance of neutrophils in circulation is orchestrated by the G-CSF and the opposing interaction between CXCR2 and

CXCR4 and any dysregulation on the expression of these molecules will impact the mobilization of neutrophils (100,103,104). Within the TME, cancer cells, immune cells and cancer-associated fibroblasts are important producers of CXCR2 chemokines, while G-CSF is release by cancer cells and macrophages (105,106). The higher concentrations of CXCR2 chemokines, such as CXCL1-3 and CXCL5-8, and G-CSF together with higher expression of CXCR2 by neutrophils play a dominant role in the recruitment of neutrophils into the TME (102,107,108). Other chemokines including interleukin-8 (IL-8 or CXCL8) and members of the IL-17 family are also key components of neutrophil recruitment by directly acting as chemo-attractants or by regulating the expression and secretion of chemokines and ligands (109–111) .

1.4 Neutrophil heterogeneity and phenotypical plasticity in cancer

Factors from tumor cells in the surrounding environment can alter the appearance and behavior of myeloid cells, resulting in distinct sub-groups (106). These sub-groups are highly adaptable and can undergo significant changes in response to cytokine signals, epigenetic modifications, and other environmental factors (106). This often leads to alterations in the structure and functions of these cells. The so called “Th1/Th2 paradigm” originally described the dichotomy on the pathogen-dependent immune response of T cells to the production of specific cytokines (107). According to this, Th1 cells lead to a pro-inflammatory response, while Th2 cells are responsible for an immunosuppressive response (107). Later, myeloid cells such as TAMs were shown to also reflect the Th1/Th2 paradigm based on their activation status: the pro-inflammatory M1 type and the anti-inflammatory or immunosuppressive M2 type, with additional subtypes added more recently (108). Within the TME, TAMs can shift from an antitumor (M1) to a protumor (M2) activation mode due to the highly active and complex secretions from the surrounding environment (109). Similarly, tumor-associated neutrophils (TANs) can exhibit varying levels of phenotypic adaptability as a response to various stimuli in the TME as will be described in the following section.

1.4.1 Tumor-associated neutrophils (TANs): N1 and N2 phenotypes

TANs can be classified into two polarization states, N1 (anti-tumor) and N2 (pro-tumor) (110, 111). Some studies have shown that when exposed to regulatory factors like G-CSF or TGFβ, neutrophils transform into the N2 phenotype (82,106,112). N2 phenotype is characterized by

increased expression of pro-tumor factors that promote immunosuppression within the TME (82,112). Examples of those actors are CCL2, CCL5, neutrophil elastase (NE) and cathepsin G, along with higher levels of arginase (ARG-1) (97,112). On the other hand, blocking TGFβ or type I IFNs signaling results in neutrophils with the N1 phenotype that are characterized by a hyper-segmented nucleus and exert cytotoxic capabilities to kill cancer cells (112,113). N1 neutrophils have higher expression of immuno-activating cytokines and chemokines such as TNF-α, ICAM-1, and FAS (112). Although functional differences of TANs within the TME are evident, there is currently no appropriate marker to distinguish between N1 and N2 neutrophils in the tumor (97).

1.4.2 Circulating neutrophils: High vs low-density neutrophils

Cancer patients have been found to have a mix of immature and mature neutrophils in their bloodstream (114). The so-called low-density neutrophils (LDNs) were first reported in 1986 as a result of the method used to separate neutrophils from blood (115). The standard procedure involves separating leukocytes through density-gradient centrifugation, where blood is layered on top of a density medium like Ficoll-Paque (115). During centrifugation, cells with higher density, including neutrophils, settle just above red blood cells on the bottom of the tube while peripheral blood mononuclear cells (PBMC) locate in the upper portion of the tube (low-density fraction) (115). Under health conditions, neutrophils are usually only found in the lower fraction and are known as high-density neutrophils (HDNs). However, during inflammatory processes, neutrophils can also be found in the PBMC fraction therefore called LDNs. Among the PBMC fraction, cells with neutrophil morphology were originally found in patients diagnosed with systemic lupus erythematosus and rheumatoid arthritis (116–118). Since then, the presence of LDNs has been reported in diverse inflammatory diseases including different kinds of cancer (114,119), psoriasis (120), asthma (121), sepsis, (ANCA)-associated vasculitis (122), and infections such as HIV (123), *Plasmodium vivax* (124) and *Mycobacterium tuberculosis* (125,126). While initially believed that LDNs were present only under pathological or inflammatory circumstances, LDNs have also been reported in the blood of pregnant women (127) and in healthy individuals (128).

Increase interest in the study of LDNs is evident, especially in the clinical context, as LDNs content often appears to correlate with disease aggressiveness and/or response to treatment (114,129–131). Despite this, the origin, composition, and function of this neutrophil population remain controversial. Differences based on the nucleus morphology, suggest that LDNs are a

mixed population of immature neutrophils with ring- or band-shaped nuclei and mature neutrophils with segmented nuclei, in comparison with HDNs that comprise a homogeneous population of mature cells (114).

In cancer, LDNs have been found in mouse models of mesothelioma, breast and lung cancer (114). Moreover, patients with breast, head and neck, lung and urologic cancers have also shown the presence of LDNs in their peripheral blood (86,114,129,132). To the resemblance of N1 and N2 TAN phenotypes, Sagiv et al. reported that HDNs exert anti-tumor capabilities, while LDNs are immunosuppressive cells with reduced anti-tumor cytotoxicity, impaired phagocytic activity, decrease migratory capacity and less oxidative burst (114). Phenotypic plasticity has been shown in tumor-bearing mice, where TGF-β can be used to switch primed HDNs into LDNs (114). Interestingly, the ratio of circulating HDNs to LDNs can change as the tumor progresses (114). Based on this, it has been suggested that HDNs and LDNs correspond to the N1 and N2 phenotypes of TANs, respectively, and presumably operate through similar mechanisms. However, there is still a lack of validated surface markers that can define LDNs and further research is necessary to characterize this neutrophil population phenotypically and functionally.

1.5 Functions of neutrophils in the TME

As previously stated, neutrophils represent a highly heterogeneous immune population. This diversity is reflected not only phenotypically but also functionally. In cancer, neutrophils play opposing roles as it will be described in the following sections.

1.5.1 Anti-tumorigenic functions of neutrophils

Neutrophils can limit tumor growth by direct or indirect mechanisms that can or cannot require physical contact of neutrophils with target cells. *In vitro* co-culture of murine neutrophils and cancer cell lines, revealed that hydrogen peroxide (H_2O_2) is secreted by neutrophils after their contact with cancer cells (133). Secretion of H_2O_2, promotes the influx of Ca^{2+} in a mechanism that involves the H_2O_2-dependent TRPM2 Ca^{2+} channel, which ultimately results in the dead of cancer cells (133). Other strong neutrophil-derived oxidants such as hypochlorous acid (HOCl) also exerts direct cytotoxic properties against cancer cells (134,135). Inducible nitric oxide synthase (iNOS) production by neutrophils has also cytotoxic effects in cancer cells (136). In some models, it has been shown that the recruitment of anti-tumor neutrophils is favored by the

hypoxic conditions of the TME (137). Moreover, the secretion of certain cytokines such as TNF-α, and proteases such as Cathepsin G by neutrophils favors the recruitment, proliferation and activation of T cells, thus indirectly fighting against the tumor (138). Moreover, in early-stage lung cancer patients, TANs can stimulate T cell proliferation and IFN-γ secretion (139). Furthermore, neutrophils can also facilitate adaptive immune response since resent research has shown that not only macrophages and DC, but also neutrophils, can present antigens to T cells (140). Antibody-dependent cellular cytotoxicity and target-specific antibody cytotoxicity are other mechanisms that neutrophils use against cancer (141–143). More recently, the role of neutrophils in positively mediating ICI has been highlighted. A study from Gungabeesoon et al. showed that, *in vivo*, neutrophils of ICI-treated mice acquired an IFN gene signature and might be crucial for the success of anti-cancer therapy (144). Moreover, Hirschhorn et al. revealed that neutrophils might have an important function by eliminating antigen loss variants in melanoma-bearing mice that were treated with a combination of adoptive T cell transfer therapy and ICI (145). They found that an anti-tumorigenic subset of neutrophils was present in treated mice and that total eradication of melanoma tumors was dependent of neutrophils in a mechanism that involves iNOS (145).

1.5.2 Pro-tumorigenic roles of neutrophils

Diverse evidence has shown that neutrophils can act as pro-tumorigenic cells though different phases of the carcinogenic pathway, from the initiation to the metastatic spread (82). Reactive oxygen species (ROS) and reactive nitrogen species as well as neutrophil-derived proteases such NE can damage epithelium and support the transformation of epithelial cells to cancer cells (82). Moreover, high neutrophil infiltration observed in inflammation-induced models of cancer, has been linked with higher rates of tumor formation, while the inhibition or deficiency of CXCR2 receptors and its ligands (necessary for neutrophil recruitment), resulted in retarded tumor formation (146–149).

In addition, neutrophils can also participate in the promotion of tumor growth by inducing proliferation of cancer cells, remodeling the ECM and inducing angiogenesis (82,150). NE can be taken up by cancer cells with the consequent degradation of insulin receptor substrate 1 (IRS1) which promotes PI3K signaling and cell proliferation (151). Furthermore, a subset of neutrophils has shown to inhibit the senescent state of cancer cells favoring their proliferation (152). Production and release of matrix metalloproteinase 9 (MMP9) has been link with the

activation of vascular endothelial growth factor (VEGFA) and subsequent induction of angiogenesis (153,154). Neutrophils can also act as immunosuppressive cells supporting tumor progression. ARG-1 and iNOS expressed by neutrophils can suppress $CD8^+$ T-cell-mediated antitumor immune response (155,156). ARG-1 metabolizes L-arginine to L-ornithine and urea. Being that arginine is an essential amino acid for the correct function of T-cell receptor, high expression and released of ARG-1 by neutrophils in the TME, with the consequent depletion of extracellular arginine, results in a profound suppression of T-cell responses in mice and humans cancers (156–158). iNOS metabolizes L-arginine to NO and citrulline and it has also been shown to be responsible of the immunosuppressive role of neutrophils (159). Interestingly, inhibition of ARG-1 and iNOS has shown to improve the response to ICI in cancer models (160,161).

Neutrophils can also facilitate the initiation of metastasis (162). Circulating cancer cells can be trapped by neutrophil-released NETs, promoting the adhesion of cancer cells at distant organs (163). There is a suggested role for neutrophils in directing cancer cells into tissues and facilitating their retention, as certain research has identified instances where cancer cells colocalize with endothelial cell-associated neutrophils (162). Additionally, the presence of neutrophil clusters alongside circulating tumor cells in the peripheral blood of breast cancer patients and in mouse models could further indicate the involvement of neutrophils in enhancing metastatic potential (164).

1.6 Neutrophil granules

As it has been already stated, neutrophils are versatile cells with contrasting roles in cancer. It is getting clearer that the composition of neutrophil cytoplasmic granules is closely linked to their functionality.

Besides their segmented nucleus (two to five lobes connected by thin chromatin filaments), neutrophil morphology is easily recognized by the presence of plentiful cytoplasmic granules that can be classified as primary or azurophilic granules, secondary or specific granules and tertiary or gelatinase granules (165). Primary granules contain MPO as well as other proteolytical and bactericidal proteins such as elastase, proteinase-3, cathepsins, glucuronidase, lysozyme, defensins, among others (166,167). Secondary granules contain lactoferrin, lipocalin/NGAL, collagenase, gelatinase, histaminase, lysozyme, membrane receptors and so on. Tertiary granules are enriched in MMP9 but also contain adhesion molecules (e.g. CD11b), plasma

proteins, alkaline phosphatase, ARG-1, membrane receptors, cytochrome b558 (NOX2), etc. (167). Besides their differences in composition, neutrophil granules are also discriminated by its hierarchy to mobilize upon an stimuli: secondary and tertiary granules become quicker incorporated into the cell surface upon neutrophil activation, while primary granules are usually mobilized later (166,168). The mobilization of secondary and tertiary granules modulates neutrophil functionality being that they contain several plasma membrane proteins that are involved in the production of superoxide anion as well as in neutrophil adhesion and diapedesis/extravasation (166). The release of primary granules is connected with priming and activation of neutrophils and triggers them to fully initiate a response against invaders (166).

The process how neutrophils degranulate and release their content into the extracellular medium is complex and involves a series of steps that initiate with neutrophil extravasation (neutrophil recruitment cascade: tethering, rolling, adhesion, crawling and transmigration) (83) which refers to the mobilization of neutrophils from the blood stream to the site of inflammation. The contact of neutrophils with the endothelium (adhesion) induces neutrophil activation. Full activation of neutrophils involves two steps: priming and activation. Priming is needed to achieve the highest level of neutrophils degranulation and activation of the NADPH oxidase pathway (83). Exposure of neutrophils to pathogen-associated molecular patterns PAMPs, damage-associated molecular patterns (DAMPs), chemoattractants or grow factors in endothelial cells, triggers neutrophil priming (83). Moreover, pro-inflammatory cytokines such as TNF-α can also trigger the priming of neutrophils (83). Neutrophil activation is mainly regulated by chemokines such as CXCL8 (IL-8) in humans and CXCL1, CXCL2 and CXCL5 in mice (169). Such chemokines signal via CXCR2 to activate neutrophils and promote their adhesion to endothelium (170). There are diverse ways in which neutrophil can resolve inflammation: phagocytosis of recognized pathogens, degranulation, oxidative burst and formation of NETs (84). Finally, neutrophils are removed from the tissue by macrophage phagocytosis (98).

Remarkably, several neutrophil granule-derived proteins have been associated with cancer progression. As it has been described in previous chapters, NE and MMP9 are some examples of neutrophil-derived molecules that contribute to tumor development. In this regard MPO has most recently emerged as a novel player that can shed light into the complex role of neutrophils in cancer.

1.7 Myeloperoxidase (MPO)

MPO is a member of the heme peroxidase family (171). MPO is a highly positive charged dimer of approximately 146 kDa formed by two functionally independent identical monomers of 73 kDa each. Each monomer consists of a light chain (14.5 kDa), a heavy chain (58.5 kDa), a heme group in the active site, at least three sites of asparagine linked glycosylation sites, and a bound calcium ion (172–175). The biosynthesis of MPO starts in the bone marrow in promyelocytic myeloid precursor cells and its expression is terminated during monocyte-to-macrophage differentiation (176). Fully differentiated neutrophils store mature MPO in their primary (azurophilic) granules and it represents up to 5% of the total dry weight of neutrophils (177). Macrophages and monocytes are also a source of MPO, although at a lesser extent (178). Once in neutrophils granules, MPO can be release to the extracellular space through various mechanisms. Inflammatory mediators such as Toll-like receptor ligands and cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF can prime and activate neutrophils and promote the secretion of MPO (179–181). Death pathways including apoptosis and necrosis also allow the release of MPO (182–184). Moreover, the extrusion of NETs has been reported as another mechanism of MPO's release (185). MPO catalytic activity includes the halogenation cycle and the peroxidase cycle (186). Many of the functions of MPO are related to its ability to use H_2O_2 as a substrate to oxidize halide and pseudohalide ions (halogenation cycle) which results in the generation of hypohalous acids including HOCl (from Cl-), HOBr (hypobromous acid from Br-), HOI (hypoiodous acid form I-) and HOSCN (hypothiocyanous acid from SCN-) (186). The high plasma concentration of chloride (Cl-, 100-140 μM) favors the generation of HOCl by MPO (186). Besides hypohalous acids, MPO also catalyzes the formation of other reactive intermediates like reactive nitrogen species and tyrosyl radicals (186).

1.7.1 Physiological functions of MPO

Killing of microorganisms is one of the main physiological functions of MPO and it is closely related to the activation of the MPO–H_2O_2–halide system with the subsequent production of oxidants such as HOCl (187). In fact, MPO highly contributes to the innate immune role of neutrophils in killing bacteria and fungus (188,189). Moreover, some studies suggest that MPO

can also regulate the release and activity of proteases such as NE and MMP9, therefore protecting pericellular tissues from uncontrolled proteolysis (190).

MPO also participates in the regulation of neutrophils recruitment and apoptosis. It has been described that MPO binds to an integrin complex formed by CD11b/CD18 (necessary for the adhesion to the endothelium and further extravasation of neutrophils) in neutrophils acting as a chemoattractant and protecting them from apoptosis (191,192). However, MPO role in regulating neutrophils apoptosis seems to be more complex since other research groups have shown that the use of the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) blocked TNF-α-induced apoptosis in neutrophils (193). Moreover, MPO-deficient neutrophils showed phorbol ester-induced apoptosis when compared with WT PMNs (194).

Other physiological functions of MPO include neutrophil-macrophage interactions, the formation of lipophilic chloramines with a subsequent suppression of pro-inflammatory pathways in macrophages, and the binding of MPO to serum proteins such albumin (2).

1.7.2 Pathological functions of MPO

MPO participates in the pathogenesis of diseases that are, usually, accompanied by high infiltration of neutrophils and involve acute or chronic inflammation. Research has especially focused in cardiovascular diseases where MPO expression has been associated with the clinical risk, severity, and outcome of diverse cardiovascular diseases (195–199). In this regard, high concentrations of active MPO has been found in circulation and arteries of patients diagnosed with stable coronary artery disease and acute coronary syndrome (200,201). Moreover, MPO and its derived oxidants are found in the endothelium of vessels of patients with atherosclerosis (202,203). The involvement of MPO in atherosclerosis development and progression is extensively documented. MPO plays a role in affecting the endothelium and promoting endothelial dysfunction, which represents an early stage preceding the formation of atherosclerotic plaques. The effects of MPO in endothelium include the alteration of ECM proteins (204–206), the decrease in the bioavailability of nitric oxide (207,208), the regulation of signaling pathways that modify vascular function (209) and the destruction of endothelial glycocalyx (210). Later, MPO can oxidize albumin and lipoproteins such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL) contributing to the progression of atherosclerotic lesions (*185*, *186*).

Besides atherosclerosis, MPO is involved in respiratory diseases. Patients with cystic fibrosis and chronic obstructive pulmonary disorder contain active MPO in sputum and bronchoalveolar fluid (213–215). Moreover, the airways of children with cystic fibrosis exhibit high levels of HOCl-derived oxidized compounds (216). High amounts of MPO in cystic fibrosis might be correlated with airway obstruction (217). Some studies suggest that the roles of MPO in respiratory diseases are linked to the action of its derived oxidants in the damage of proteins and protease inhibitors (218,219), however more studies are needed to better understand the actions of MPO in respiratory diseases.

MPO has also been related to neurodegenerative diseases in which increased amounts of MPO have been found in patients suffering of Alzheimer´s disease, Parkinson´s disease and multiple sclerosis particularly in the brain regions affected with neurodegeneration (220–222). On the context of Parkinson's disease, MPO is found within neurons in the substantia nigra, which is associated with elevated nitration and aggregation of α-synuclein, ultimately worsening motor impairment (223). Additionally, in mice, the abnormal expression of MPO in astrocytes and microglia may account for certain impacts of MPO in the process of neurodegeneration.(224). Some of the effects of MPO in neurodegeneration have been linked to the action of MPO-derived HOCl; in this sense, necrosis or apoptosis or neurons due to high concentrations of HOCl can be a cause of neurodegeneration (225). Moreover, damage of myelin sheath and neuronal injury by HOCl could explain the neurodegenerative action of MPO in multiple sclerosis (226). Remarkably, MPO and its derived oxidants can enhance the permeability of the blood/brain barrier by promoting endothelial damage (227).

1.7.3 MPO in cancer

In recent years, there has been a growing interest in exploring the involvement of MPO in cancer, leading to dedicated research efforts. Although the field is still relatively new, several reports have discussed the involvement of this enzyme in cancer regulation. MPO exhibits both pro- and anti-tumor properties, but the majority of evidence emphasizes its role in promoting tumor initiation and progression (2). MPO contributes to tumor initiation by creating a hyper-mutagenic environment through the action of MPO-derived oxidants that oxidize and modify DNA. Additionally, the presence of MPO influences cancer progression by affecting tumor growth, apoptosis, cell migration, and metastasis. Some studies also suggest that MPO may

play a role in regulating adaptive immunity in cancer. The following sections provide evidence that sheds light on the emerging roles of MPO in cancer development and progression.

1.7.3.1 MPO polymorphism

The connection between cancer risk and MPO expression has been extensively studied, particularly concerning the MPO polymorphism MPO-463G > A. (228,229). This polymorphism (situated in the promoter region of the MPO gene) negatively affects the mRNA expression and transcription of MPO. Less expression of MPO could be linked with reduced oxidative stress and dismiss risk of cancer. Several case-control and meta-analysis have been performed in lung cancer, breast cancer, gastric carcinoma, prostate cancer, cervical cancer, among others to clarify the relation between the MPO polymorphism and cancer risk. Data suggest that MPO polymorphism MPO-463G > A may represent a reduced risk for certain cancers (230,231) . This could be particularly relevant in cancers in which a decreased metabolic activation of procarcinogens is relevant, such as lung cancer (230,231). Besides the MPO polymorphism, some studies have reported higher expression of MPO in serum and bronchoalveolar fluid of patients with NSCLC (232) and MPO-positive cells in tumors have been used as a prognostic factor in some cancers (233–237).

1.7.3.2 MPO in tumor initiation

The participation of MPO in the promotion of tumor initiation seems to have especial importance is the context of lung cancer. It is well known that long exposure to ROS can result in DNA damage and increased genomic instability which can lead to alteration in cell proliferation and apoptosis and contribute to the formation of tumors (238). In this sense, lungs are the main target for many carcinogenic compounds (e.g., tobacco smoke, asbestos, ozone) and neutrophils are usually recruited to the lungs as a response to the contact of such compounds with lung tissue. Once in lung tissue, neutrophils are activated and can contribute to tumor formation and progression due to the release of ROS (239). In this sense, it has been reported that MPO-derived HOCl was able to induce mutations in genes of the LUAD cell line A549 (240). Moreover, a higher number of DNA adducts have been observed in mice with induced pulmonary inflammation and high influx of neutrophils (240). Other studies have shown that the exposure of A549 cells to extracellular MPO promoted the formation of early intermediates of DNA oxidation known as DNA-centered radicals (241). Interestingly, DNA-centered radicals

were also observed in co-culture experiments of PMA-activated neutrophils and A549 cells, suggesting that this process could also occurs in more complex scenarios (241). MPO (through the generation of excessive HOCl and HOBr) can also contribute to the oxidation of nitrogenous bases and generate 5-chlorouracil, 5-chlorocytosine and 5-bromouracil which can be mutagenic (242,243).

MPO has also been linked to the transformation of environmental pollutants and procarcinogens to their carcinogenic form (244,245). In this sense, MPO can interact with benzo(a)pyrene (BaP, a common component of cigarette smoke) and generate reactive metabolites such as BaP-diol-epoxide which is capable to form DNA adducts (244). Irreversible binding of arylamine carcinogens to DNA strands has been also documented to be mediated by MPO (246) and some reports suggest that DNA repair mechanisms may be inhibited MPO-derived compounds (247,248).

1.7.3.3 MPO in tumor progression

MPO might be implicated in regulation of tumor growth as has been reported by some research groups. Increased tumor growth was observed in primary breast tumors of mice that were intra-tumoral treated with MPO when compared with tumors of mice without MPO treatment (249). Similarly, in an inflammation-induced mouse lung cancer model, the use of an MPO inhibitor, KYC (N-acetyl lysyltyrosylcysteine), reduced tumor burden when compared to control mice that did not receive the inhibitor (250). A reduction in tumor size has also been observed in a tumor graft model in MPO knockout mice using Lewis lung carcinoma (LLC) cells (250). Besides these interesting reports, there are still no data regarding a putative mechanism that explains how MPO positively regulate tumor growth. On the other hand, a recent study showed that orthotopic melanomas in aged mice grew faster in MPO knockout mice and mice treated with the MPO inhibitor ABAH when compared with the respective controls (251). The suggested mechanism implicated the trans-inhibition of the NF-κB kinase activity by MPO followed a reduction in the transcriptional activity of NF-κB with subsequent changes in the expression of genes that regulate metabolic activity, cell cycle and DNA replication in melanoma cells (251).

Besides its role tumor growth, MPO might be also implicated in the regulation of apoptosis in cancer cells. On one side, MPO expression by ovarian cancer cells and ovarian cancer tissue correlated with a reduction of apoptosis in a mechanism that involves the S-nitrosylation of caspase-3 by nitric oxide (252). On the other side, high amounts of HOCl (the most common

MPO-derived oxidant) can induce tumor cell death (253,254). In this context, malignant cells produce high amounts of extracellular superoxide anions (due to the actions of NOX-1) which react with HOCl to generate apoptosis-inducing hydroxyl radicals ultimately resulting in cell dead (255). However, cancer cells have found a way to overcome this process by preventing the synthesis of HOCl using membrane-associated catalase (256,257). Other study revealed that MPO can potentiate the apoptotic effect of the polyphenol (-)-epigallocatechin-3-gallate (EGCG) in myeloid leukemia cells, effect that was blocked with the MPO inhibitor ABAH, or when an enzymatically inactive MPO was used (258). Further research is necessary to elucidate the mechanisms by which MPO and its derived oxidants regulate apoptosis of cancer cells.

MPO might regulate migration of cancer cells since the mobility of mammary carcinoma cells and fibroblast was increased by MPO (249). Besides, mouse breast cancer cells treated with MPO resulted in an enhanced expression of MMP1 mRNA (ECM protein that is associated with organ specific metastasis to the lungs) as well as an increased expression of other ECM components such as MMP1, MMP3, collagen I and collagen IV in fibroblasts, which are important components of the TME (249). These effects have been linked to increased tumor cell adhesion and invasion of breast cancer cells *in vitro* (Panagopoulos et al., 2017).

MPO is also an important component of NETs and contribute to NET formation by driving chromatin decondensation (259,260). In this sense, MPO could also indirectly contribute to cancer development through its involvement in NET formation. NETs have been linked to some of the neutrophils actions in cancer and several reports remark NETs participation in with cancer progression, metastatic spread, cancer associated thrombosis, cancer immunoediting and awaking of dormant cancer cells (163,261–263).

1.7.3.4 MPO and regulation of adaptive immunity

MPO could also indirectly modulate cancer development through the regulation of adaptive immunity. The oxidation of proteins by HOCl could enhance their immunogenicity as have been shown with oxidized proteins and glycoproteins that bind with higher affinity to receptors of murine DCs and macrophages (264) or oxidized ovoalbumin that is processed and presented to T cells by DCs more efficiently when compared to native ovoalbumin (265). Other research revealed that the oxidation of proteins in ovarian cancer cells by HOCl facilitated the phagocytosis of the malignant cells by monocyte-derived DCs (266). Moreover, co-culture of T cells and DCs with oxidized ovarian cancer cells resulted in an increase of IFN-γ production by

T-cells (266). Further studies revealed that DCs that were triggered by HOCl-oxidized ovarian cancer cells generated a polyclonal anti-tumor response and were able to recognize autologous tumor (267).

MPO has been observed to have a detrimental effect on the activation ofDCs in the lymph nodes whereas the deletion of MPO resulted in an improved activation and proliferation $CD4^+$ T cell activation (183). On the other hand, a recent study showed that MPO-derived HOCl encourages myeloid-derived cell and CD8 cytotoxic T cell migration towards the periphery of melanomas, resulting in a reduction in tumor size(251). Moreover, when $CD8^+$ T-cells were exposed to HOCl, it had a positive impact on the expression of genes related to T-cell activation, including those involved in the MAPK and PI3K-AKT signaling pathways.(251)

Remarkably, a report from 2023 found that both MPO deficiency enhanced the repose to ICI in an *in vivo* model of melanoma using aged mice (268). Moreover, the use of specific MPO inhibitors in combination with ICI therapy enhanced the response rates when compared with the ICI therapy alone (268). In the same study, a significant increase of myeloid cells and lymphoid populations was observed in the TME of MPO-deficient mice when compare to wild type mice (268).

1.8 Hypothesis and aims of the thesis

The importance of neutrophils in controlling lung cancer development has been established by large evidence. However, neutrophils are complex cells that exhibit diverse phenotypes and secrete a variety of molecules which play varied roles in cancer. In the end, further investigation of the impact of neutrophils in regulating NSCLC development is needed.

The first aim of the thesis was to identify specific surface markers that allow us to discriminate between LDNs and HDNs in patients with NSCLC. Based on previous reports, we hypothesized that LDNs are a neutrophil population found in NSCLC patients and that they express surface markers that are not found in HDNs. To prove this, we used an unbiased high-dimensional flow cytometry screening, which together with bioinformatic tools, allowed us to identify surface proteins specifically expressed by LDNs. We finally validated the results of the top overexpressed markers using flow cytometry.

The second goal of this thesis was to unravel the role of MPO, a neutrophil-derived enzyme, in the development of NSCLC. Our hypothesis was that MPO would positively contribute to tumor growth. To prove this, we used MPO KO mice and WT mice and compared tumor growth and

immune cell infiltration. We next treated T cells with MPO and investigated how the enzyme affects lymphocyte proliferation and activation *in vitro*.

In detail, identifying specific surface markers that allow us to discriminate between distinct neutrophil populations as well as understanding the role of neutrophil-derived molecules in the pathogenesis of lung cancer is crucial to develop better strategies to fight against this disease. In this sense LDNs and MPO might represent novel biomarkers and potential therapeutic targets, respectively.

2 MATERIALS AND METHODS

As parts of this dissertation have previously been published as an original research article in Frontiers in Immunology (1) and in a preprint in BioRxiv (3) the Materials and Methods section has been partially adapted from the article and any resemblances in regards to content and phrasing are to be expected.

2.1 Study approval for working with clinical samples

The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Medical University of Graz (EK-numbers: 30-105 ex17/18, 29-593 ex 16/17 and 17-291 ex 05/06).

2.2 Human NSCLC tissue samples

Patients with NSCLC were recruited from the Department of Internal Medicine, Division of Oncology and Department of Surgery, Division of Thoracic and Hyperbaric Surgery, Medical University of Graz (Graz, Austria) (**Table 4**). All participants signed an informed consent. Healthy volunteers were recruited as control group.

2.3 Ethical issues for animal studies

All *in vivo* protocols were granted by the Austrian Federal Ministry of Science and Research (BMWF-66.010/0041-V/3b/2018 and GZ 2022-0.748.851).

2.4 Preparation of human peripheral blood leukocytes

Blood from NSCLC patients was obtained prior to treatment and during the study and blood from healthy donors was also drawn to serve as control. The processing of the blood was performed within 4 hours after blood draw. Blood was collected in EDTA-containing or sodium citrate-containing tubes and platelet-rich plasma was separated by centrifugation of the samples (300 ×g, 20 min). To remove erythrocytes, an equal volume of 3% dextran T-500 (Sigma-Aldrich) in saline was added, tubes were well mixed and let them repose. After 30 min, dextran-crosslinked erythrocytes sedimented on bottom of the tubes. The upper layer containing non-sedimented leukocytes was layered on top of 15 mL Histopaque (Sigma-Aldrich). Finally, the samples were centrifugated (300 ×g, 20 min) to separate high-density polymorphonuclear leukocytes (PMNLs)

from peripheral blood mononuclear cells (PBMCs). After this centrifugation, PBMCs were in the interphase while PMNLs localized in the bottom of the tube. PBMCs were carefully removed from the interphase with the use of a pipette and washed in Ca^{2+} and Mg^{2+}-free assay buffer and resuspended in Dulbecco's Phosphate Buffered Saline (PBS, Gibco). On the other hand, the supernatant was removed from the tube and the pellet was resuspended in NH_4Cl to lyse erythrocytes. Finally, remained PMNLs were washed twice in PBS without Ca^{2+} and Mg^{2+} and resuspended in PBS. An EVE automated cell counter (NanoEntek) was used to measure cell viability and cell numbers.

2.5 Cytospins

$1x10^5$ PBMCs were centrifuge (600 ×g, 5 min) onto a glass slide using a Shandon Cytospin 3 Cells were stained immediately using Hemacolor® rapid staining of blood smear according to manufacturer´s instructions (Merck).

2.6 LEGENDScreen Neutrophil Surface Marker Screening

Table 1. *LEGENDScreen panels*

PBMC Panel	PMNL panel
CD45- AF700	CD45-AF700
CD3-PECy5	CD66b-APC
CD4-BUV395	Siglec8-PeCy7
CD8-BUV496	
CD19-FITC	
CD14-BUV605	
CD66b-APC	
Siglec8-PECy7	

We used the LEGENDScreen™ Human PE Kit (Biolegend) to assess the surface expression profile of 361 markers (including 10 isotype controls) in PBMCs and PMNLs. For this purpose, PBMCs and PMNLs from 6 NSCLC patients were isolated as previously described and stained for flow cytometry analysis. Fixable viability dye (FVD) eFluor™ 780 (eBioscience) was used to discriminate live from dead cells (incubation for 30 min at 4°C in the dark). Cells were washed twice with staining buffer (SB, PBS+2 % FBS) and FcBlock (Biolegend) was added to the cells (incubation for 10 min at 4°C). Next, cells were stained with the corresponding antibody master mix for PBMCs or PMNLs, respectively (**Table 1**, antibody and clone details see **Table 7**). 3 x 10^5 cells per well were distributed in the respective plates from the LEGENDScreen™ Human PE Kit and stained according to the manufacturer´s protocol. Cells were measured on a BD LSR II Fortessa (BDBiosciences). After measuring samples, some showed low neutrophil viability or abnormal FSC/SSC properties and were therefore excluded (in total 33 markers were excluded). Based on their quality, 328 samples (markers) were included in the final analysis.

2.7 Bioinformatics

Bioinformatic analysis were performed by Oliver Kindler. The R packages (R-Version 4.0.3) used are listed in **Table 2**. Further information regarding bioinformatic analysis has been previously described in (1,3).

Table 2. *R packages used for bioinformatic analysis*

R package	Use
InfinityFlow without background correction	Prediction of marker co-expression Two dimensional projection of the data using Uniform Manifold Approximation and Projection (UMAP) plots (269)
ggplot2_3.3.3, pheatmap_1.0.12, corrplot_0.84	Drawing of graphs
pheatmap	Heat map of significantly upregulated genes of CPTAC LUAD data
ggpubr	$CD45^+/CD11b^+/CD11c^{-/+}/Siglec\text{-}F^-/Ly6G^-/Ly6C^-/F4/80^+$
survminer, surv_cutpoint function	Survival curve (MPO) and cutpoint Kaplan–Meier analysis
Hacksig (z-score method)	MPO signature (86,269)

Data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) lung adenocarcinoma (LUAD) cohort (protein, transcript, clinical, and survival data) were obtained by accessing the cptac python package (https://pypi.org/project/cptac/).

Data from The Cancer Genome Atlas (TCGA)-LUAD cohort (transcriptomic, clinical, and survival data) were accessed via the pan-cancer atlas hub on the UCSC Xena browser.

Software: Python 3.10.6, R 4.2.1, Platform: x86_64-conda-linux-gnu (64-bit), operating system: Debian GNU/Linux 11 (bullseye).

2.8 Mice and cell lines

C57/B6-J/$MPO^{-/-}$ (KO) and C57/B6-J/$MPO^{+/+}$ (WT) mice were bred in our facilities. All *in vivo* experiments were performed using mice from 6-14 weeks of age from both sexes. The murine lung carcinoma cell lines KP (tumor cells isolated and maintained from mouse LUADs from a KrasLSL-G12DTrp53Fl/FL model on a C57BL/6 background, established in our lab) and LLC (Gibco/Life Technologies) were culture in Dulbecco's Modified Eagle Medium (DMEM, Life

Technologies) with 10% of fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (P/S, PAA Laboratories) (complete DMEM) at 37°C and 5% CO2 in a humidified atmosphere.

2.9 Murine tumor model

On day 0, MPO KO and WT mice were subcutaneously (s.c.) administered with KP cells or LLC cells (5 x 10^5 in 450 µl PBS) into the lower right flanks. Inhaled isoflurane was used as anesthesia. Once tumors were palpable (after ~5 days), mice were shaved and length and width of tumors were measured every other day using a digital caliper. Mice were sacrificed on day 12 (KP tumors-bearing mice) or day 19 (LLC tumors-bearing mice) and tumors and spleens were collected. A digital caliper was used to measure the tumors, and a scale was use to weight the tumors and the spleens. Tumor volume was calculated by using the formula (v = length x width x height x π/6). Tumors were used for subsequent downstream experiments.

2.10 Single-cell suspensions

Tumors tissues from mice. Tumors were minced with surgical scissors and digested in Roswell Park memorial Institute (RPMI) medium with collagenase IV (4.5 U/ml; Worthington) and DNase I (160 mU/mL, Worthington) (30 min, 37°C) while rotating at 1000 rpm. A 40-µm strainer was used to filter the tumor digests and tumor cells were collected in a 50 ml tube. Cells were resuspended in SB, centrifuged (500 ×g, 5 min, 4°C) and washed in cold PBS. An EVE automated cell counter was used to measure cell viability and cell numbers.

Spleen tissues from mice. A syringe plunger was used to mince spleens. A 40-µm strainer was used to filter the spleen tissue and then suspended in SB and centrifuged (500 ×g, 5 min, 4°C). 1x RBC (BioLegend, # 420301) buffer was used to lyse erythrocytes (5 ml per sample, 5 min, 4°C with occasional shaking). Four volumes of PBS were added to the samples to neutralize RBC and samples were centrifugated (500 ×g, 5 min, 4°C) and washed another two times with PBS. An EVE automated cell counter was used to measure cell viability and cell numbers

*Tumor samples from NSCLC patients. NSCLC t*umors were weighted and immediately transfer to a tube containing pre-warmed RPMI medium with collagenase IV (40 U/ml; Worthington) and DNase I (150 U/mL, Worthington) collagenase (max. 2g tissue per 20 ml medium per tube).

Tubes with tissue were incubated in a water bath (20 min, 37 °C). Tissue was then homogenized with a 5 ml pipette and incubated again in the water bath (10 min,37 °C). Tissue was homogenized with a 5 ml pipette and passed through a 19-gauge needle on 10ml syringe (when possible). A 100-µm strainer was used to filter the tissue digests and centrifuged (500 ×g, 5 min, 4°C). Cells were resuspended in 1x RBC lysis buffer (3 min on ice, occasional shaking). Four volumes of PBS were added to the samples to neutralize RBC. Cells were passed through a 40-µm strainer, centrifuged (500 ×g, 5 min, 4°C) and washed once more with PBS. An EVE automated cell counter was used to measure cell viability and cell numbers.

2.11 Flow cytometry

Table 3. *Mice and human flow panels*

Lymphoid Panel (mice, surface)	**Myeloid panel (mice, surface)**	**IFN-γ panel (mice, surface)**	**MPO panel (human, surface)**
CD45-AF700	CD45-BV785	CD45-FITC	CD45-BV510
CD3-BUV395	Ly6C-APC	CD3-BV421	CD66b-APC
CD8-PerCPCy5.5	Ly6G-PE /Dazzle	CD4-PE-Cy7	CD163-BV605
CD4-BUV496	CD11c-BV605	CD8-PerCP-Cy5.5	MPO-FITC
CD19-FITC	PD-L1-PeCy7	IFN-γ-PE	CD45-BV510
NKp46-BV510	CD206-FITC		
gdTCR-PECF594	MHCII-PerCP-Cy5.5		
CD62L-BV605	CD103-BV510		
CD44-BUV737	CD11b-BUV737		
PD-1-APC	F4/80-BUV395		
Lymphoid (mice, intracellular)	Siglec-F-PE		
FoxP3-PE			

Single cells from mice tumors. FVD eFluor™ 780 (eBioscience) was used to discriminate live from dead cells (1:2000, 30 min, 4°C in the dark). Next, samples were incubated with 1 µg TruStain FcX™ (FcBlock, Biolegend; 10 min, 4°C). For the analysis of infiltrating immune populations, cells were divided into two groups and stained (30 min, 4°C protected from light) with two different pre-mixed panels of antibodies, respectively (**Table 3**, antibody and clone details see **Tables 7 and 8**). The first panel included antibodies to recognize lymphoid populations (lymphoid panel) and the second panel was to identify myeloid populations (myeloid panel). After staining of surface antigens for the lymphoid panel, cells were permeabilized with Transcription Factor Buffer Set (BD Biosciences) and then cells were stained with an antibody against FoxP3 (intracellular antigen). Some samples were also stained with an antibody to

detect expression of IFN-γ. For this purpose, 2 × 10^6 single-cells of tumors or spleens per well were seeded into 96-well U-bottomed plates. Cells were incubated (4 h, 37°C) in complete RPMI (RPMI supplemented with 10% FBS and 1% P/S) media containing 1.5 µl/ml GolgiStop (BD Biosciences). Phorbol 12-myristate 13-acetate/ionomycin (PMA, 100 ng/ml, Sigma-Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) were used to stimulate T cells. Afterwards, surface and intracellular staining (BD Cytofix/CytopermTM Kit) was performed as previously described using a pre-mixed antibody panel **(Table 3)**.

Single cells from human tumors. Single cells obtained from lung tumors from patients were stained to detect MPO expression. In brief, cells were incubated with FVD eFluor™ 780 (30 min, 4°C in the dark) and 0.5 µg human of FcBlock per sample was added and incubated (10 min, 4°C). Cells were stained with the corresponding pre-mixed panel of antibodies (**Table 3**, antibody and clone details see **Tables 7 and 8**).
Finally, all stained cells were washed, fixed with IC fixation buffer (ThermoFisher Scientific, 10 min, 4°C), washed, suspended in SB and stored at 4°c until analysis. Samples were analyzed on a BD LSRFortessa™ or a BD Canto™ flow cytometer with FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA). The data were compensated and analyzed using FlowJo software (TreeStar, Ashland, OR, USA) and gates were defined using fluorescence-minus-one (FMO) strategy (1).

2.12 T cell isolation and MPO treatments

The EasySep™ Human T-cell Isolation Kit (Stemcell Technologies) was used to enriched T cells from previously isolated PBMCs of healthy donors. The enrichment of T cells is based on negative selection. In brief, 50 × 10^6 PBMCs/mL were resuspended in the recommended buffer (SB + 1 mM EDTA) in 5 ml FACS tube. An isolation cocktail was used to label unwanted cells with antibody complexes (5 min, RT). Next, RapidSphere magnetic particles were added to the tube. An EasySep™ magnet was used to separate the undesired magnetically-labeled cells (tube placed in magnet for 3 min, RT). After incubation time, unlabeled T-cells were collected in a fresh tube and washed once with PBS. A Neubauer chamber and trypan blue were used to count the cells. Complete RPMI media was used to culture T cells and MPO (Elastin Products, catalog no. MY862) or/and heparin were added when required.

2.13 T cell proliferation

To asses T cell proliferation, we used the fluorescent proliferation dye eFluorTM 450 (eF450, Thermo Fisher) that monitors individual cell divisions. For this purpose, 10 mio/ml T cells suspended in PBS were labeled with 10 µM eF450 (10 min, RT). Cells were washed twice with SB and resuspended in X-VIVO 15 media (Lonza) supplemented with 1% P/S, 50-µM β-mercaptoethanol, 2-mM L-Glutamine, 25-mM HEPES, 5-ng/mL interleukin-2, and 1-µg/mL αCD28. 96-well plates were pre-coated with 5 µg/ml αCD3 in PBS (overnight, 4 °C) and 2.5 × 10^5 T cells were seeded and incubated (72 h, 37°C). After incubation time, cells were incubated with 0.5 µL of FcX-SB in FACS tubes (10 min, 4°C). Cells were stained with the pre-mixed panel of antibodies (CD4-PE and CD8-FITC, 30 min, 4°C in the dark; antibody and clone details see **Table 8**). Finally, T cells were washed twice with SB, fixed with IC fixation buffer (10 min, 4°C in the dark), centrifuged and resuspended in SB until measuring in a flow cytometer.

2.14 Statistical analysis

Statistical analysis of all data was performed with GraphPad Prism 6.1 (GraphPad Software, La Jolla, CA, USA). Data were tested for normal distribution (when possible) with the Shapiro–Wilk normality test. Data obtained from the same patient were analyzed with paired t-test (parametric) or Wilcoxon matched-pairs test (non-parametric). The level of significance between two experimental groups was assessed by unpaired student's t-tests with Welch´s correction, or Mann–Whitney test for normal-distributed or non-normal distributed groups, respectively. Comparison for more than two experimental groups was performed by one-way analysis of variance (ANOVA), with the indicated post hoc test. Pearson's correlation coefficient (r) and Spearman's correlation coefficient rho (rs) were used to determine the correlation between neutrophil content and MPO+ lymphocytes. Standard deviation (SD) and standard error of the mean (SEM) were used to express the results, as is indicated in each figure legend. A *p*-value of <0.05 was considered statistically significant.

3 RESULTS

The Results section of this thesis is divided in two main parts. The first part corresponds to the project "LDNs display a distinctive immune signature in NSCLC patients" and the second part correspond to the project "MPO favors tumor growth and acts an immunosuppressive factor in NSCLC" Although Part 1 and Part 2 describe the results of two independent projects, all the findings presented in this dissertation are closely linked to the main research area of our laboratory in where we study the role of neutrophils in the development of lung cancer.

3.1 Part 1. LDNs display a distinctive immune signature in NSCLC patients

As parts of this dissertation have previously been published as an original research article in Frontiers in Immunology (1), the Results section has been partially adapted from the article and any resemblances in regards to content and phrasing are to be expected.

3.1.1 Patients with NSCLC have increased content of LDNs

Table 4. *Participants characteristics*

	All NSCLC
N	n = 54
Age	66 (45 – 81)
Sex	Female n = 21 Male n = 33
Smoking	Yes n = 44 No n = 9 Unknown n = 1
Stages	I-IIa n = 18 IIIa n = 8 Ib n = 6 IIb n = 6 IIIb n = 4 IV n = 0 Undefined n = 12
Histology	Adenocarcinoma n = 31 Squamous cell carcinoma n = 15 Undefined NSCLC n = 8
Therapy	No prior treatment n = 54 Treatment n = 0

LDNs have been found in the peripheral blood of patients with renal carcinoma, head and neck cancer, pancreatic cancer, colon cancer breast adenocarcinoma and lung cancer (86,114,129,132). To confirm the presence of LDNs in our NSCLC patient cohort, we collected blood from healthy volunteers and patients with NSCLC regardless of disease stage (**Table 4**).

To examine the content of neutrophils in the blood from NSCLC patients and healthy donors, PBMCs and PMNLs were separated using density gradient centrifugation. The percentage of neutrophils (identify as $CD66b^{+}$ cells) in each fraction was analyzed using flow cytometry. Moreover, whole blood of patients and

healthy donors was also analyzed. We found an increased content of LDNs, identify as CD66b$^+$ cells, in the PBMC fraction of NSCLC patients (median=20.4%, range 0.3-76.1%; n=26) as compared with healthy controls (median=0.3%, range 0.1-3.9%; n=14) (**Figure 1 A-B**).

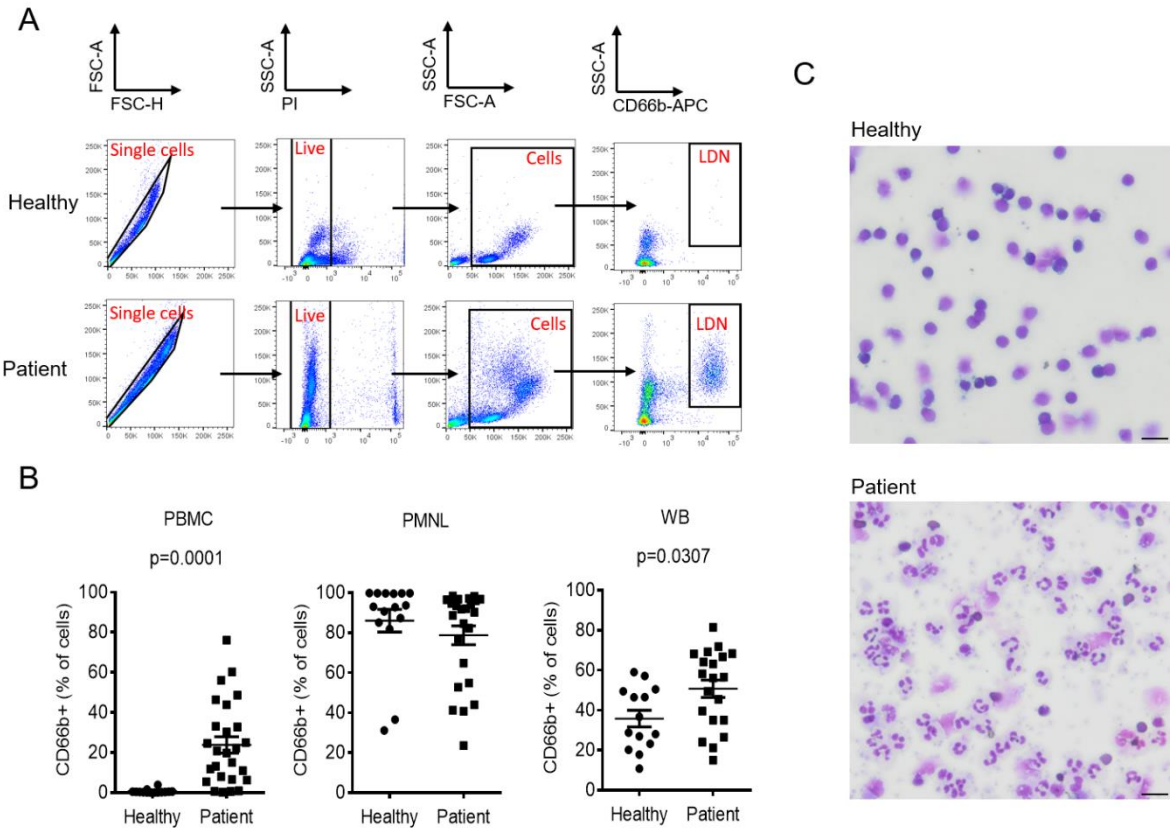


Figure 1. *Low-density neutrophils (LDNs) are increased in patients with NSCLC.*
(A) Gating strategy to identify LDNs in blood from patients and healthy donors. Doublets were eliminating in the first gate by plotting forward scatter height (FSC-H) vs forward scatter area (FSC-A). Propidium iodide (PI) was used to discriminate live (PI$^-$) vs. dead (PI$^+$) cells. Next, total cells were gated base on their forward scatter (FSC-A) and side scatter (SSC-A) properties. Finally, CD66b was used to identify neutrophils (LDNs). **(B)** LDN (CD66b$^+$) content was significative increased in the PMBC fraction and whole blood (WB) of NSCLC patients (n=26) when compared to healthy donors (n=14). No differences were observed in CD66b$^+$ content in the PMNL fractions. **(C)** Cytospin images show no neutrophils in the PBMC fraction of a healthy donor (upper image), while many neutrophils with heterogenous morphology were found in the PBMC fraction of a NSCLC patient (lower image). Pictures were acquired with a 40X objective, bar length 20μM. **(B)** Symbols represent an individual donor, and bars show the mean. The p values (when statistically significant) are also disclosed. Data are shown as means ± SD. This figure has been adapted from (1).

Similarly, whole blood of NSCLC patients also had an increased percentage of CD66b$^+$ cells (median=56.4%, range 15.1-81.5%) in comparison to healthy donors (median=32.9%, range

10.9-59.1%) (**Figure 1 A-B**). This result might be related with the increased number of LDNs in patients. On the other hand, we did not observe significant differences when compared the amount of $CD66b^+$ cells in the PMNL fraction of NSCLC patients vs healthy donors (**Figure 1 A-B**). Previous studies have reported that LDNs are an heterogeneous population (114). We also observed morphological varieties within the LDNs from in patients. Some LDNs were segmented (mature) while others were banded and ring-shaped (immature) (**Figure 1 C**). As expected, no LDNs were detected in the PBMC fraction of healthy donors (**Figure 1 C**).

3.1.2 Comprehensive screening of surface markers in LDNs of patients with NSCLC

Some studies have already aimed to identify markers that can be used to discriminate between LDNs and HDNs, although mainly focused in already known myeloid cell and neutrophil maturation markers (99,270–272). With the purpose to better characterize the phenotype LDNs in NSCLC patients, we decided to analyze a broader spectrum of surface markers, including also markers that have not been previously reported to be expressed by neutrophils.

For this purpose, we used the human LEGENScreen (Biolegend) and analyzed the results using Infinity Flow (269). The LEGENScreen kit contains 354 monoclonal antibodies that are conjugated to a PE fluorophore allowing the identification of a broad diversity of antigens. In our experimental approach, PBCM and PMNL fractions from patients were stained with a cocktail of antibodies to identify neutrophils (CD45-AF700, Siglec8-PECy7 and CD66b-APC = Backbone). Then the sample was distributed across 354 wells (each well containing one single monoclonal antibody conjugated to PE fluorophore) and were acquired in a flow cytometer as individual samples. Following the staining phase, each well held a portion of the overall sample, and all cells within it were labeled with all the Backbone markers along with a single PE-conjugated marker. After flow cytometric analysis, each well's unique dataset, which was saved in its individual FCS file, comprised actual measurements of forward scatter (FSC) and side scatter (SSC) parameters, all the Backbone markers, and an actual measurement of one of the specific panel markers. Using this information, Infinity Flow (269) was performed. Infinity Flow is a computational tool that uses machine learning to automate the process of filling in missing information for cell surface protein expression at the individual cell level, utilizing experimental flow cytometry data. Briefly, the intensities of PE-conjugated markers were used to predict the specific expression of each marker of interest on all single-cell events acquired through the

LEGENDScreen (328 fcs files were used as imput in the current study) via non-linear regression using the intensities of the backbone markers.

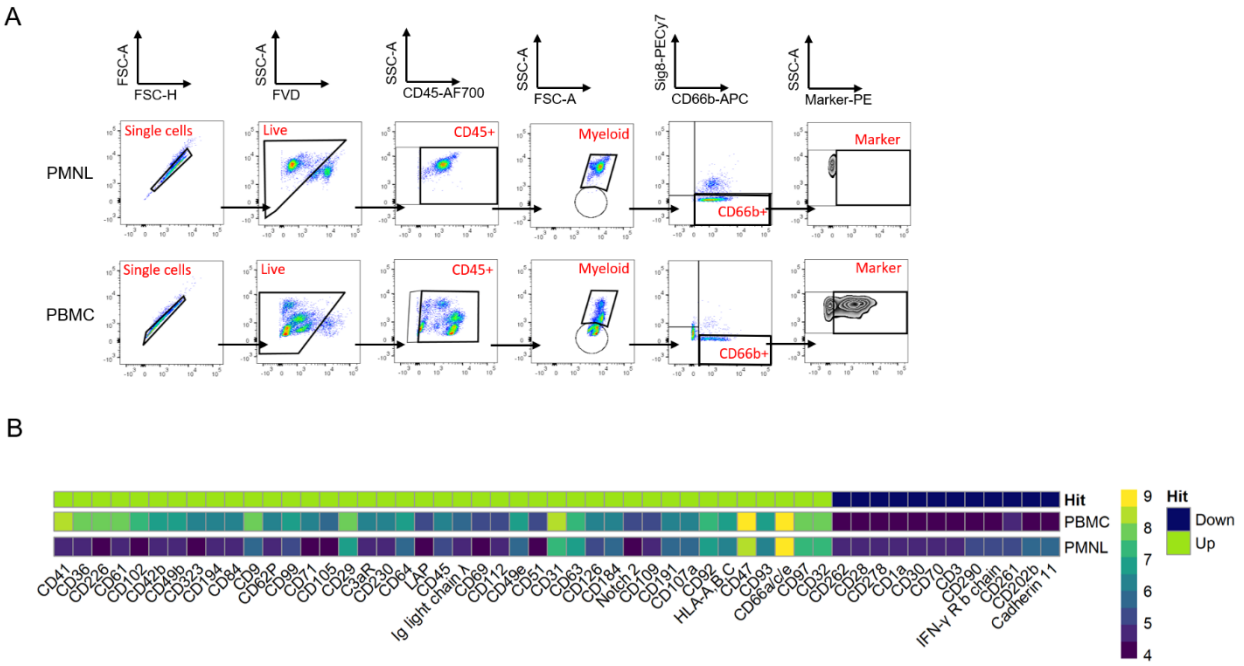


Figure 2. *Comprehensive screening of surface markers in PMNLs and PBMCs from NSCLC patients.*
(A) Gating strategy for surface screening: doublets and dead cells (FVD^+) were excluded. Myeloid population (containing neutrophils and eosinophils) was gated based on the forward FSC-) and SSC-A. Neutrophils, categorized as HDNs in the PMNL fraction and LDNs in the PBMC fraction were distinguished as $CD66b^+$ $Siglec8^-$ cells, and the PE channel was utilized to assess the specific surface markers. **(B)** Heatmap displaying marker hit expressions. Marker hits were determined by comparing the fold change in marker expression (GeoMean) with a fold change greater than 2 and less than 0.5 (LDNs/HDNs ratio) (refer to **Table 9** for further information). The heatmap scale represents the GeoMean, and the 'hits' indicate the markers that are differentially regulated in LDNs (PBMC gated) compared to HDNs (PMNL gated). In the heatmap, green indicates overexpression, while dark blue indicates downregulation. This figure has been adapted from (1).

After analysis of the flow cytometry data, some samples were excluded due to low neutrophil live counts or abnormal FCS/SSC properties. At the end, 328 samples (individual antigens) were included in the analysis. LDNs (in the PBMC fraction) and HDNs (in the PMNL fraction) were identified as $CD66b^+$ $Siglec8^-$ cells (**Figure 2 A**). Being that eosinophils also express CD66b, we use Siglec8 to discriminate between neutrophils ($Siglec8^-$) and eosinophils ($Siglec8^+$). We then determined a LDNs/HDNs fold change in GeoMean lower than 0.5 or higher than 2 to select

differentially expressed markers. According to this, we identified 53 markers that were up or downregulated in LDNs when compared to HDNs (**Figure 2 B and Table 9**). The top four upregulated hits (CD36 fold change=31.1, CD41 fold change=6.7, CD61 fold change=20.1, CD226 fold change=27.8) showed a fold change above 5 (**Figures 2 A-B and Table 9**).

3.1.3 Identification of specific surface markers in LDNs of NSCLC patients

We next aimed to validate the findings of the surface marker screening. Based on the results obtained from the screening panel, we chose CD36, CD41, CD61 and CD226 as part of the highest expressed markers in the LDN fraction (**Figure 3 A**). Moreover, we also included Lox-1 in the validation panel because it has been previously pointed out as a specific marker of LDNs in cancer patients (273).

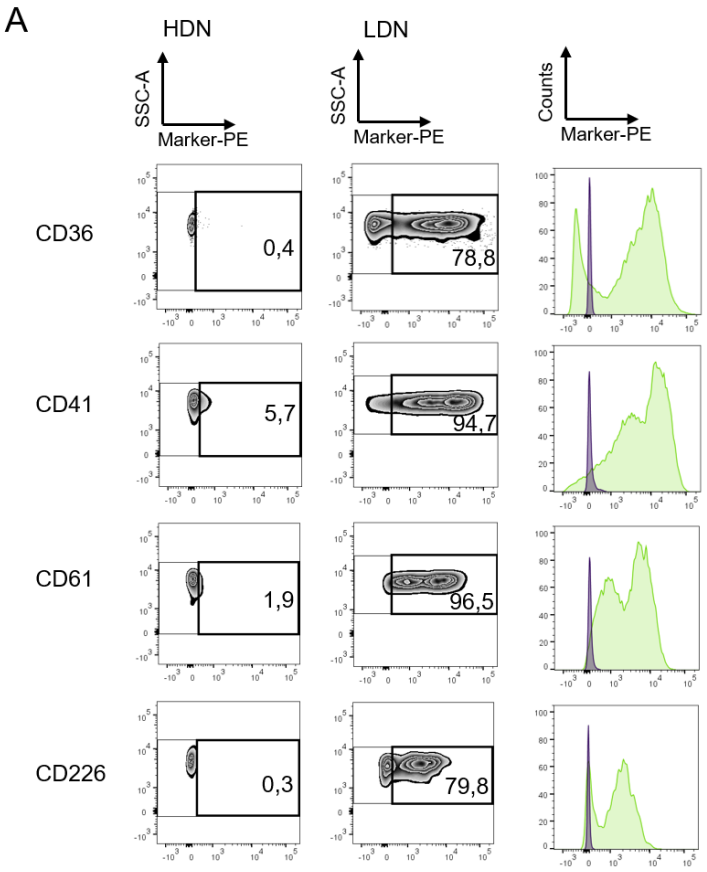


Figure 3. *Top screening hits in LDNs.*
(A) Surface antigens that exhibit the most significant variations in expression levels between HDNs and LDNs. Zebra plots illustrate CD36, CD41, CD61, and CD226 in the HDN and LDN fractions. Additionally, histograms display the fluorescence intensity in HDNs (depicted in violet) and LDNs (depicted in green). This figure has been adapted from (1).

Our next goal was to validate the expression of the four selected markers. To do this, we designed a new flow cytometry panel including CD36, CD41, CD61, CD226 and Lox-1 and stain PBMCs and PMNLs of 13 NSCLC patients (**Figures 4 A-B**). Using this strategy, we validated a significantly higher proportion of CD36 (median=48.2%, range 5.1-87.4%), CD41 (median=29.2%, range 5.3-70.8%), CD61 (median=44.4%, range 16.8-90.2%) and Lox1 (median=22.1%, range 7.7-58.3%) in the LDN fraction compared to the HDN fraction (**Figures 4 B and 5 A**).

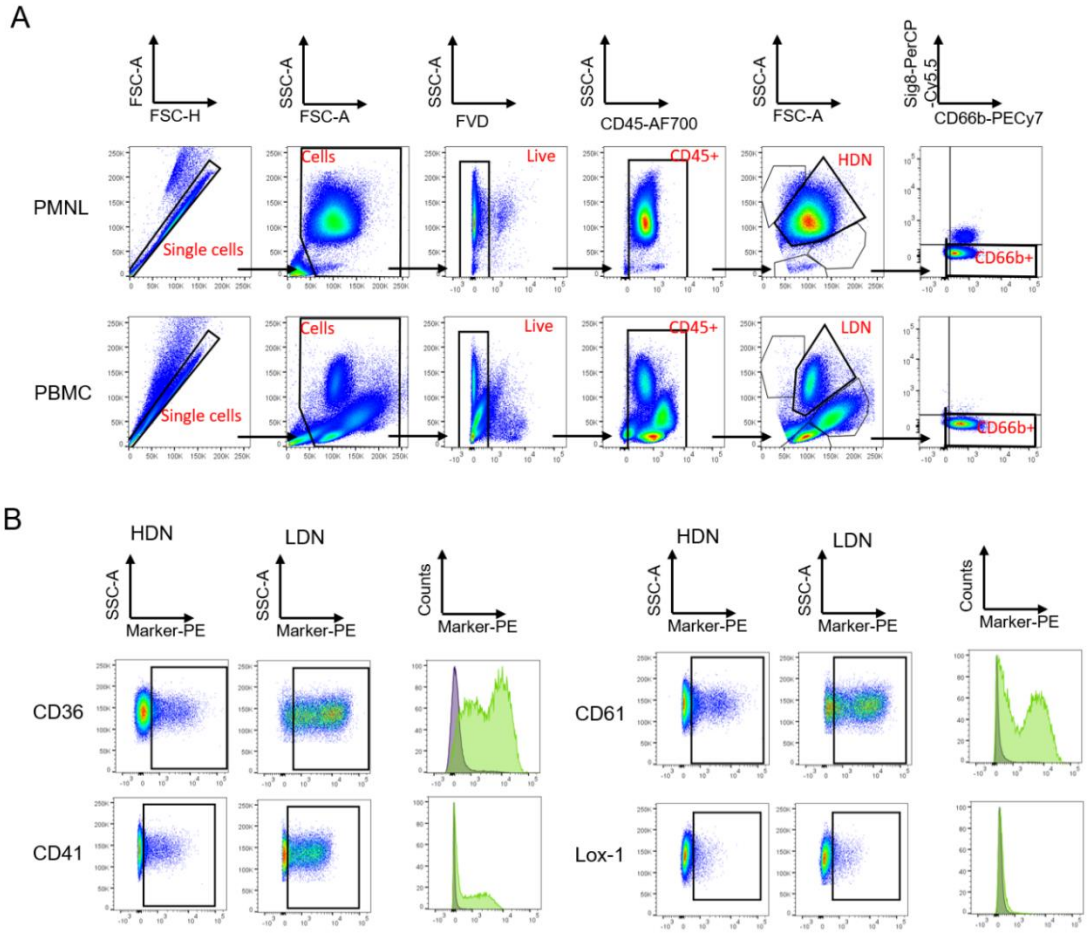


Figure 4. *Validation panel.*
(A) Gating strategy of the validation panel. Initial gating was for single cells and total of cells. To exclude nonviable cells, FVD stain was used, and subsequent gating was carried out to identify $CD45^+$ cells. HDNs and LDNs were distinguished based on their forward and side scatter characteristics. Neutrophils were identified as cells that were $CD66b^+$ and $Siglec8^-$. **(B)** CD36, CD41, CD61 and Lox-1 exhibited an increased expression in LDNs when compared to HNDs as it is shown with the representative dot plots and histograms. This figure has been adapted from (1).

Accordingly, the GeoMean of the chosen markers also exhibited a significant increase in LDNs as compared to HDNs (**Figure 5 B**). Furthermore, we noticed an elevated ratio of LDNs to HDNs for all the corresponding markers, expressed as a percentage of $CD66b^+$ cells and GeoMean (**Figure 5 C**).

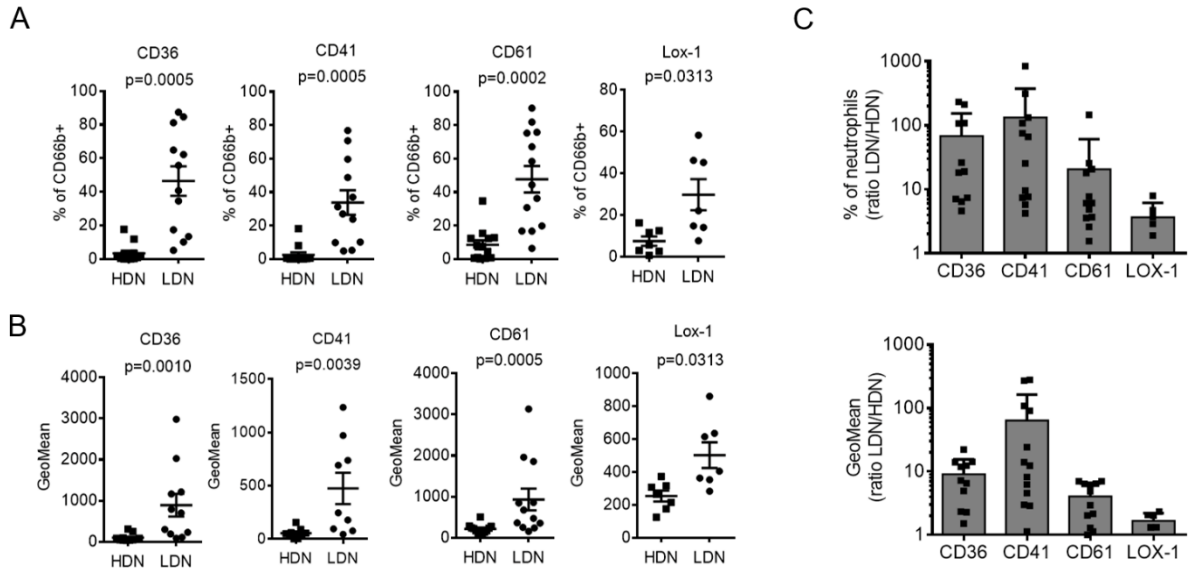


Figure 5. *LDNs overexpress CD36, CD41, CD61 and Lox1.*
(A) LDNs (n=13) exhibited an increased percentage of CD36, CD41, CD61 and Lox-1 vs. HDNs (n=7). **(B)** CD36, CD41, CD61 and Lox-1 showed a higher GeoMean in LDNs vs HDNs. **(C)** LDNs/HDNs ratio analyzed as % of neutrophils and GeoMean. **(A-B)** The p values (when statistically significant) are also disclosed. Data are shown as means ± SEM. This figure has been adapted from (1).

Although CD226 (median=4.6%, range 0.6-35.2%, n=6) also showed augmented expression in LDNs vs HDNs, it had to be excluded due to a weak separation of negative and positive populations **(Figure 6 A-B).**

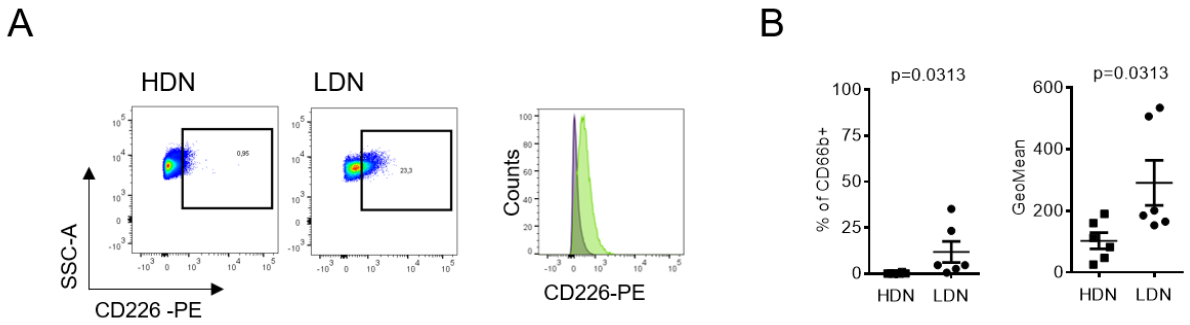


Figure 6. *LDNs overexpress CD226*
(A) CD226 is over expressed in LDNs (depicted in green) from cancer patients (n=6) as compared to HDNs (depicted in violet). **(B)** Quantitative analysis of the markers expression as % of $CD66b^+$ cells and GeoMean in the HDN and LDNs subsets in NSCLC patients (n=6). Data are shown as means ± SEM.

3.1.4 Maturation status of LDNs and HDNs

Previous reports have shown that LDNs consist in neutrophils with different maturation status. CD10, CD16, CD15 and CD11b are classical myeloid and maturation markers and we chose them to compare the maturation status of LDNs and HDNs isolated from NSCLC patients (**Figure 7 A**). When compared the expression of those markers in LDNs and HDNs, we found no significant differences (**Figure 7 A**). On the other hand, we observed that vast majority of the HDN fraction expressed CD10 indicating that HDNs are mature neutrophils (**Figure 7 B**). On the other hand, LDNs displayed greater diversity in the ratio of $CD10^-$ vs $CD10^+$ among various patients (**Figure 7 B**).

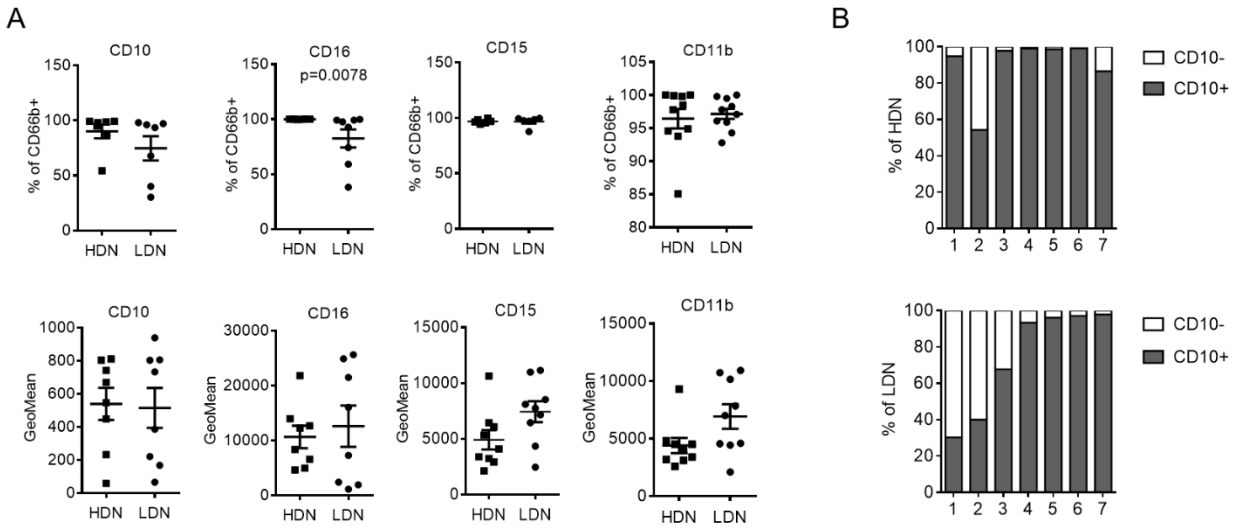


Figure 7. *Neutrophil maturation markers in LDNs and HDNs.*
(A) Expression of maturation markers in HDN and LDN from a subset (n=7-9) of NSCLC patients. Results are represented as percentage of $CD66b^+$ cells and GeoMean. **(B)** Proportions of $CD10^+$ and $CD10^-$ cells out of the HDNs and LDNs of a subset of NSCLC patients (n=7). Data are shown as means ± SEM

3.1.5 LDNs markers are co-expressed in LDNs of NSCLC patients

Our ultimate objective was to examine whether the newly identified markers in LDNs were simultaneously expressed in a group of NSCLC patients. To do this, we used t-distributed stochastic neighbor embedding (tSNE) plots and correlation analysis. We selected one healthy donor and one NSCLC patient as representative samples and created tSNE plots from the respective HDN and LDN subsets. As we expected, CD36, CD41 and CD61 were not present in the HDN fraction of the healthy control (**Figure 8 A**). However, we detect Lox-1 expression in some cells of the HDN fraction (**Figure 8 A**). Additionally, we noted a varied expression pattern of the studied markers within the LDN fraction. Some cells did not exhibit any of the antigens, while others were positive for certain markers, and a subset of cells displayed simultaneous expression of all the markers (**Figure 8 A**). Additional analysis unveiled a robust correlation between the expression of CD36, CD41, and CD61, whereas Lox-1 did not exhibit any correlation with the other markers (**Figure 8 B**). Moreover, we noticed that by using the specified markers we could capture around 80% of all LDNs (**Figure 8 C**).

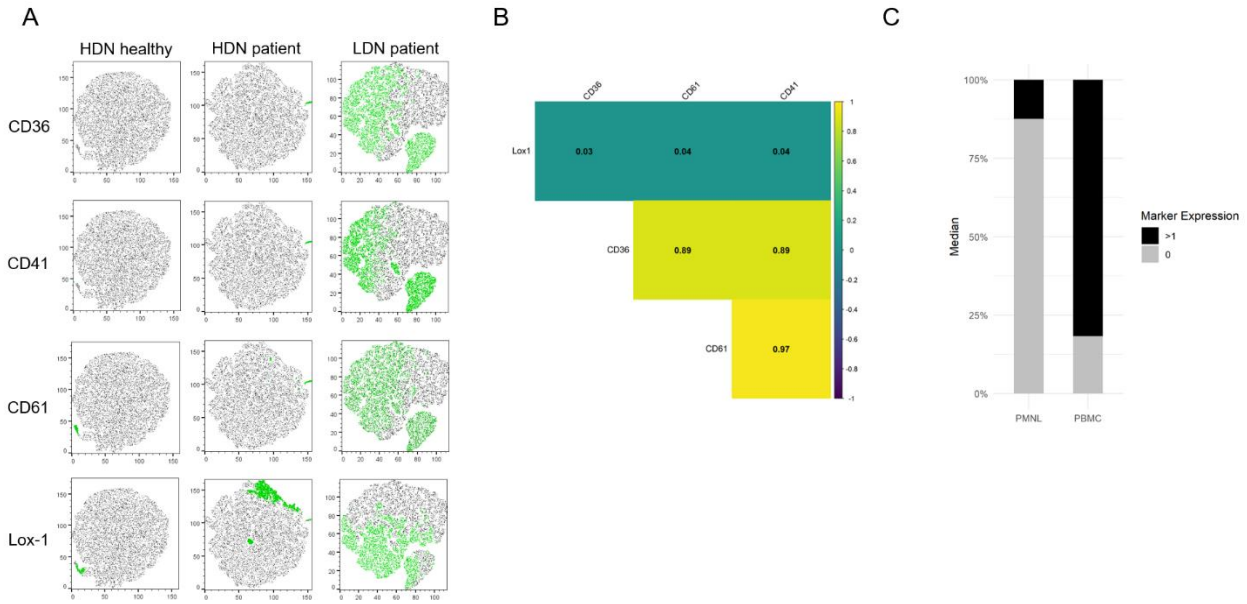


Figure 8. *Co-expression of CD36, CD41, CD61 and Lox-1 in LDNs of patients with NSCLC.*
(A) Representative tSNE visualization of HDNs and LDNs. Green spots show the fraction of cells that expressed CD36, CD41, CD61 and Lox-1, respectively. **(B)** Examination of the relationship between the four examined markers in one representative patient. Results revealed a lack of correlation between Lox-1 and the other markers, but a substantial correlation between CD36, CD41, and CD61 (all results statistically significant/$p<0.01$). **(C)** Percentages of marker expression (in black) and non-expression (in grey) in the PMNL and PBMC portions of NSCLC patients.

3.2 Part 2. MPO favors tumor growth and acts as an immunosuppressive factor in NSCLC

As parts of this dissertation have previously been published as a preprint article in BioXriv (3), the Results section has been partially adapted from the preprint and any resemblances in regards to content and phrasing are to be expected.

3.2.1 MPO expression negatively correlates with survival in patients with NSCLC

Our initial objective was to assess the prognostic significance of MPO protein expression NSCLC. We utilized the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database and conducted Kaplan-Meier analysis to explore how MPO protein expression in primary tumor tissues affected patient survival. The findings indicated that patients with low MPO protein expression experienced more favorable outcomes (**Figure 9 A**). To further delve into the impact of MPO, we categorized patients into MPO-high and MPO-low groups based on the median value of MPO protein expression (**Figure 9 B, Table 10)**. We employed Welch's t-test to identify differentially expressed genes, using an adjusted p-value threshold of 0.05. (**Figure 9 B**). Significantly upregulated genes were used to calculate an MPO signature score in the TCGA-LUAD dataset (**Figure 9 B**).

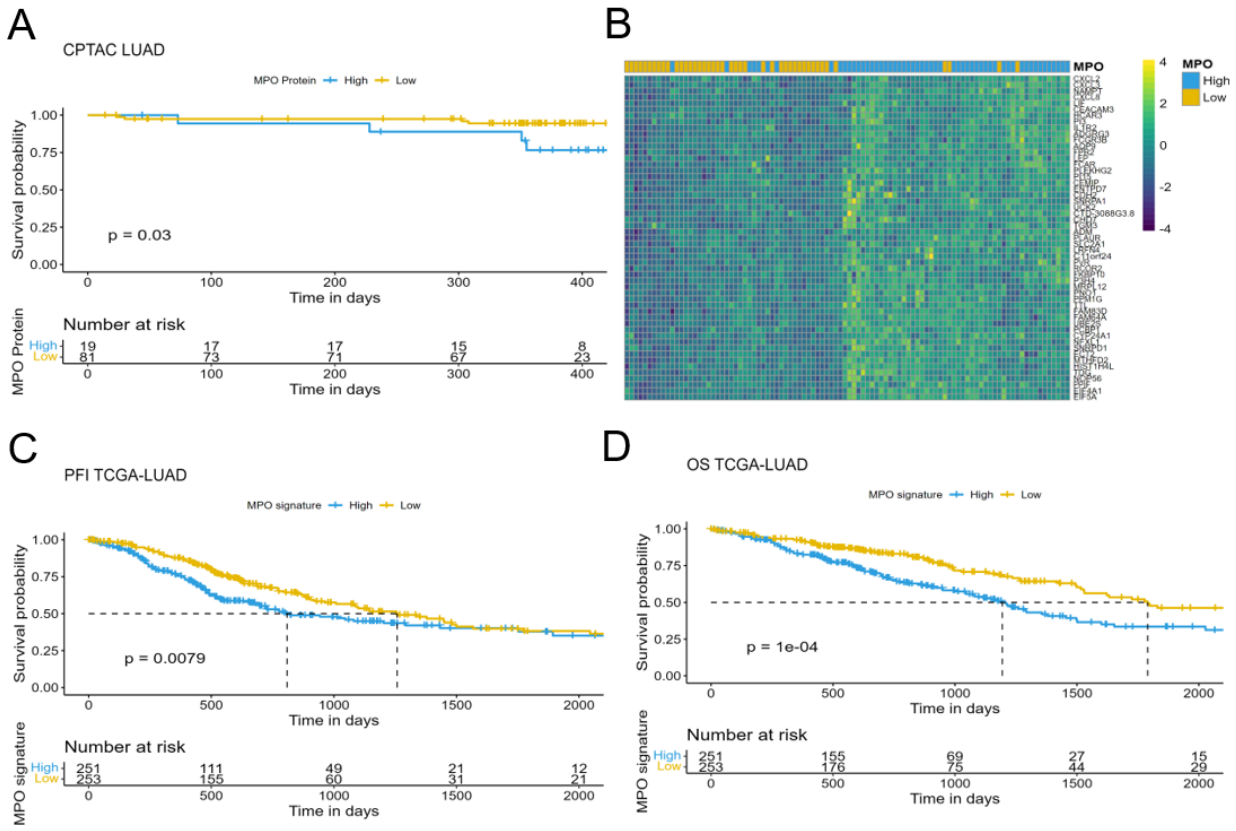


Figure 9. *MPO expression is clinically relevant in patients with NSCLC.*
(A) Kaplan-Meier analysis was employed to assess survival outcomes among individuals with NSCLC who exhibited varying levels of MPO expression.). **(B)** A heatmap depicting genes that showed differential expression in the MPO-high and MPO-low subgroups. **(C-D)** TCGA patients were segregated into MPO-high and -low subgroups, and their progression-free and overall survival rates were analyzed showing a survival benefit in patients with low MPO signature. Data generated by Oliver Kindler. This figure has been adapted from (3).

The scores of upregulated and downregulated genes were strongly negatively correlated, indicating a biological reason for this expression profile, and therefore validating the use of this signature (**Figures 10 A-B**). We then evaluated the effect of the MPO signature on progression-free interval (PFI, **Figure 9 C**) and overall survival (OS, **Figure 9 D**) and found that the low MPO signature group had higher survival rates.

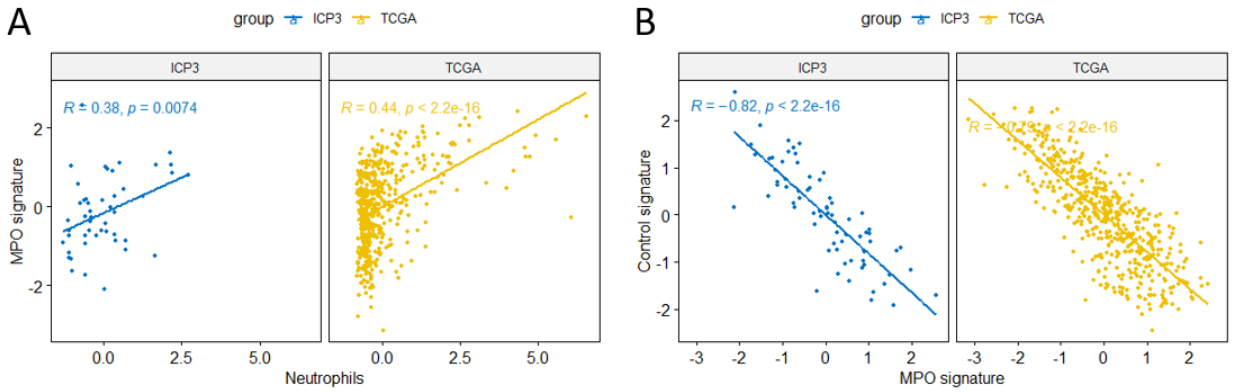


Figure 10. *Validation of MPO signature*
(A) Scatterplot showing Pearson correlation between scaled MPO signature and scaled neutrophil content in two different datasets (ICP3: Neutrophils in % CD45+ cells; TCGA-LUAD: Percentage of Neutrophils derived from CASSANDRA website). **(B)** Scatterplot showing Pearson correlation between scaled MPO signature and Control signature in ICP3 and TCGA LUAD dataset. Data generated by Oliver Kindler. This figure has been adapted from (3).

3.2.2 Genetic depletion of MPO reduces tumor growth *in vivo*

Next, we aimed to investigate the role of MPO on tumor growth using a heterotopic lung tumor engraftment mouse model. We used C57BL/6 $MPO^{+/+}$ (WT) and age-matched $MPO^{-/-}$ (KO). To induce tumor growth, mice were subcutaneously injected into the flanks with KP cells (**Figure 11 A**) or LLC cells (**Figure 11 C**). We observed that lacking of MPO resulted in slower tumor growth and reduced weight and volume of the tumors at end point (**Figure 11 B and D**). By assessing tumor weight and volume, mice with MPO deficiency demonstrated a 35 % reduction in KP tumor growth (**Figure 11 B**) and 40% reduction in LLC tumor growth (**Figure 11 D**) when compared with their WT littermates.

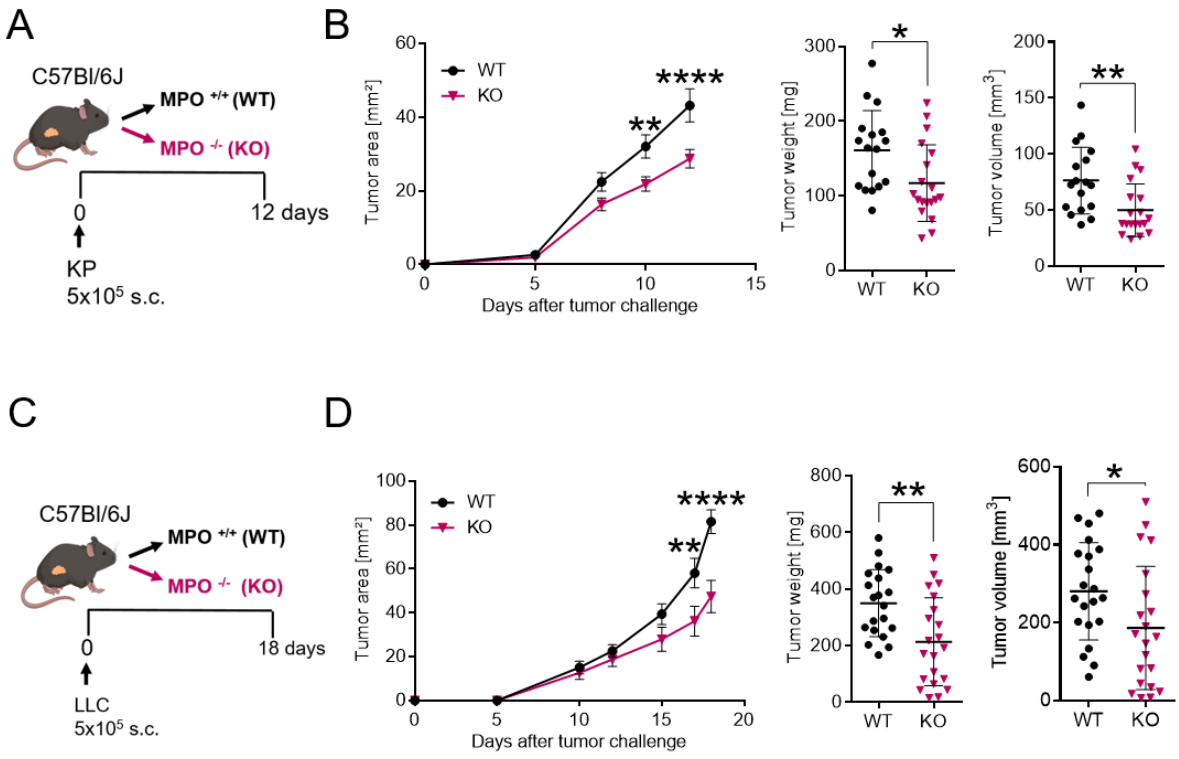


Figure 11. *KO of MPO causes tumor growth reduction in vivo*
(A, C) Experimental design: $MPO^{-/-}$ (knock-out, KO) and $MPO^{+/+}$ (wild-type, WT) mice (n=18-21) were subcutaneously (s.c) injected with KP or LLC cells on day 0. Tumor development was observed throughout the duration of the study. Mice were euthanized on day 12 (KP-bearing) or 18 (LLC-bearing) and the tumor mass and volume were measured *ex-vivo*. **(B)** MPO KO mice had significantly less KP tumors that their WT littermates. **(D)** MPO KO mice had significantly less LLC tumors that their WT littermates. **(B, D)** The experiment was done two times in total, with male mice for the KP cells and female mice for the LLC cells. Data of KP and LLC -bearing mice were combined, respectively. Symbols depict data from individual mice, and bars show the mean. Data are shown as mean ± SD *p < 0.05 and **p < 0.01 This figure has been adapted from (3).

3.2.3 Lacking of MPO in mice results in an anti-tumorigenic TME

Next, we analyzed the immune cell profile in the tumors of MPO KO mice compared to WT mice. We used two flow cytometric panels to explore the infiltration of myeloid and lymphoid populations (**Figure 12, Tables 5 and 6**) into KP tumors.

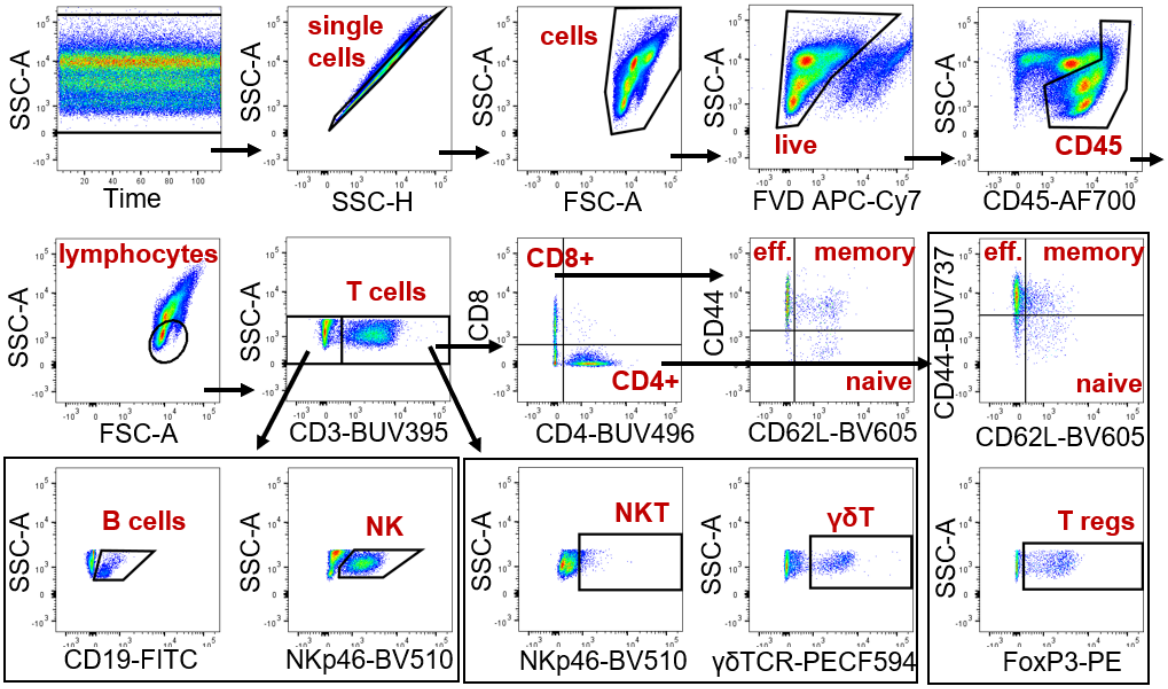
Table 5. *Markers of myeloid and populations*

Myeloid population	**Markers**
Eosinophils	$CD45^+/CD11b^+/CD11c^-/Siglec\text{-}F^+$
Neutrophils	$CD45^+/CD11b^+/CD11c^-/Siglec\text{-}F^-/Ly6G^+$
Monocytes	$CD45^+/CD11b^+/CD11c^-/Siglec\text{-}F^-/Ly6G^-/Ly6C^+$
Macrophages	$CD45^+/CD11b^+/CD11c^{-/+}/Siglec\text{-}F^-/Ly6G^-/Ly6C^-/F4/80^+$
Myeloid dendritic cells (mDCs)	$CD45^+/CD11b^+/ F4/80^{+/-}/CD11c^+/MHCII^+$
Plasmacytoid DCs (panDCs)	$CD45^+/CD11b^-/ F4/80^-/MHCII^+$
Dendritic cells (DCs2)	$CD45^+/CD11b^-/ F4/80^-/MHCII^+/CD103^+$.

Table 6. *Markers of myeloid populations*

Lymphoid population	**Markers**
T cells	$CD45^+/CD3^+$
NK cells	$CD45^+/CD3^-/NKp46^+$
B cells	$CD45^+/CD3^-/CD19^+$
$CD8^+$ T cells	$CD45^+/CD3^+/CD8^+$
$CD4^+$ T cells	$CD45^+/CD3^+/CD4^+$
$CD8^+$ T effector (eff.)	$CD45^+/CD3^+/CD8^+/CD44^+$
$CD8^+$ T naïve cells	$CD45^+/CD3^+/CD8^+/CD62L^+$
$CD8^+$ T memory cells	$CD45^+/CD3^+/CD8^+/CD44^+/CD62L^+$
$CD4^+$ T effector (eff.)	$CD45^+/CD3^+/CD4^+/CD44^+$
$CD4^+$ T naïve cells	$CD45^+/CD3^+/CD4^+/CD62L^+$
$CD4^+$ T memory cells	$CD45^+/CD3^+/CD4^+/CD44^+/CD62L^+$
$CD4^+$ T regulatory (T regs)	$CD45^+/CD3^+/CD4^+/FoxP3^+$

A


SSC-A
Time
single cells
SSC-H
cells
FSC-A
live
FVD APC-Cy7
CD45
CD45-AF700
lymphocytes
FSC-A
T cells
CD3-BUV395
CD8
CD8+
CD4+
CD4-BUV496
CD44
eff.
memory
naive
CD62L-BV605
CD44-BUV737
B cells
CD19-FITC
NK
NKp46-BV510
NKT
NKp46-BV510
γδT
γδTCR-PECF594
T regs
FoxP3-PE

B

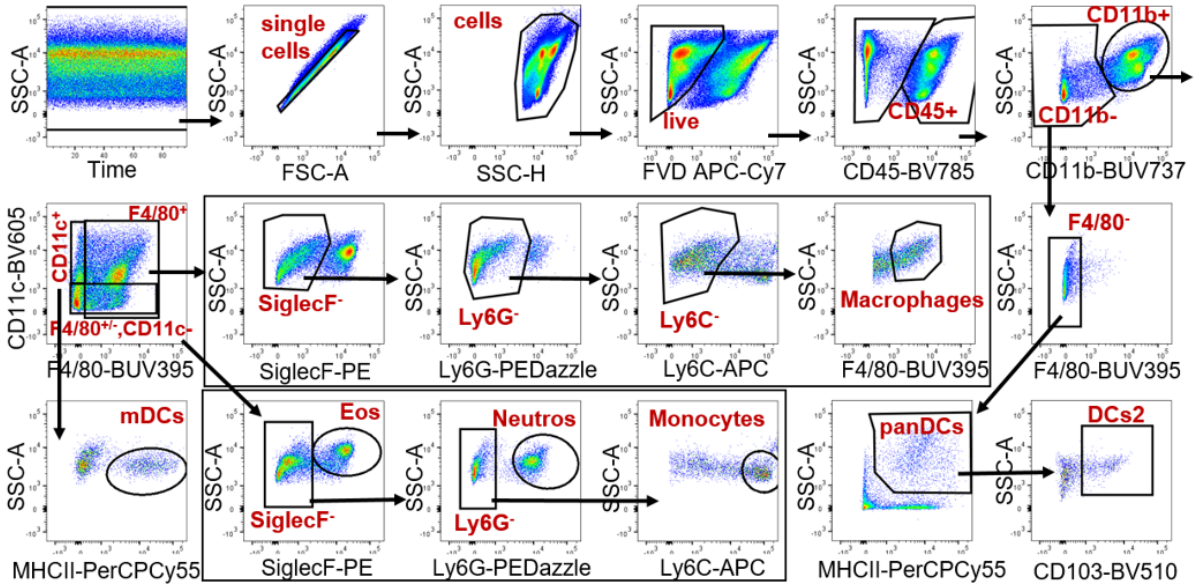

Time
single cells
FSC-A
cells
SSC-H
live
FVD APC-Cy7
CD45+
CD45-BV785
CD11b+
CD11b-
CD11b-BUV737
CD11c-BV605
CD11c+
F4/80+
F4/80+/-,CD11c-
F4/80-BUV395
SiglecF-
SiglecF-PE
Ly6G-
Ly6G-PEDazzle
Ly6C-
Ly6C-APC
Macrophages
F4/80-
mDCs
MHCII-PerCPCy55
Eos
Neutros
Monocytes
panDCs
DCs2
CD103-BV510

Figure 12. *Flow cytometry gating strategies of mice lymphoid and myeloid panels*

(A-B) Gating strategies to identify lymphoid and myeloid populations in KP and LLC tumors. Initial two gates are to select time and single cells. Total cells were gated based in their FSC-A and side scatter SSC-A properties. Live cells were identified as FVD^- and CD45 was used to gate immune infiltrating cells. An additional lymphocyte gate was used to exclude myeloid cells from the lymphoid panel. The specific immune populations were then defined as is described in **Table 5** and **Table 6**. This figure has been adapted from (3).

The infiltration of live cells and leukocytes into KP tumors appeared to be comparable in both the WT and MPO KO mice (**Figure 13 A-B**). However, significant differences in immune infiltration were noticed in the TME of MPO-deficient mice when compared to WT mice, between the tumor of mice lacking of MPO and WT mice, predominantly within the lymphoid cell populations. We observed a significantly higher number of monocytes and eosinophils in the tumors of MPO KO mice than in WT mice (**Figure 13 C**). Other myeloid cell populations, including neutrophils, macrophages, DCs, mDCs, and panDCs, showed no changes between the two groups (**Figure 13 C**). Regarding lymphoid immune populations, we identified a significant increase in the infiltration of γδT cells, NK cells, NKT cells, $CD3^+$ T cells, and $CD8^+$ T cells into tumors of MPO KO mice (**Figure 13 D-E**). We also identified differences in the relative abundance in subtypes of $CD4^+$ and $CD8^+$ T cells, as memory $CD4^+$ and $CD8^+$ T cells and effector $CD8^+$ T cells were increased in MPO KO mice when compared to WT mice (**Figure 14 A-B**). Additionally, the inhibitory checkpoint receptor PD-1 on tumor-infiltrating $CD4^+$ and $CD8^+$ T cells was increased in MPO KO mice (**Figure 14 C**), suggesting increased immune activity of these cells.

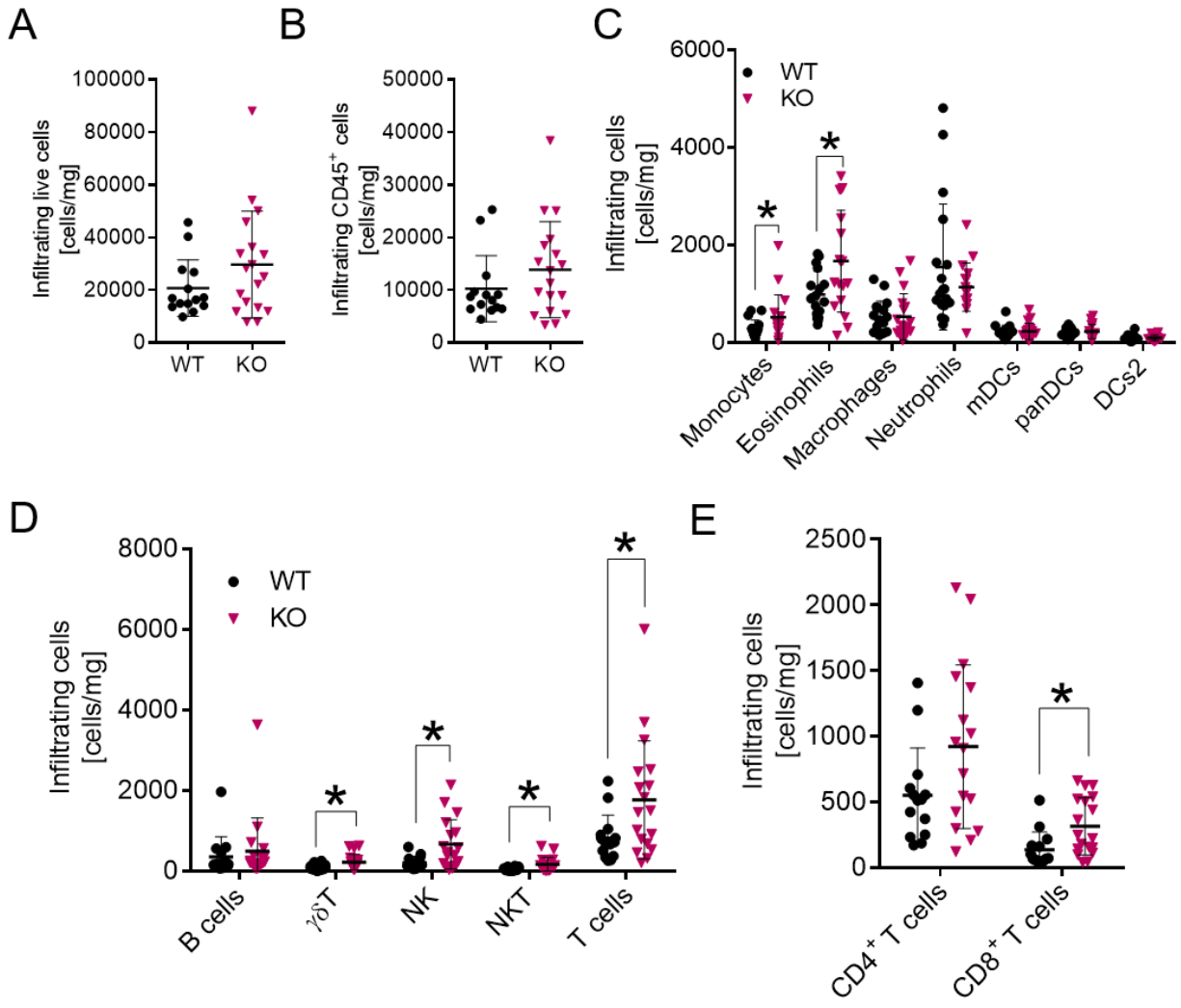


Figure 13. *MPO influences the immune cell profile of the TME*

Single cell suspension of KP tumors collected from MPO KO and WT (n=18-20) were stained with two flow cytometric panels and analyzed by flow cytometry. The data is expressed as cells per mg. **(A, B)**. No differences were observed in the live and $CD45^+$ cells when compared tumors from MPO KO vs WT. **(C)** A higher number of infiltrating monocytes and eosinophils was observed in the tumors of MPO KO mice when compared to the WT littermates. **(D)** An increased amount of infiltrating γδT cells, NK, NK T cells and T cells was observed in tumors of MPO KO vs WT. **(E)** Tumors from MPO KO mice exhibited more $CD8^+$ T cells as compared to tumors from WT mice. **(A-E)** Experiment was performed two times in total with male mice and data were combined. Data are shown as mean ± SD *p < 0.05 and **p < 0.01. Dendritic cells, DCs2, myeloid dendritic cells, mDCs; plasmacytoid DCs, panDC; NK, natural killer cells; NKT, natural killer T cells. This figure has been adapted from (3).

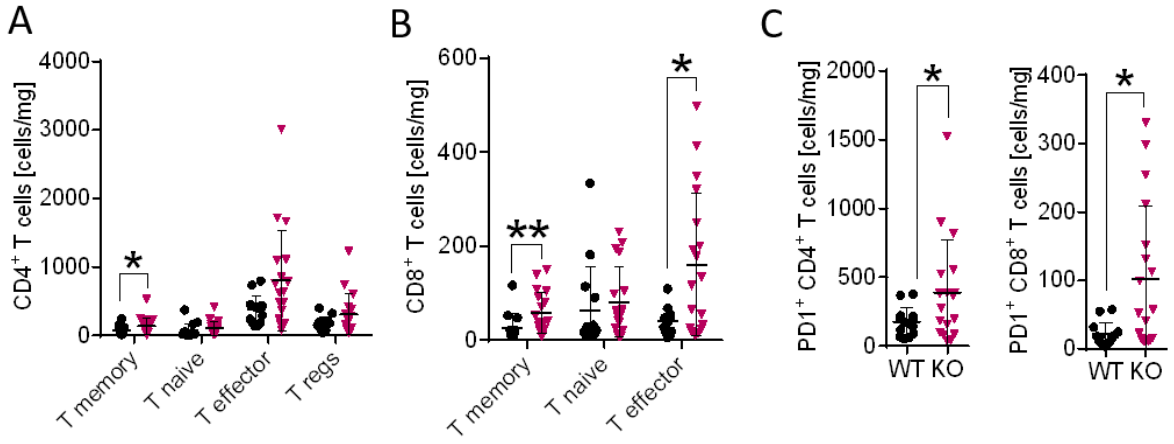


Figure 14. *KO of MPO influences the content of T cells subtypes*
(A-B) Graphical representation of the absolute content (cell/mg) of subtypes of $CD4^+$ and $CD8^+$ T cells. **(A)** In the $CD4^+$ T cell subset, a higher number of memory cells was observed in tumors from MPO KO mice vs. WT. **(B)** In the $CD8^+$ T cell subset, a higher number of memory and effector cells was observed in tumors from MPO KO mice vs. WT. **(C)** The absolute content (cells/mg) of $PD1^+/CD4^+$ and $PD1^+/CD8^+$ T cells was also increased in the tumors from MPO KO mice when compared vs WT mice. **(A-C)** Experiment was performed two times in total with male mice (n=18-20) and data were combined. Data are shown as mean ± SD *p < 0.05 and **p < 0.01 This figure has been adapted from (3).

The analysis of lymphoid populations within spleens of non-bearing and bearing mice revealed no differences between MPO KO and WT mice (**Figure 15 A-C and Figure 16 A-C**), indicating that the observed differences in immune infiltration into the tumors are likely linked to the tumor growth.

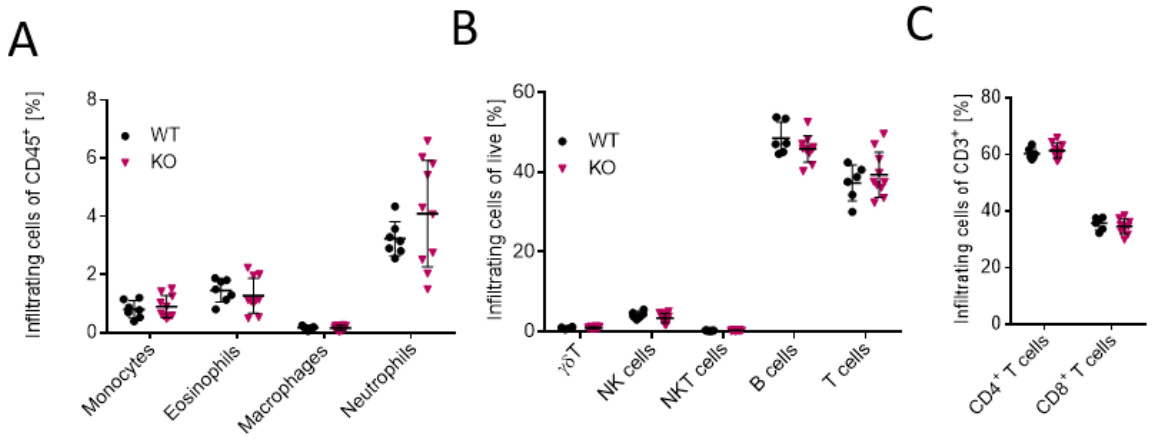


Figure 15. *Baseline levels of splenic lymphoid immune cells in tumor-free mice*
(A-C) No significant differences were found in lymphoid and myeloid populations in the spleens of tumor-free MPO KO and WT mice. Experiment was performed one time in total with male mice (n=6–10). Data are shown as mean ± SD. This figure has been adapted from (3).

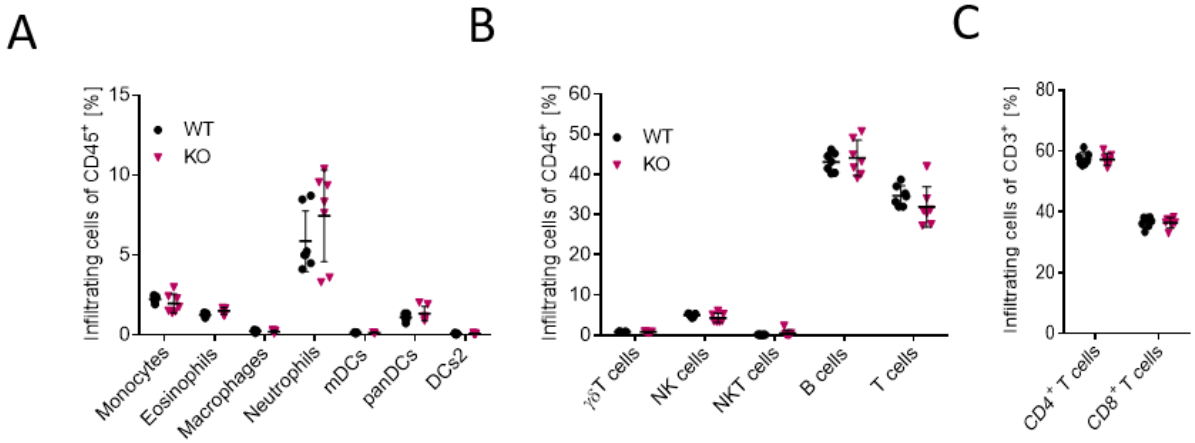


Figure 16. *Baseline levels of splenic lymphoid immune cells in tumor-bearing mice*
(A-C) No significant differences were found in lymphoid and myeloid populations in the spleens of tumor-bearing MPO KO and WT mice. Experiment was performed one time in total with male mice (n=7). Data are shown as mean ± SD. This figure has been adapted from (3).

3.2.4 Tumor infiltrating T cells exhibit enhanced local activation in tumors of MPO-depleted mice

To evaluate the cytotoxic function tumor-infiltrating T cells, T cells derived from MPO KO and WT mice were stimulated with PMA/Ionomycin *ex vivo* and flow cytometry was used to measure their activity. In comparison to WT mice, tumors of MPO KO mice exhibited enhanced expression levels of IFN-γ on $CD8^+$ (**Figure 17 A-B**) and $CD4^+$ T cells (**Figure 17 C**), indicating local activation and increased tumoricidal activity of T cells.

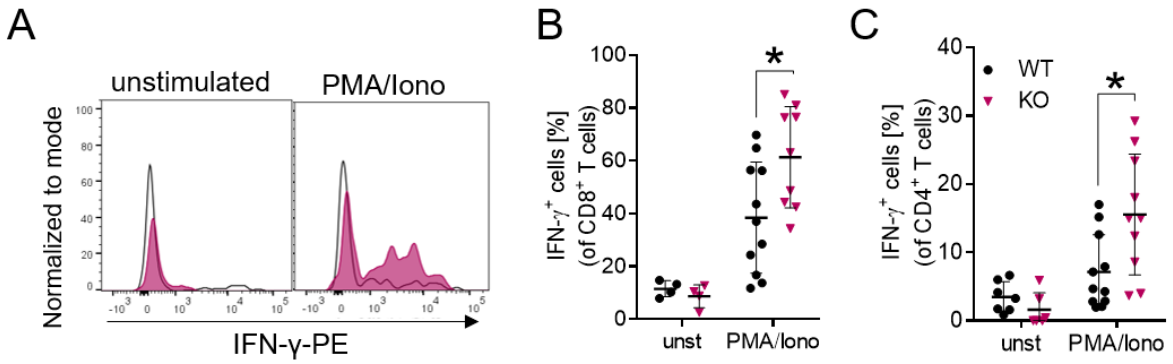


Figure 17. *MPO absence results in an increased cytotoxic activity of T cells ex vivo*
(A) Representative histograms showing the percentage of IFN-γ expression in tumor-infiltrating $CD8^+$ T cells before (unstimulated) and after PMA/Iono stimulation *ex vivo*. **(B-C)** $CD8^+$ and $CD4^+$ cells from MPO KO mice (in pink) showed increased expression of IFN- γ as compared to the WT littermates (in black). Experiment was performed one time in total with male mice (n=5-10). Data are shown as mean ± SD. *p < 0.05 Iono, ionomycin; PMA, phorbol 12-myristate 13-acetate/ionomycin. This figure has been adapted from (3).

3.2.5 Reduction of tumor size in MPO KO mice is dependent on $CD8^+$ T cells

To explore whether the reduction in tumor size observed in MPO KO mice is associated with the presence of $CD8^+$ T cells, mice bearing KP tumors were subjected to i.p. injections of either anti-CD8 antibodies or control IgG isotypes. (**Figure 18 A**). $CD8^+$ content was monitored during the course of the experiment demostrating the efficacy of the anti-CD8 antibodies (**Figure 18 A**). At the end of the experiment, tumors were collected, weighed, measured, and analyzed using flow cytometry. On average, the $CD8^+$ T cell pool was reduced by 90% (**Figure 18 C**). In the isotype-treated groups, MPO KO mice exhibited decreased tumor weight as compared with tumors of the WT mice (**Figure 18 B**). Remarcably, we observed no differences in tumor weight between

the MPO KO and WT mice in the anti-CD8 antibody-treated groups (**Figure 18 B**). $CD8^+$ T-cell content was higher in isotype-treated MPO KO mice compared to WT mice (**Figure 18 C**). These findings suggest that $CD8^+$ T cells are necessary for the reduction in tumor growth observed in MPO KO mice.

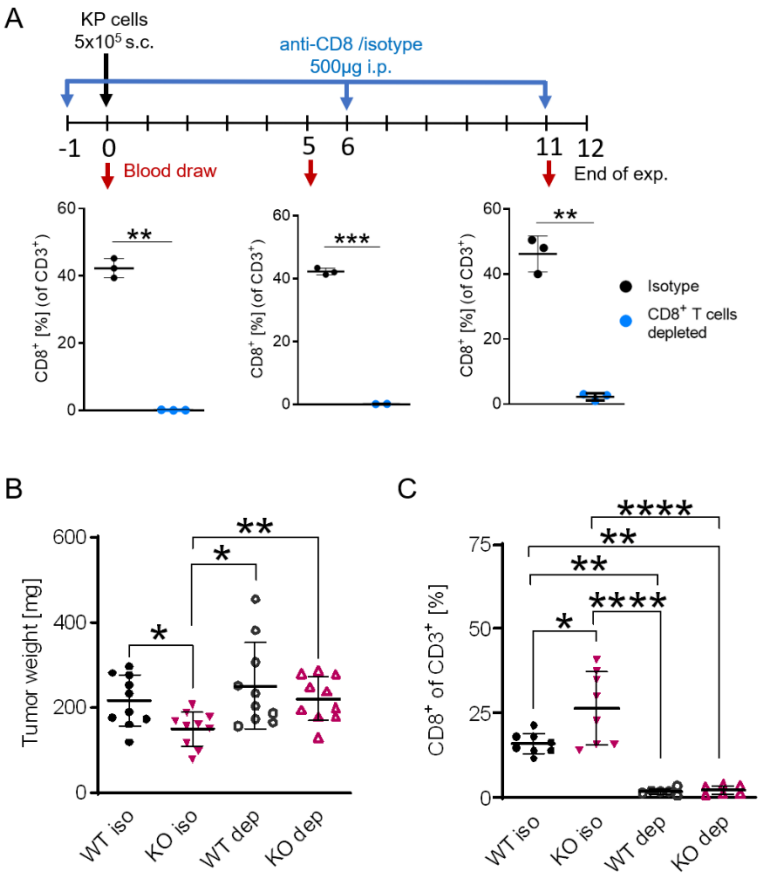


Figure 18. *$CD8^+$ T cells are necessary to reduce tumor growth in MPO KO mice.*
(A) Experimental design: Mice were i.p. administered with anti-CD8 antibody (500 µg per mouse) or isotype control on day 0. On day 1 KP cells were s.c. administered to the mice. Mice received another two dosages of anti-CD8 antibody or isotype control on days 6 and 11. On days 0, 5 and 11 blood was drawn for mice and the content of $CD8^+$ cells was analyzed by flow cytometry. At the end of experiment (on day 12), mice were euthanized and tumors were measured and weighted. **(B)** In the isotype-treated mice, a significant reduction of tumor weight was observed the MPO KO mice when compared to WT mice. In contrast, in the anti CD8-treated mice, no differences in tumor weight were observed between the two groups. **(C)** $CD8^+$ cells were depleted in WT and MPO KO mice treated with anti-CD8 antibody. **(B-C)** Experiment was performed one time in total with male mice (n=6-10). Data are shown as mean ± SD. *p < 0.05, **p < 0.01, and ****p < 0.0001.This figure has been adapted from (3).

3.2.6 MPO decreases proliferation and function of T cells

To comprehend the mechanisms through which MPO influences T cell behavior, we conducted *in vitro* experiments. We used the proliferation dye eFlour™ 450 to stain T cells previously isolated from healthy donors PBMCs. T cells were exposed to different concentrations of MPO (0, 5, 10, and 20 µg/mL) for 72 hours and their proliferation was examined using flow cytometry. Following 72 hours of MPO treatment, the proliferation of both $CD4^+$ and $CD8^+$ T cells exhibited a reduction of approximately 20% (**Figure 19 A-B**).

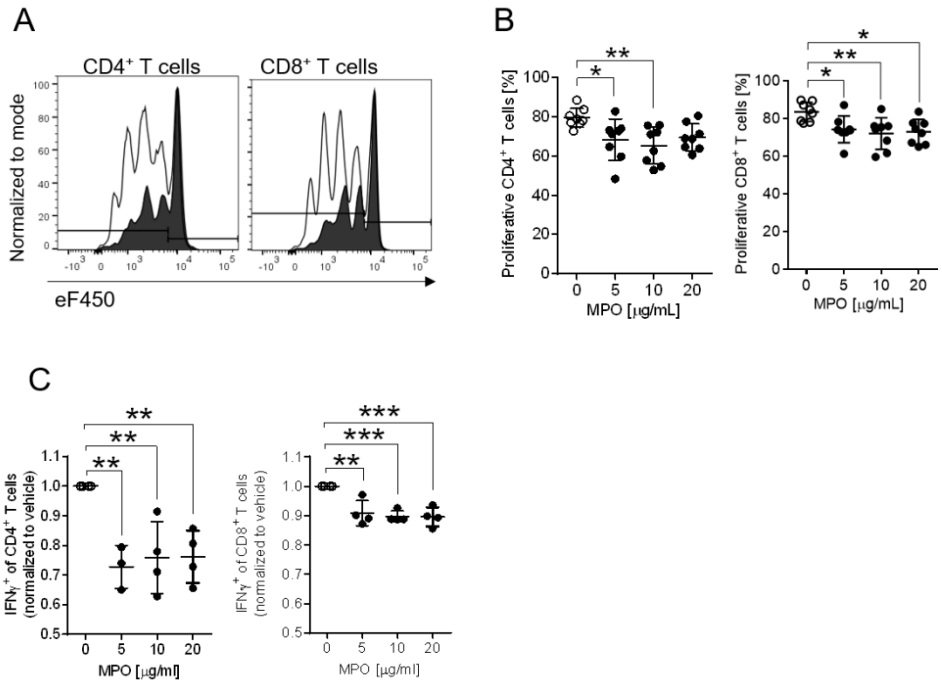


Figure 19. *T cells exposed to MPO have decreased proliferation and function*
(A) Enriched T cells (from healthy donors' PBMCs) were labeled with the proliferation dye eFlourTM 450 and incubated with MPO for 72 h. The monitoring of individual cell divisions was evaluated in $CD4^+$ and $CD8^+$ T cells using flow cytometry. Each peak corresponds to different cell divisions in MPO-treated (black) and untreated (white) cells. **(B)** $CD4^+$ and $CD8^+$ T cells treated with MPO showed a reduced proliferation when compared to untreated cells (n=8). **(C)** T cells were incubated with MPO for 24 h, iono/PMA was used to stimulate IFN-γ production and its expression was evaluated in $CD4^+$ and $CD8^+$ T cells by flow cytometry. A reduction in the expression of IFN-γ was observed in T cells that were treated with MPO (n=4). Data are shown as mean ± SD. n= 8. *p < 0.05, **p < 0.01, and ***p < 0.001 This figure has been adapted from (3).

Furthermore, we explored the impact of MPO on T cell activation. Human $CD4^+$ and $CD8^+$ T cells were subjected to varying MPO concentrations for 24 hours, subsequently stimulated with PMA/Iono, and their capacity to produce IFN-γ was evaluated using flow cytometry. In comparison to the control group, MPO-treated T cells displayed diminished levels of IFN-γ expression, indicative of reduced activation (**Figure 19 C**).

3.2.7 MPO internalizes into T cells

The ability of MPO to internalize into endothelial and epithelial cells has been broadly reported (274,275). To investigate whether MPO is also able to bind and internalize into T cells, T cells were exposed to MPO (0, 5, 10, and 20 µg/mL) for 2 hours and flow cytometry was used to assess its binding and internalization into T cells (**Figure 20 A**). After 2 hours of treatment, more than 50% of $CD4^+$ and $CD8^+$ T cells were MPO-positive (**Figure 20 A-B**).

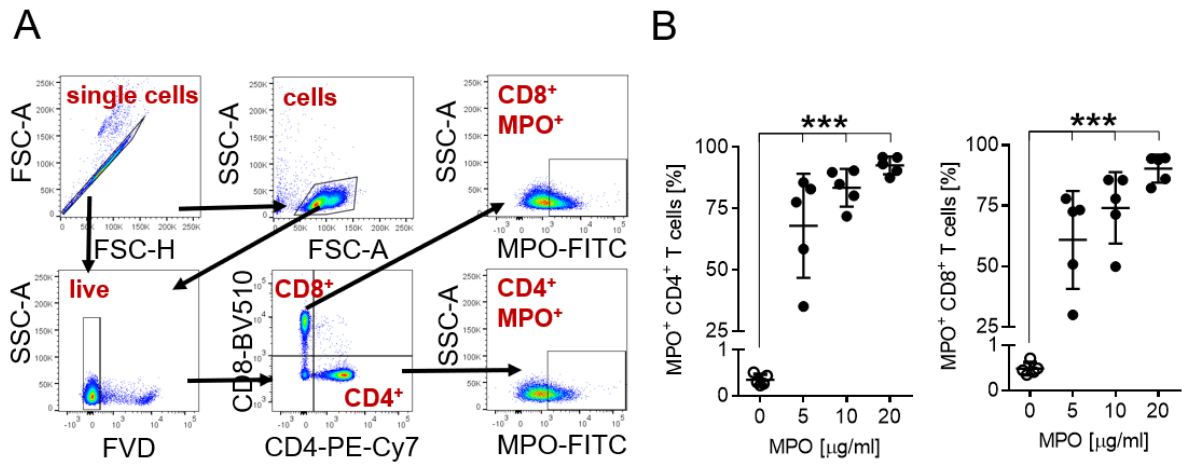


Figure 20. *MPO binds and internalizes into T cells*
(A) Gating strategy: Initial gates was to select single cells. Total cells were gated based in their FSC-A and (SSC-A) properties. Live cells were identified as FVD^-. Cells were further analyzed for their CD4 and CD8 expression and finally MPO expression was determined within the $CD8^+$ and $CD4^+$ populations. **(B)** MPO-treated $CD8^+$ and $CD4^+$ T cells showed positive MPO staining (n=5). Data are shown as mean ± SD. ***$p < 0.001$ This figure has been adapted from (3).

3.2.8 Heparin blocks MPO internalization and prevents MPO effects on T cells

It has been reported that interactions between cell surface glycosaminoglycan and MPO are required for MPO internalization into cells and soluble glycosaminoglycans such as heparin can block MPO cellular uptake (274). Accordingly, we treated T cells with heparin for 45 minutes followed by treatment with MPO for 2 hours which resulted in the blocking of MPO uptake by T cells (**Figure 21 A-B**). Interestingly, MPO's effect on T cell proliferation was reversed by heparin pretreatment (**Figure 21 C**), implying that MPO binding and internalization to T cells are required for its functional role.

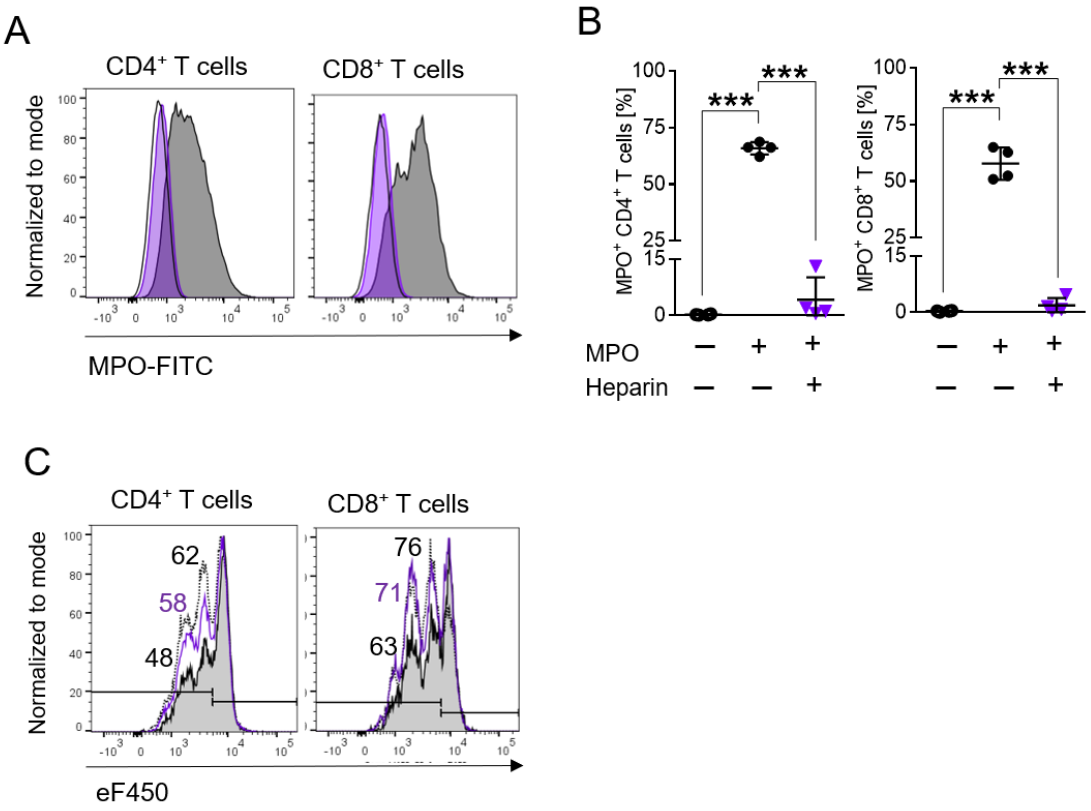


Figure 21. *MPO's uptake by T cells is prevented by heparin*
(A-B) T cells were pretreated with heparin for 45 minutes followed by MPO treatment for 2 hours and positive staining for MPO was evaluated using flow cytometry. **(A)**Histograms showing the fluorescence intensity in the untreated (white), MPO-treated (grey) and heparin-MPO-treated (violet) T cells samples. **(B)** The percentage of MPO^+ $CD4^+$ and MPO^+ $CD8^+$ T cells was reduced when cells were pre-treated with heparin (n=5). Data are shown as mean ± SD. ***p < 0.001 **(B)** Cell proliferation was determined in $CD4^+$ and $CD8^+$ T cells that were pretreated with heparin and next treated with MPO. Numbers represent the percentage of proliferative cells in the vehicle (dotted line), MPO-treated (black line), and heparin-MPO-treated (purple line) cells (n = 1). This figure has been adapted from (3).

3.2.9 MPO is found in the lymphocytes of tumor samples from patients with NSCLC

Ultimately, we sought to validate whether our findings concerning MPO binding and uptake by T cells were consistent in clinical samples. To accomplish this, we employed human LUAD tissue specimens and utilized flow cytometry to investigate the presence of MPO within lymphocytes in NSCLC tissues (**Figure 22 A**).

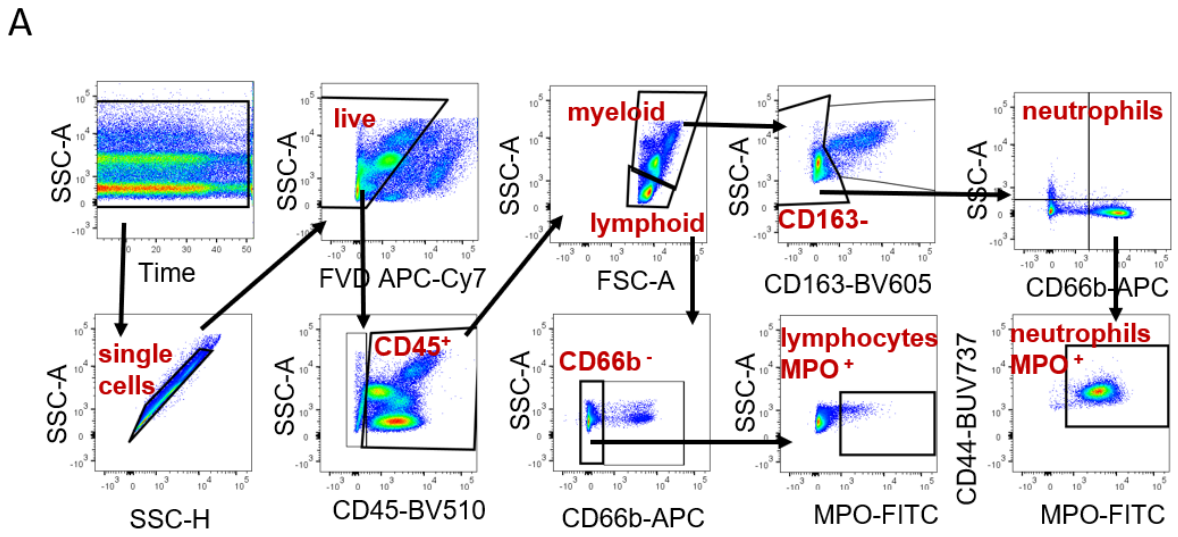


Figure 22. *Gating strategy to analyze MPO in tumors from NSCLC patients*

(A) First two gates were to select time and singlets. Live cells were identified as FVD^-. Cells were further analysed for their CD45 and myeloid and lymphoid cells were gated based on their FSC-A and SSC-A properties and finally MPO expression was determined within the $CD8^+$ and $CD4^+$ populations. CD66b was used to exclude myeloid cells within the lymphoid gate and MPO presence in lymphocytes was determined as $CD66b^-/MPO^+$. MPO^+ neutrophils were gated as $CD163^-/CD66b^+/MPO^+$. This figure has been adapted from (3).

Immunohistochemistry staining demonstrated the presence of MPO within lymphocytes in LUAD tissues (**Figure 23 A**), This observation aligns with our earlier in vitro findings. Furthermore, flow cytometry analysis of several NSCLC samples revealed the existence of a lymphocyte MPO^+ population (**Figure 23 B-D**). It is noteworthy that there was a positive correlation between the proportion of infiltrating neutrophils (characterized as $CD163^-$ $CD66b^+$), which are the primary source of MPO in NSCLC, and the proportion of MPO-positive lymphocytes in NSCLC samples (**Figure 23 E**).

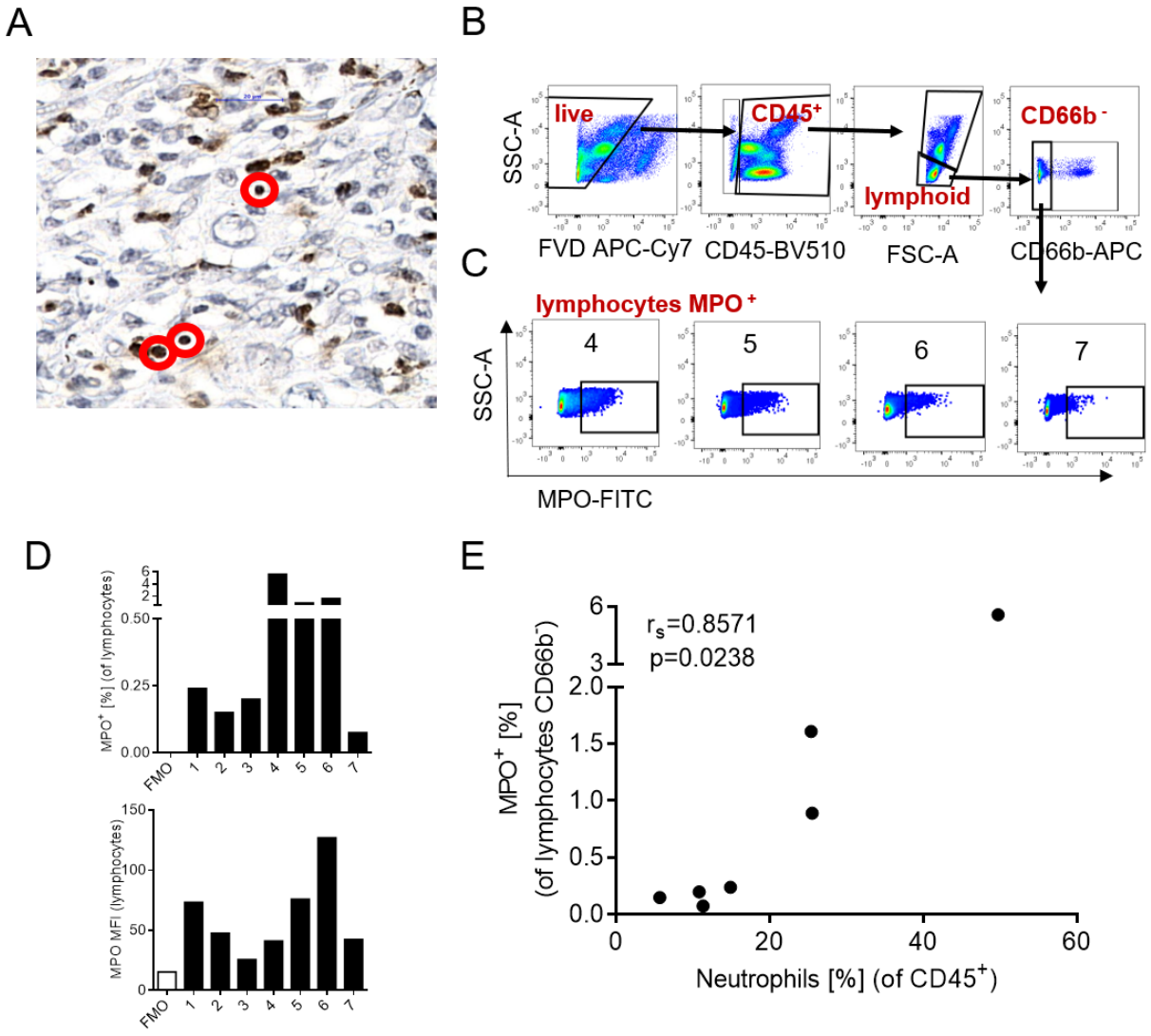


Figure 23. *MPO is found in lymphocytes from NSCLC tumor samples.*
(A) Immunohistochemistry staining of LUAD samples showing MPO presence in inflammatory cells, including lymphocytes (circle) (digital magnification: ×80). **(B)** Gating strategy to recognize MPO^+ lymphocytes in NSCLC patients **(C)** Representative dot plots of MPO^+ lymphocytes in NSCLC patients. **(D)** Graphs showing the percentages of MPO^+ lymphocytes and MPO median fluorescence intensity (MFI). **(E)** A positive correlation (determined by Spearman's correlation coefficient rho, rs, n=7) between MPO^+ lymphocytes and neutrophil content in a cohort of NSCLC patients was found. This figure has been adapted from (3).

4 DISCUSSION

The immune system plays a pivotal role in tumor development. On the one hand, prolonged inflammation is linked to the development of malignant tumors and on the other hand, properly regulated immune cells can contribute to fight against cancer. Moreover, immune cells can also be useful as biomarkers to detect cancer in earlier stages, monitor treatment response, and predict patient survival. Cells such as T lymphocytes have been broadly studied in the context of cancer and many of their roles are already unraveled. For their part, neutrophils have come to the spotlight more recently showing to be phenotypically and functionally diverse and playing divergent roles in cancer development. During my PhD I investigated the impact of neutrophils in NSCLC from two different perspectives. In the first project we combined high dimensional flow cytometry screening of clinical samples and bioinformatic tools to define new surface markers that allowed us to discriminate between two neutrophil subpopulations known as LDNs and HDNs. We validated the overexpression of four novel markers in LDNs from patients with NSCLC and confirmed that the LDN subpopulation is increased in the blood of NSCLC patients when compared to healthy people. In the second project, we used *in vivo* and *in vitro* models to explore the role of a neutrophil-derived enzyme, MPO, in NSCLC. We found that MPO absence in mice results in smaller tumors and higher lymphocyte infiltration. Moreover, *in vitro* data showed that MPO modifies the function of T cells towards an immunosuppressive role.

Despite the obvious differences in the approaches and findings of both projects, our results emphasize the importance of neutrophils and neutrophil-derived enzymes in the biology of lung cancer and contribute to the understanding of the complex roles of neutrophils in cancer. In the end this dissertation provides suggestions of how neutrophils and neutrophil-derived molecules are potential candidates that can be used as diagnostic tools and therapeutic targets.

4.1 LDNs display a distinctive immune signature in NSCLC patients.

The diverse and contrasting roles of neutrophils in cancer could be explained by their phenotypical heterogeneity. In fact, it is likely that not only the nature but also the ratio of the diverse neutrophil subpopulations in tumors and in circulation define the overall role of these cells in cancer. According to this, it is critical to characterize the distinct neutrophils subpopulations based on reliable surfaces markers that allow us to isolate them, functional characterize them and explore their potential as diagnostic and prognostic tools.

Among the diversity of neutrophil subpopulations, LDNs have become increasingly interesting being their presence in patients with inflammatory conditions and their usual absence in healthy people. Our research showed that LDNs are increased in patients with NSCLC (independently of their disease stage) when compared to healthy volunteers. Our findings are in accordance with other reports that have shown a higher number of LDNs in patients with breast, head and neck, lung and urologic cancers (86,114,129,132). It has been suggested that LDN content might correlate with the state of the disease (129,276) being that their abundance is heterogeneous within patients. The fact that the percentages of LDNs in our NSCLC cohort comprised a broad range (0.3-76.1% of all cells in the PBMC fraction) supports this theory.

To validate the potential utility of LDN content as a biomarker for tracking cancer progression or treatment effectiveness, additional research is needed. These studies should involve larger and more diverse patient cohorts, encompassing various stages of the disease and individuals undergoing different treatments. Unfortunately, exploring LDNs in the clinical setting as diagnostic and prognostic tools is complicated due to some limitations. Such limitations include the high workload required to isolate them (which involves sucrose gradient centrifugation and flow cytometry staining), as well as the requirement to handle blood samples shortly after their collection. On this regard, further characterization of neutrophil subpopulations in the blood stream is needed. Most studies have focused on myeloid cell and neutrophil maturation markers including CD10, CD11b, CD15, CD16, CXCR4 and PDL-1 (129,277,278). In human cancers, LDNs overexpress CD66b, CD11b and CD15 compared to HDNs (114,132). CD10, CXCR4 and PDL-1 were found to be differentially expressed in LDNs vs HDNs in patients with advanced lung cancer (129). Despite these reports, a comprehensive examination of neutrophil diversity in the bloodstream, especially in the context of cancer, is limited by the challenge of conducting unbiased gene expression analysis on neutrophils, primarily owing to their limited RNA content. Regardless, using a gene expression array, Lox1 (lectin-type oxidized LDL receptor 1) has been identified as a surface marker exclusively expressed on LDNs but not in HDNs, both in the peripheral blood and in tumors of cancer patients (273). .

To overcome some of these limitations and with the purpose of identify markers that allow us to discriminate between LDNs and HDNs, we used blood samples of NSCLC patients and performed an unbiased high-dimensional flow cytometry surface marker screen on LDNs and HDNs isolated from the PBMC and PMNL fractions, respectively. Using this approach, we aimed to analyze a broader variety of surface markers, most of which have not been formerly described

as neutrophil markers. We defined circulating neutrophils (LDNs and HDNs) as $CD66b^{+}Siglec8^{-}$ and used flow cytometry and bioinformatic analysis (UMAP and correlation) to validate our results. Using this approach, we overcome the limitation of a small sample size and found several cell surface markers that were differentially expressed in LDNs vs HDNs. We validated the findings of the screen with the top four overexpressed markers (CD36, CD41, CD61 and CD226). However, CD226 had to be excluded of the final analysis being the weak separation of the positive and negative populations. At the end we could confirmed that CD36, CD41, CD61 are overexpressed in LDN fraction of NSCLC patients when compared to the HDN fraction.

It is to note that none of the four identified surface markers have been previously reported to be expressed by neutrophils, however, CD36 and CD61 are expressed by other immune cells such as macrophages (CD36), monocytes (CD36) and DCs (CD36 and CD61) (279–282). Moreover, the expression of CD36 has been also reported in microvascular endothelial cells, retinal epithelial cells, adipocytes, platelets, enterocytes, microglial cells and podocytes, as well as in tumor cells, stromal cells and immune cells of tissues from cancer patients (279,283). In the case of CD41, its expression has been reported in platelets and megakaryocytes (280,284,285). A broad diversity of functions has been described for these surface markers; CD36 is a transmembrane glycoprotein that acts as a scavenger receptor and its involved in lipid uptake, immunological recognition, inflammation, molecular adhesion, and apoptosis (279,283); CD61 (integrinβ3) is involved in the uptake of apoptotic cells and induction of immune tolerance and together with CD41 (αIIb integrin) form a complex that acts as a receptor for adhesion molecules required for platelet aggregation and clotting (280–282,285). In the context of cancer, CD36 has been reported to participate in lipid homeostasis, immune response, angiogenesis, adhesion, and metastasis (283). Therefore, further research is necessary to elucidate the function of these markers in the biology of LDNs.

It is crucial to bear in mind that LDNs have been found not only in cancer patients but also in individuals with other inflammatory conditions such as asthma, HIV, dermatomyelosis and malaria and moreover also in non-pathological conditions like during pregnancy and in newborns (116,117,122–124,126,132,270,286–289). Therefore, further studies to evaluate the expression of CD36, CD41 and CD61 in the LDN fraction of patients suffering from other pathologies as well as in pregnant women and newborns are necessary to better understand LDNs diversity and function.

Our data showed that some patients exhibited low marker expression on HDNs, which support the concept that neutrophils can change their phenotype (neutrophil plasticity), and that HDNs and LDNs represent a spectrum of neutrophil phenotypes. Recent discoveries indicate that LDNs from cancer patients can be found in the HDN fraction and vice versa. This aligns with earlier research demonstrating that in mice with tumors, HDNs can transition into LDNs and vice versa, both *in vivo* and *ex vivo.* Further research incorporating novel LDNs markers such as CD36, CD41, and CD61, in addition to neutrophil maturation markers, would enhance our understanding of the origin and function of LDNs.

One of the long-term goals of this project is the use of LDNs as biomarkers for lung cancer early detection and to monitor therapy response. The use of low-dose computed tomography scan is still broadly use as the method for early lung cancer screening, although quick, painless and non-invasive (no dyes, no injections, and requires nothing to swallow by mouth), this method is expensive and has a high false positive rate which demonstrate the need of alternative (or complementary) diagnostic test that are minimally invasive and that harbor the ability to identify malignant pulmonary nodules. Moreover, minimally invasive tests to identify biomarkers that allow us to identify patients that will positively respond to targeted therapies (e.g. immune checkpoint inhibitor, ICI, therapy) are highly valuable to avoid side-effects and enable fast clinical decisions. In this context, the present study contributes to the identification of blood-based markers for lung cancer early detection in patients with high risk of developing lung cancer, as well as for the prediction of response to therapy. Further efforts should include research to establish a protocol that allow the identification of LDNs in whole blood of patients with the objective of develop rapid, easy, and affordable clinical routine method to use LDNs as biomarkers. The identification of specific LDNs surface markers is one of the first steps towards this goal.

Other relevant aspects of the role of LDNs in cancer development and progression need further exploration. Although LDNs have been reported to have immunosuppressive effects on T cell proliferation, activation and function, further studies are necessary to confirm this. The identification of specific LDNs markers is crucial to study them. Surface markers can be used for cell sorting and co-culture experiments and further exploration of the LDNs role not only as immunosuppressive cells but also to study their impact in cancer cells proliferation, migration and invasion.

4.2 MPO favors tumor growth and acts as an immunosuppressive factor in NSCLC

The TME is comprised not only by different kind of cells but also by a variety of molecules that support tumor development. Cells within the TME can secrete soluble factors as cytokines, chemokines, growth factors and metabolites, among others (290). Communication between the different cells in the TME is orchestrated in a complex way that include cell-cell contact and autocrine or paracrine signaling. At the end, the intricate network of cells and soluble factors will facilitate a pro- or anti-tumorigenic phenotype in the TME (290).

In NSCLC, neutrophil-derived molecules as NE, have been recently shown to possess pro-tumorigenic properties (151). Based on this, during my PhD I aimed to understand whether another neutrophil-derived protein called MPO contributed to NSCLC carcinogenesis.

MPO is a heme peroxidase highly expressed by neutrophils (177). Physiologically, MPO helps to the clearance of pathogens once neutrophils arrive at the site of infection where it can be released upon neutrophil activation or neutrophil dead (184). Moreover, MPO also plays a role in triggering and advancing inflammatory diseases, with particular significance in the pathophysiology of cardiovascular diseases (291,292). More recently, MPO has been also shown to participate in the regulation of other inflammatory conditions including cancer (2).

In the present study, using public available data bases and bioinformatic analysis, we showed that low MPO protein expression correlated with better survival prognosis of NSCLC patients in comparison with patients with higher MPO protein expression. In this context, higher amounts of MPO have been detected in the serum and bronchoalveolar fluid of patients with lung cancer (232). Other research has revealed that patients with lower MPO levels have a reduced vulnerability to develop different kinds of cancer such as lung (293), ovary (294) and breast cancer (295). Lower MPO expression is particularly linked to the MPO polymorphism MPO-463G > A which negatively influences the expression at mRNA levels and subsequent transcription of MPO (228). Some studies show that a lower MPO expression is linked to reduced oxidative stress which could explain the lower susceptibility to develop certain kinds of cancer. DNA-damage induced by MPO-derived oxidants could also contributed to carcinogenesis (240) . In any case, further studies are necessary to elucidate the possible use of MPO as a biomarker since not conclusive data is available yet.

We next observed that mice that lack MPO and that were s.c. injected with lung cancer cells (either KP cells or LLC), developed smaller flank tumors in comparison with WT mice. Previous studies in other laboratories have also shown that MPO KO mice developed smaller tumors in comparison with WT controls (250). However, in a model using aged-mice (23 week-old), researchers found that orthotopic melanomas grew slowly in WT mice (MPO+/+) when compared to MPO KO mice and interestingly, this effect was also observed with the MPO inhibitor ABAH, being that ABAH-treated mice developed bigger tumors when compare to WT mice (251). The discrepancies between the findings of our study and the last-mentioned study could be explained by the differences in the age of the mice. Regarding the efforts to investigate the potential of MPO as a druggable target, the administration of the MPO inhibitor KYC in an inflammatory-driven lung tumor model, resulted in a reduction of tumor burden (250). Moreover, a report from 2023, revealed that either MPO deficiency or pharmacological inhibition of MPO (using ABAH) enhanced the response to ICI therapy in models of melanoma in aged mice (268). Further research with focus in the development of specific MPO inhibitors is necessary to better explore the potential of MPO as a therapeutic target.

We also found that the TME shifted towards an anti-tumorigenic profile in the tumors of the MPO-KO mice being that we observed higher numbers NK, NKT cells, γδ T cells, and $CD3^+CD8^+$ T cells when compared to tumors of the WT mice. When further exploring the infiltrating $CD8^+$ T cells of MPO KO vs. WT mice, we discovered an increased activation status in the KO's, which revealed in elevated levels of IFN-γ. Moreover, the numbers of memory and effector $CD8^+$ T cells were also enhanced in the KO's. In general, enhanced infiltration of cytotoxic lymphocytes ($CD8^+$ T cells and NK cells) into the TME is associated with a good prognosis (296). However, in NSCLC tissue, it has been reported that the number of cytotoxic T cells are reduced and have a decrease in IFN-γ expression (297,298). Accordingly, the higher infiltration of cytotoxic cells such as $CD8^+$ T cells and NK cells as well as the improved activity of $CD8^+$ T cells observed in our model might have supported the reduction in tumor growth observed in the MPO-KO mice vs WT. To test this hypothesis, we depleted $CD8^+$ T cells in MPO KO and WT mice and observed no differences in tumor weight and volume between the $CD8^+$ T cells-depleted groups. With this experiment we could confirm that $CD8^+$ T cells contribute to the reduction in tumor growth observed in the MPO KO mice. In contrast to our findings, Liu et al. reported that MPO depletion resulted significant decrease in $CD8^+$ cytotoxic T cells at the periphery of s.c. melanoma tumors of aged mice (251) . The differences between our results and

the literature suggest that the effect of MPO deficiency on the state of the TME is dependent of tumor entity but can also be a result of the age of the mice used in the studies. Moreover, our results show the content of $CD8^+$ T cells in homogenates of the tumors which we cannot compare to histological staining reported in the study from Liu et al. In any case further research is necessary to better characterize the influence of MPO in the immune infiltration of cytotoxic T cells into the TME. Being that an enhanced infiltration of NK cells into the TME has been also reported to correlate with a good prognosis in lung cancer (299), further studies should also focus in investigate the relevance of NK cells in the MPO KO model.

Being the increasing interest in ICI therapy as a therapeutic strategy to fight against NSCLC, we also measured the expression of the checkpoint inhibitory receptor PD-1 in the TME of MPO deficient mice. We discovered increased expression of PD-1 on both $CD4^+$ and $CD8^+$ T cells. It has been reported that only PD-1^+-adoptively transferred $CD8^+$ T cells managed to regulate tumor progression when compared to PD-1 negative $CD8^+$ T cells (300).

Besides lymphoid population we also observed an increase in infiltrating monocytes and eosinophils in tumors of the MPO KO mice vs. WT. Although eosinophils are usually a rare population in human NSCLC (0.3 % of all leukocytes) (301) and their role in cancer is ambivalent, they have shown to possess tumoricidal effects in various solid tumors (77). Hence, the rise in eosinophil count in our model could have facilitated an anti-cancer impact. Some cautions that we need to take in consideration regarding our tumor model are related with the relatively poor immunogenicity of our mice model being the lacking of a tumor antigen in the KP cells that were used to generate the tumors. This means that that the immune infiltration into the tumor mass as well as the recruitment of immune cells to the periphery of the tumors is in general minimal. Further studies should include models that are more immunogenic for example using (or generating) cell lines in where a tumor antigen is present.

To identify the ways in which the absence of MPO in the TME might influence T cells, we performed proliferation and activation assays *in vitro*. T cells that were treated with MPO exhibit a reduction in their proliferation as well as a decrease in the expression of IFN-γ when compared vs controls. These data suggest that MPO might directly regulate T cell behavior. Some immunosuppressive effects of MPO have been reported previously. *In vivo* studies Odobasic et al. showed that neutrophil-released MPO can interact with DCs in lymph nodes and suppress DCs function with a subsequent inhibition of $CD4^+$ T cells activation, proliferation and

differentiation (183). Moreover, it has also been reported that MPO can impair antigen-cross presentation in tumors via a mechanism that involves the peroxidation of lipids (302). More studies are needed to understand the details of how MPO specifically regulate T cell function. Co-culture experiments of activated neutrophils and T cells are necessary to study MPO impact on T cells in a more complex context. Moreover, the use of specific MPO inhibitors is needed to discriminate between enzymatically-dependent and independent mechanisms of action of MPO.

We finally demonstrated that, *in vitro*, MPO bound and internalized into T cells and that heparin pre-treatment blocked such interaction. Interestingly, preliminary data suggested that the effect of MPO in impairing T cell proliferation can be reversed when T cells are pre-treated with heparin which suggests that MPO binding to T cells is essential for its functional role. Moreover, we also observed MPO presence in lymphocytes of tumors from NSCLC patients which indicates that MPO binding can also be relevant in a clinical context. The uptake of MPO by endothelial cells, neutrophils and macrophages has been broadly documented (303,304). In neutrophils and macrophages MPO uptake seems to be mediated by mechanisms that involve the integrins CD11b/CD18 and the manose receptor (303) however, the exact mechanism by which MPO is internalized into endothelial cells is not well understood. Being the highly cationic nature of MPO, it can rapidly bind into negative-charged surfaces such cellular membranes, bacterial surfaces and extracellular matrix components, and it has been suggested that in endothelial cells MPO internalization is carried out by a mechanism called transcytosis (274), however more studies are needed to fully understand the mechanisms by which MPO is internalized into different types of cells. Further research should be focused in unravel the functional implications of MPO binding and internalization into T cells.

4.3 Conclusions

Our findings highlight neutrophils as important players in the development of NSCLC. We showed four new surface markers (CD36, CD41, CD61 and CD226) that are differentially express in human LDNs when compared to HDNs supporting the concept that LDNs represent a specific neutrophil subset. This data help to the characterization of this neutrophil population in cancer and will support further research to better understand the role of LDNs in tumor progression as well as their potential as biomarkers to monitor disease progression and treatment response. Moreover, we demonstrated the effect of MPO on impairing T cell behavior

and promoting tumor growth *in vitro* contributing to our knowledge of the pro-tumorigenic role of neutrophil-derived molecules. These results point out MPO as a putative therapeutic target for NSCLC treatment.

5 BIBLIOGRAPHY

1. Valadez-Cosmes P, Maitz K, Kindler O, Raftopoulou S, Kienzl M, Santiso A, et al. Identification of Novel Low-Density Neutrophil Markers Through Unbiased High-Dimensional Flow Cytometry Screening in Non-Small Cell Lung Cancer Patients. Front Immunol. 2021;12:703846.

2. Valadez-Cosmes P, Raftopoulou S, Mihalic ZN, Marsche G, Kargl J. Myeloperoxidase: Growing importance in cancer pathogenesis and potential drug target. Vol. 236, Pharmacology and Therapeutics. 2022.

3. Valadez-Cosmes P, Maitz K, Kindler O, Cosic Mujkanovic N, Lueger A, Raftopoulou S, et al. Myeloperoxidase promotes a tumorigenic microenvironment in non-small cell lung cancer. bioRxiv. 2023;

4. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209–49.

5. Bade BC, Dela Cruz CS. Lung Cancer 2020: Epidemiology, Etiology, and Prevention. Vol. 41, Clinics in Chest Medicine. 2020. p. 1–24.

6. Inamura K. Lung cancer: understanding its molecular pathology and the 2015 wHO classification. Front Oncol. 2017;7(AUG).

7. Zappa C, Mousa SA. Non-small cell lung cancer: Current treatment and future advances. Transl Lung Cancer Res. 2016;5(3):288–300.

8. Couraud S, Zalcman G, Milleron B, Morin F, Souquet PJ. Lung cancer in never smokers - A review. Eur J Cancer. 2012;48(9):1299–311.

9. Malhotra J, Malvezzi M, Negri E, La Vecchia C, Boffetta P. Risk factors for lung cancer worldwide. Eur Respir J. 2016;48(3):889–902.

10. Gettinger S, Horn L, Jackman D, Spigel D, Antonia S, Hellmann M, et al. Five-year follow-up of nivolumab in previously treated advanced non–small-cell lung cancer: Results from the CA209-003 study. J Clin Oncol. 2018;36(17):1675–84.

11. Muller M, Schouten RD, De Gooijer CJ, Baas P. Pembrolizumab for the treatment of non-

small cell lung cancer. Expert Rev Anticancer Ther. 2017;17(5):399–409.

12. Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. J Cell Sci. 2012;125(23):5591–6.

13. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Vol. 9, Nature Reviews Cancer. 2009. p. 239–52.

14. Hanahan D. Hallmarks of Cancer: New Dimensions. Vol. 12, Cancer Discovery. 2022. p. 31–46.

15. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Vol. 19, Nature Medicine. 2013. p. 1423–37.

16. Hanahan D, Coussens LM. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. Vol. 21, Cancer Cell. 2012. p. 309–22.

17. Wei R, Liu S, Zhang S, Min L, Zhu S. Cellular and Extracellular Components in Tumor Microenvironment and Their Application in Early Diagnosis of Cancers. Vol. 2020, Analytical Cellular Pathology. 2020.

18. Anderson NM, Simon MC. The tumor microenvironment. Curr Biol. 2020;30(16):R921–5.

19. Swann JB, Smyth MJ. Immune surveillance of tumors. Vol. 117, Journal of Clinical Investigation. 2007. p. 1137–46.

20. Ribatti D. The concept of immune surveillance against tumors. The first theories. Vol. 8, Oncotarget. 2017. p. 7175–80.

21. Hinshaw DC, Shevde LA. The tumor microenvironment innately modulates cancer progression. Vol. 79, Cancer Research. 2019. p. 4557–67.

22. Lei X, Lei Y, Li JK, Du WX, Li RG, Yang J, et al. Immune cells within the tumor microenvironment: Biological functions and roles in cancer immunotherapy. Vol. 470, Cancer Letters. 2020. p. 126–33.

23. Carstens JL, De Sampaio PC, Yang D, Barua S, Wang H, Rao A, et al. Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer. Nat Commun. 2017;8.

24. Miotto D, Cascio N Lo, Stendardo M, Querzoli P, Pedriali M, De Rosa E, et al. CD8+ T cells expressing IL-10 are associated with a favourable prognosis in lung cancer. Lung Cancer. 2010;69(3):355–60.

25. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. Clin Cancer Res. 2008;14(16):5220–7.

26. Schulze AB, Evers G, Görlich D, Mohr M, Marra A, Hillejan L, et al. Tumor infiltrating T cells influence prognosis in stage I-III non-small cell lung cancer. J Thorac Dis. 2020;12(5):1824–42.

27. Sharma P, Shen Y, Wen S, Yamada S, Jungbluth AA, Gnjatic S, et al. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. Proc Natl Acad Sci U S A. 2007;104(10):3967–72.

28. Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AHS, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol. 2011;29(15):1949–55.

29. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science (80-). 2006;313(5795):1960–4.

30. Zhang N, Bevan MJ. CD8+ T Cells: Foot Soldiers of the Immune System. Vol. 35, Immunity. 2011. p. 161–8.

31. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: Human memory T-cell subsets. Vol. 43, European Journal of Immunology. 2013. p. 2797–809.

32. Verma K, Ogonek J, Varanasi PR, Luther S, Bünting I, Thomay K, et al. Human CD8+ CD57- TEMRA cells: Too young to be called "old." PLoS One. 2017;12(5).

33. Wherry EJ, Ahmed R. Memory CD8 T-Cell Differentiation during Viral Infection. J Virol. 2004;78(11):5535–45.

34. Tay RE, Richardson EK, Toh HC. Revisiting the role of CD4+ T cells in cancer

immunotherapy—new insights into old paradigms. Vol. 28, Cancer Gene Therapy. 2021. p. 5–17.

35. Nersesian S, Schwartz SL, Grantham SR, MacLean LK, Lee SN, Pugh-Toole M, et al. NK cell infiltration is associated with improved overall survival in solid cancers: A systematic review and meta-analysis. Transl Oncol. 2021;14(1).

36. Tachibana T, Onodera H, Tsuruyama T, Mori A, Nagayama S, Hiai H, et al. Increased intratumor Vα24-positive natural killer T cells: A prognostic factor for primary colorectal carcinomas. Clin Cancer Res. 2005;11(20):7322–7.

37. Screpanti V, Wallin RPA, Grandien A, Ljunggren HG. Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. Vol. 42, Molecular Immunology. 2005. p. 495–9.

38. Orange JS. Formation and function of the lytic NK-cell immunological synapse. Vol. 8, Nature Reviews Immunology. 2008. p. 713–25.

39. Wylie B, Macri C, Mintern JD, Waithman J. Dendritic cells and cancer: From biology to therapeutic intervention. Cancers (Basel). 2019;11(4).

40. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol. 2006;24(34):5373–80.

41. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942–9.

42. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res. 2006;12(18):5423–34.

43. Fridman WH, Pagès F, Sautѐs-Fridman C, Galon J. The immune contexture in human tumours: Impact on clinical outcome. Vol. 12, Nature Reviews Cancer. 2012. p. 298–306.

44. Hsieh CS, Lee HM, Lio CWJ. Selection of regulatory T cells in the thymus. Vol. 12, Nature Reviews Immunology. 2012. p. 157–67.

45. Campbell DJ, Koch MA. T reg cells: Patrolling a dangerous neighborhood. Nat Med. 2011;17(8):929–30.

46. Facciabene A, Motz GT, Coukos G. T-Regulatory cells: Key players in tumor immune escape and angiogenesis. Vol. 72, Cancer Research. 2012. p. 2162–71.

47. Qian BZ, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. Vol. 141, Cell. 2010. p. 39–51.

48. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. Vol. 196, Journal of Pathology. 2002. p. 254–65.

49. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Vol. 104, Blood. 2004. p. 2224–34.

50. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. Vol. 233, Journal of Cellular Physiology. 2018. p. 6425–40.

51. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res. 2006;66(23):11238–46.

52. Zumsteg A, Christofori G. Corrupt policemen: Inflammatory cells promote tumor angiogenesis. Vol. 21, Current Opinion in Oncology. 2009. p. 60–70.

53. Condeelis J, Pollard JW. Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. Vol. 124, Cell. 2006. p. 263–6.

54. Zabuawala T, Taffany DA, Sharma SM, Merchant A, Adair B, Srinivasan R, et al. An Ets2-driven transcriptional program in tumor-associated macrophages promotes tumor metastasis. Cancer Res. 2010;70(4):1323–33.

55. Ojalvo LS, Whittaker CA, Condeelis JS, Pollard JW. Gene Expression Analysis of Macrophages That Facilitate Tumor Invasion Supports a Role for Wnt-Signaling in Mediating Their Activity in Primary Mammary Tumors. J Immunol. 2010;184(2):702–12.

56. Cassetta L, Baekkevold ES, Brandau S, Bujko A, Cassatella MA, Dorhoi A, et al. Deciphering myeloid-derived suppressor cells: isolation and markers in humans, mice and non-human primates. Cancer Immunol Immunother. 2019;68(4):687–97.

57. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Vol. 12, Nature Reviews Immunology. 2012. p. 253–68.

58. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. Vol. 117, Journal of Clinical Investigation. 2007. p. 1155–66.

59. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni V, Mocellin S, et al. IL4Rα+ Myeloid-Derived Suppressor Cell Expansion in Cancer Patients. J Immunol. 2009;182(10):6562–8.

60. Martens A, Wistuba-Hamprecht K, Foppen MG, Yuan J, Postow MA, Wong P, et al. Baseline peripheral blood biomarkers associated with clinical outcome of advanced melanoma patients treated with ipilimumab. Clin Cancer Res. 2016;22(12):2908–18.

61. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Vol. 24, Trends in Immunology. 2003. p. 301–5.

62. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res. 2006;66(2):1123–31.

63. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Krüger C, Manns MP, et al. A New Population of Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma Patients Induces CD4+CD25+Foxp3+ T Cells. Gastroenterology. 2008;135(1):234–43.

64. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-Talk between Myeloid-Derived Suppressor Cells and Macrophages Subverts Tumor Immunity toward a Type 2 Response. J Immunol. 2007;179(2):977–83.

65. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell. 2004;6(4):409–21.

66. Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szeliga W, et al. Myeloid-derived suppressor cells endow stem-like qualities to breast cancer cells through IL6/STAT3 and NO/NOTCH cross-talk signaling. Cancer Res. 2016;76(11):3156–65.

67. Yuen GJ, Demissie E, Pillai S. B Lymphocytes and Cancer: A Love–Hate Relationship. Vol. 2, Trends in Cancer. 2016. p. 747–57.

68. Nedergaard BS, Ladekarl M, Nyengaard JR, Nielsen K. A comparative study of the cellular immune response in patients with stage IB cervical squamous cell carcinoma. Low numbers of several immune cell subtypes are strongly associated with relapse of disease within 5 years. Gynecol Oncol. 2008;108(1):106–11.

69. Riemann D, Wenzel K, Schulz T, Hofmann S, Neef H, Lautenschläger C, et al. Phenotypic Analysis of T Lymphocytes Isolated from Non-Small-Cell Lung Cancer. Int Arch Allergy Immunol. 1997;114(1):38–45.

70. Sharonov G V., Serebrovskaya EO, Yuzhakova D V., Britanova O V., Chudakov DM. B cells, plasma cells and antibody repertoires in the tumour microenvironment. Vol. 20, Nature Reviews Immunology. 2020. p. 294–307.

71. Tao H, Lu L, Xia Y, Dai F, Wang Y, Bao Y, et al. Antitumor effector B cells directly kill tumor cells via the Fas/FasL pathway and are regulated by IL-10. Eur J Immunol. 2015;45(4):999–1009.

72. Kemp TJ, Moore JM, Griffith TS. Human B Cells Express Functional TRAIL/Apo-2 Ligand after CpG-Containing Oligodeoxynucleotide Stimulation. J Immunol. 2004;173(2):892–9.

73. Zhang H, Jing X, Shi Y, Xu H, Du J, Guan T, et al. N-acetyl lysyltyrosylcysteine amide inhibits myeloperoxidase, a novel tripeptide inhibitor. J Lipid Res. 2013;54(11):3016–29.

74. Pucci F, Garris C, Lai CP, Newton A, Pfirschke C, Engblom C, et al. SCS macrophages suppress melanoma by restricting tumor-derived vesicle-B cell interactions. Science (80-). 2016;352(6282):242–6.

75. Kumar S, Mohan A, Guleria R. Prognostic implications of circulating anti-p53 antibodies in lung cancer - A review. Vol. 18, European Journal of Cancer Care. 2009. p. 248–54.

76. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, et al. Tumor-

evoked regulatory B cells promote breast cancer metastasis by converting resting CD4+ T cells to T-regulatory cells. Cancer Res. 2011;71(10):3505–15.

77. Varricchi G, Galdiero MR, Loffredo S, Lucarini V, Marone G, Mattei F, et al. Eosinophils: The unsung heroes in cancer? Vol. 7, OncoImmunology. 2018.

78. Grisaru-Tal S, Itan M, Klion AD, Munitz A. A new dawn for eosinophils in the tumour microenvironment. Vol. 20, Nature Reviews Cancer. 2020. p. 594–607.

79. Kienzl M, Hasenoehrl C, Valadez-Cosmes P, Maitz K, Sarsembayeva A, Sturm E, et al. IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils. Oncoimmunology. 2020 Jan;9(1):1–12.

80. Carretero R, Sektioglu IM, Garbi N, Salgado OC, Beckhove P, Hämmerling GJ. Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8 + T cells. Nat Immunol. 2015;16(6):609–17.

81. Lucarini V, Zicchedda G, Macchia I, La Sorsa V, Peschiaroli F, Buccione C, et al. IL-33 restricts tumor growth and inhibits pulmonary metastasis in melanoma-bearing mice through eosinophils. Oncoimmunology. 2017;6(6).

82. Coffelt SB, Wellenstein MD, De Visser KE. Neutrophils in cancer: Neutral no more. Vol. 16, Nature Reviews Cancer. 2016. p. 431–46.

83. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Vol. 13, Nature Reviews Immunology. 2013. p. 159–75.

84. Lehman HK, Segal BH. The role of neutrophils in host defense and disease. J Allergy Clin Immunol. 2020;145(6):1535–44.

85. Xiong S, Dong L, Cheng L. Neutrophils in cancer carcinogenesis and metastasis. Vol. 14, Journal of Hematology and Oncology. 2021.

86. Kargl J, Busch SE, Yang GHY, Kim KH, Hanke ML, Metz HE, et al. Neutrophils dominate the immune cell composition in non-small cell lung cancer. Nat Commun. 2017;8(14381).

87. Diem S, Schmid S, Krapf M, Flatz L, Born D, Jochum W, et al. Neutrophil-to-Lymphocyte ratio (NLR) and Platelet-to-Lymphocyte ratio (PLR) as prognostic markers in patients with non-small cell lung cancer (NSCLC) treated with nivolumab. Lung Cancer. 2017;111:176–

81.

88. Szor DJ, Dias AR, Pereira MA, Ramos MFKP, Zilberstein B, Cecconello I, et al. Prognostic role of neutrophil/lymphocyte ratio in resected gastric cancer: A systematic review and meta-analysis. Vol. 73, Clinics. 2018.

89. Schmidt H, Bastholt L, Geertsen P, Christensen IJ, Larsen S, Gehl J, et al. Elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma: A prognostic model. Br J Cancer. 2005;93(3):273–8.

90. Hirahara T, Arigami T, Yanagita S, Matsushita D, Uchikado Y, Kita Y, et al. Combined neutrophil-lymphocyte ratio and platelet-lymphocyte ratio predicts chemotherapy response and prognosis in patients with advanced gastric cancer. BMC Cancer. 2019;19(1).

91. Dirican A, Kucukzeybek BB, Alacacioglu A, Kucukzeybek Y, Erten C, Varol U, et al. Do the derived neutrophil to lymphocyte ratio and the neutrophil to lymphocyte ratio predict prognosis in breast cancer? Int J Clin Oncol. 2015;20(1):70–81.

92. Ethier JL, Desautels D, Templeton A, Shah PS, Amir E. Prognostic role of neutrophil-to-lymphocyte ratio in breast cancer: A systematic review and meta-analysis. Breast Cancer Res. 2017;19(1).

93. Suzuki R, Takagi T, Hikichi T, Konno N, Sugimoto M, Watanabe K, et al. Derived neutrophil/lymphocyte ratio predicts gemcitabine therapy outcome in unresectable pancreatic cancer. Oncol Lett. 2016;11(5):3441–5.

94. Graziano V, Grassadonia A, Iezzi L, Vici P, Pizzuti L, Barba M, et al. Combination of peripheral neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio is predictive of pathological complete response after neoadjuvant chemotherapy in breast cancer patients. Breast. 2019;44:33–8.

95. Nakamatsu K, Anami S, Fukuda K, Inada M, Tatebe H, Ishikawa K, et al. Neutrophil-to-lymphocyte ratio predicts survival after whole-brain radiotherapy in non-small cell lung cancer. In Vivo (Brooklyn). 2019;33(1):195–201.

96. Alessi J V., Ricciuti B, Alden SL, Bertram AA, Lin JJ, Sakhi M, et al. Low peripheral blood derived neutrophil-to-lymphocyte ratio (dNLR) is associated with increased tumor T-cell

infiltration and favorable outcomes to first-line pembrolizumab in non-small cell lung cancer. J Immunother Cancer. 2021;9(11).

97. Wu L, Saxena S, Awaji M, Singh RK. Tumor-associated neutrophils in cancer: Going pro. Cancers (Basel). 2019;11(4).

98. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. Vol. 125, Immunology. 2008. p. 281–8.

99. Elghetany MT. Surface antigen changes during normal neutrophilic development: A critical review. Blood Cells, Mol Dis. 2002;28(2):260–74.

100. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. J Clin Invest. 2010;120(7):2423–31.

101. Capucetti A, Albano F, Bonecchi R. Multiple Roles for Chemokines in Neutrophil Biology. Vol. 11, Frontiers in Immunology. 2020.

102. Bajrami B, Zhu H, Kwak HJ, Mondal S, Hou Q, Geng G, et al. G-CSF maintains controlled neutrophil mobilization during acute inflammation by negatively regulating CXCR2 signaling. J Exp Med. 2016;213(10):1999–2018.

103. Martin C, Burdon PCE, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity. 2003;19(4):583–93.

104. Strydom N, Rankin SM. Regulation of circulating neutrophil numbers under homeostasis and in disease. Vol. 5, Journal of Innate Immunity. 2013. p. 304–14.

105. Girbl T, Lenn T, Perez L, Rolas L, Barkaway A, Thiriot A, et al. Distinct Compartmentalization of the Chemokines CXCL1 and CXCL2 and the Atypical Receptor ACKR1 Determine Discrete Stages of Neutrophil Diapedesis. Immunity. 2018;49(6):1062–76.e6.

106. Casbon AJ, Reynau D, Park C, Khu E, Gan DD, Schepers K, et al. Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. Proc Natl Acad Sci U S A. 2015;112(6):E566–75.

107. Raftopoulou S, Valadez-Cosmes P, Mihalic ZN, Schicho R, Kargl J. Tumor-Mediated Neutrophil Polarization and Therapeutic Implications. Vol. 23, International Journal of Molecular Sciences. 2022. p. 3218.

108. Sharma B, Nawandar DM, Nannuru KC, Varney ML, Singh RK. Targeting CXCR2 enhances chemotherapeutic response, inhibits mammary tumor growth, angiogenesis, and lung metastasis. Mol Cancer Ther. 2013;12(5):799–808.

109. Wu L, Awaji M, Saxena S, Varney ML, Sharma B, Singh RK. IL-17–CXC Chemokine Receptor 2 Axis Facilitates Breast Cancer Progression by Up-Regulating Neutrophil Recruitment. Am J Pathol. 2020;190(1):222–33.

110. Li TJ, Jiang YM, Hu YF, Huang L, Yu J, Zhao LY, et al. Interleukin-17-producing neutrophils link inflammatory stimuli to disease progression by promoting angiogenesis in gastric cancer. Clin Cancer Res. 2017;23(6):1575–85.

111. David JM, Dominguez C, Hamilton DH, Palena C. The IL-8/IL-8R axis: A double agent in tumor immune resistance. Vol. 4, Vaccines. 2016.

112. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: “N1” versus “N2” TAN. Cancer Cell. 2009;16(3):183–94.

113. Andzinski L, Kasnitz N, Stahnke S, Wu CF, Gereke M, Von Köckritz-Blickwede M, et al. Type i IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. Int J Cancer. 2016;138(8):1982–93.

114. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. Cell Rep. 2015;10(4):562–73.

115. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Investig Suppl. 1968;97:77–89.

116. Hacbarth E, Kajdacsy-Balla A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. Arthritis Rheum. 1986;29(11):1334–42.

117. Midgley A, Beresford MW. Increased expression of low density granulocytes in juvenile-onset systemic lupus erythematosus patients correlates with disease activity. Lupus. 2016;25(4):407–11.

118. Ramanathan K, Glaser A, Lythgoe H, Ong J, Beresford MW, Midgley A, et al. Neutrophil activation signature in juvenile idiopathic arthritis indicates the presence of low-density granulocytes. Rheumatol (United Kingdom). 2018;57(3):488–98.

119. Brandau S, Trellakis S, Bruderek K, Schmaltz D, Steller G, Elian M, et al. Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. J Leukoc Biol. 2011;89(2):311–7.

120. Wang WM, Jin HZ. Role of Neutrophils in Psoriasis. Vol. 2020, Journal of Immunology Research. 2020.

121. Fu J, Tobin MC, Thomas LL. Neutrophil-like low-density granulocytes are elevated in patients with moderate to severe persistent asthma. Ann Allergy, Asthma Immunol. 2014;113(6):635–40.e2.

122. Grayson PC, Carmona-Rivera C, Xu L, Lim N, Gao Z, Asare AL, et al. Neutrophil-related gene expression and low-density granulocytes associated with disease activity and response to treatment in antineutrophil cytoplasmic antibody-associated vasculitis. Arthritis Rheumatol. 2015;67(7):1922–32.

123. Cloke T, Munder M, Taylor G, Müller I, Kropf P. Characterization of a Novel Population of Low-Density Granulocytes Associated with Disease Severity in HIV-1 Infection. PLoS One. 2012;7(11).

124. Rocha BC, Marques PE, Leoratti FM de S, Junqueira C, Pereira DB, Antonelli LR do V, et al. Type I Interferon Transcriptional Signature in Neutrophils and Low-Density Granulocytes Are Associated with Tissue Damage in Malaria. Cell Rep. 2015;13(12):2829–41.

125. Morisaki T, Goya T, Ishimitsu T, Torisu M. The increase of low density subpopulations and CD10 (CALLA) negative neutrophils in severely infected patients. Surg Today. 1992;22(4):322–7.

126. Deng Y, Ye J, Luo Q, Huang Z, Peng Y, Xiong G, et al. Low-density granulocytes are

elevated in mycobacterial infection and associated with the severity of tuberculosis. PLoS One. 2016;11(4).

127. Ssemaganda A, Kindinger L, Bergin P, Nielsen L, Mpendo J, Ssetaala A, et al. Characterization of neutrophil subsets in healthy human pregnancies. PLoS One. 2014;9(2).

128. Blanco-Camarillo C, Alemán OR, Rosales C. Low-Density Neutrophils in Healthy Individuals Display a Mature Primed Phenotype. Front Immunol. 2021;12.

129. Shaul ME, Eyal O, Guglietta S, Aloni P, Zlotnik A, Forkosh E, et al. Circulating neutrophil subsets in advanced lung cancer patients exhibit unique immune signature and relate to prognosis. FASEB J. 2020;34(3):4204–18.

130. Saraiva DP, Correia BF, Salvador R, de Sousa N, Jacinto A, Braga S, et al. Circulating low density neutrophils of breast cancer patients are associated with their worse prognosis due to the impairment of T cell responses. Oncotarget. 2021;12(24):2388–403.

131. Arasanz H, Bocanegra AI, Morilla I, Fernández-Irigoyen J, Martínez-Aguillo M, Teijeira L, et al. Circulating low density neutrophils are associated with resistance to first line anti-PD1/PDL1 immunotherapy in non-small cell lung cancer. Cancers (Basel). 2022;14(16):3446.

132. Liu Y, Hu Y, Gu F, Liang J, Zeng Y, Hong X, et al. Phenotypic and clinical characterization of low density neutrophils in patients with advanced lung adenocarcinoma. Oncotarget. 2017;8(53):90969–78.

133. Gershkovitz M, Fainsod-Levi T, Zelter T, Sionov R V., Granot Z. TRPM2 modulates neutrophil attraction to murine tumor cells by regulating CXCL2 expression. Cancer Immunol Immunother. 2019;68(1):33–43.

134. Dissemond J, Weimann TK, Schneider LA, Schneeberger A, Scharffetter-Kochanck K, Goos M, et al. Activated Neutrophils Exert Antitumor Activity Against Human Melanoma Cells: Reactive Oxygen Species-Induced Mechanisms and Their Modulation by Granulocyte-Macrophage-Colony-Stimulating Factor [2]. Vol. 121, Journal of Investigative Dermatology. 2003. p. 936–8.

135. Ulfig A, Leichert LI. The effects of neutrophil-generated hypochlorous acid and other

hypohalous acids on host and pathogens. Vol. 78, Cellular and Molecular Life Sciences. 2021. p. 385–414.

136. Finisguerra V, Di Conza G, Di Matteo M, Serneels J, Costa S, Thompson AAR, et al. MET is required for the recruitment of anti-tumoural neutrophils. Nature. 2015;522(7556):349–53.

137. Blaisdell A, Crequer A, Columbus D, Daikoku T, Mittal K, Dey SK, et al. Neutrophils Oppose Uterine Epithelial Carcinogenesis via Debridement of Hypoxic Tumor Cells. Cancer Cell. 2015;28(6):785–99.

138. Minns D, Smith KJ, Findlay EG. Orchestration of Adaptive T Cell Responses by Neutrophil Granule Contents. Vol. 2019, Mediators of Inflammation. 2019.

139. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. J Clin Invest. 2014;124(12):5466–80.

140. Beauvillain C, Delneste Y, Scotet M, Peres A, Gascan H, Guermonprez P, et al. Neutrophils efficiently cross-prime naive T cells in vivo. Blood. 2007;110(8):2965–73.

141. Van Egmond M, Bakema JE. Neutrophils as effector cells for antibody-based immunotherapy of cancer. Vol. 23, Seminars in Cancer Biology. 2013. p. 190–9.

142. Matlung HL, Babes L, Zhao XW, van Houdt M, Treffers LW, van Rees DJ, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. Cell Rep. 2018;23(13):3946–59.e6.

143. Behrens LM, van Egmond M, van den Berg TK. Neutrophils as immune effector cells in antibody therapy in cancer. Immunological Reviews. 2022.

144. Gungabeesoon J, Gort-Freitas NA, Kiss M, Bolli E, Messemaker M, Siwicki M, et al. A neutrophil response linked to tumor control in immunotherapy. Cell. 2023;186(7):1448–64.e20.

145. Hirschhorn D, Budhu S, Kraehenbuehl L, Gigoux M, Schröder D, Chow A, et al. T cell immunotherapies engage neutrophils to eliminate tumor antigen escape variants. Cell. 2023;186(7):1432–47.e17.

146. Jamieson T, Clarke M, Steele CW, Samuel MS, Neumann J, Jung A, et al. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. J Clin Invest. 2012;122(9):3127–44.

147. Katoh H, Wang D, Daikoku T, Sun H, Dey SK, DuBois RN. CXCR2-Expressing Myeloid-Derived Suppressor Cells Are Essential to Promote Colitis-Associated Tumorigenesis. Cancer Cell. 2013;24(5):631–44.

148. Tazzyman S, Barry ST, Ashton S, Wood P, Blakey D, Lewis CE, et al. Inhibition of neutrophil infiltration into A549 lung tumors in vitro and in vivo using a CXCR2-specific antagonist is associated with reduced tumor growth. Int J Cancer. 2011;129(4):847–58.

149. Keane MP, Belperio JA, Xue YY, Burdick MD, Strieter RM. Depletion of CXCR2 Inhibits Tumor Growth and Angiogenesis in a Murine Model of Lung Cancer. J Immunol. 2004;172(5):2853–60.

150. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci U S A. 2006;103(33):12493–8.

151. Houghton AMG, Rzymkiewicz DM, Ji H, Gregory AD, Egea EE, Metz HE, et al. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. Nat Med. 2010;16(2):219–23.

152. Di Mitri D, Toso A, Chen JJ, Sarti M, Pinton S, Jost TR, et al. Tumour-infiltrating Gr-1 + myeloid cells antagonize senescence in cancer. Nature. 2014;515(7525):134–7.

153. Deryugina EI, Zajac E, Juncker-Jensen A, Kupriyanova TA, Welter L, Quigley JP. Tissue-Infiltrating Neutrophils Constitute the Major In Vivo Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment. Neoplasia (United States). 2014;16(10):771–88.

154. Bekes EM, Schweighofer B, Kupriyanova TA, Zajac E, Ardi VC, Quigley JP, et al. Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. Am J Pathol. 2011;179(3):1455–70.

155. Navasardyan I, Bonavida B. Regulation of t cells in cancer by nitric oxide. Vol. 10, Cells. 2021. p. 2655.

156. Munder M, Schneider H, Luckner C, Giese T, Langhans CD, Fuentes JM, et al. Suppression of T-cell functions by human granulocyte arginase. Blood. 2006;108(5):1627–34.

157. Rotondo R, Bertolotto M, Barisione G, Astigiano S, Mandruzzato S, Ottonello L, et al. Exocytosis of azurophil and arginase 1-containing granules by activated polymorphonuclear neutrophils is required to inhibit T lymphocyte proliferation. J Leukoc Biol. 2011;89(5):721–7.

158. Romano A, Parrinello NL, Vetro C, Tibullo D, Giallongo C, Cava P La, et al. The prognostic value of the myeloid-mediated immunosuppression marker Arginase-1 in classic Hodgkin Lymphoma. Oncotarget. 2016;7(41):67333–46.

159. Vanini F, Kashfi K, Nath N. The dual role of iNOS in cancer. Vol. 6, Redox Biology. 2015. p. 334–43.

160. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. Proc Natl Acad Sci U S A. 2014;111(32):11774–9.

161. Orillion A, Hashimoto A, Damayanti N, Shen L, Adelaiye-Ogala R, Arisa S, et al. Entinostat neutralizes myeloid-derived suppressor cells and enhances the antitumor effect of PD-1 inhibition in murine models of lung and renal cell carcinoma. Clin Cancer Res. 2017;23(17):5187–201.

162. Spicer JD, McDonald B, Cools-Lartigue JJ, Chow SC, Giannias B, Kubes P, et al. Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells. Cancer Res. 2012;72(16):3919–27.

163. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest. 2013;123(8):3446–58.

164. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. Nature. 2019;566(7745):553–7.

165. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate

immunity proteins. Trends Immunol. 2007;28(8):340–5.

166. Mollinedo F. Neutrophil Degranulation, Plasticity, and Cancer Metastasis. Vol. 40, Trends in Immunology. 2019. p. 228–42.

167. Bedouhène S, Dang PMC, Hurtado-Nedelec M, El-Benna J. Neutrophil Degranulation of Azurophil and Specific Granules. In: Methods in Molecular Biology. 2020. p. 215–22.

168. Sengeløv H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol. 1995;154(8):4157–65.

169. Williams MR, Azcutia V, Newton G, Alcaide P, Luscinskas FW. Emerging mechanisms of neutrophil recruitment across endothelium. Vol. 32, Trends in Immunology. 2011. p. 461–9.

170. Pruenster M, Mudde L, Bombosi P, Dimitrova S, Zsak M, Middleton J, et al. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. Nat Immunol. 2009;10(1):101–8.

171. Zamocky M, Jakopitsch C, Furtmüller PG, Dunand C, Obinger C. The peroxidase-cyclooxygenase superfamily: Reconstructed evolution of critical enzymes of the innate immune system. Proteins Struct Funct Genet. 2008;72(2):589–605.

172. Andrews PC, Krinsky NI. The reductive cleavage of myeloperoxidase in half, producing enzymically active hemi-myeloperoxidase. J Biol Chem. 1981;256(9):4211–8.

173. Fiedler TJ, Davey CA, Fenna RE. X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution. J Biol Chem. 2000;275(16):11964–71.

174. Olsen RL, Little C. Purification and some properties of myeloperoxidase and eosinophil peroxidase from human blood. Biochem J. 1983;209(3):781–7.

175. Zeng J, Fenna RE. X-ray crystal structure of canine myeloperoxidase at 3 Å resolution. J Mol Biol. 1992;226(1):185–207.

176. Tobler A, Miller CW, Johnson KR, Selsted ME, Rovera G, Koeffler HP. Regulation of gene expression of myeloperoxidase during myeloid differentiation. J Cell Physiol.

1988;136(2):215–25.

177. Schultz J, Kaminker K. Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. Arch Biochem Biophys. 1962;96(3):465–7.

178. Bos A, Wever R, Roos D. Characterization and quantification of the peroxidase in human monocytes. BBA - Enzymol. 1978;525(1):37–44.

179. Chatham WW, Turkiewicz A, Blackburn WD. Determinants of neutrophil HOCl generation: Ligand-dependent responses and the role of surface adhesion. J Leukoc Biol. 1994;56(5):654–60.

180. Dang Y, Lowe GM, Edwards SW, Galvani DW. The effects of GM-CSF on myeloperoxidase release in normal and myelodysplastic neutrophils. Leuk Res. 1993;17(12):1037–44.

181. Holle JU, Windmöller M, Lange C, Gross WL, Herlyn K, Csernok E. Toll-like receptor TLR2 and TLR9 ligation triggers neutrophil activation in granulomatosis with polyangiitis. Rheumatol (United Kingdom). 2013;52(7):1183–9.

182. Bentwood BJ, Henson PM. The sequential release of granule constitutents from human neutrophils. J Immunol [Internet]. 1980;124(2):855–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6153206

183. Odobasic D, Kitching AR, Yang Y, O'Sullivan KM, Muljadi RCM, Edgtton KL, et al. Neutrophil myeloperoxidase regulates T-cell2driven tissue inflammation in mice by inhibiting dendritic cell function. Blood. 2013;121(20):4195–204.

184. van der Veen BS, de Winther MPJ, Heeringa P. Myeloperoxidase: Molecular Mechanisms of Action and Their Relevance to Human Health and Disease. Antioxid Redox Signal. 2009;11(11):2899–937.

185. Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009;15(6):623–5.

186. Davies MJ, Hawkins CL. The Role of Myeloperoxidase in Biomolecule Modification, Chronic Inflammation, and Disease. Vol. 32, Antioxidants and Redox Signaling. 2020. p. 957–81.

187. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. J Leukoc Biol. 2013;93(2):185–98.

188. Aratani Y, Koyama H, Nyui SI, Suzuki K, Kura F, Maeda N. Severe impairment in early host defense against Candida albicans in mice deficient in myeloperoxidase. Infect Immun. 1999;67(4):1828–36.

189. Yamamoto K, Miyoshi-Koshio T, Utsuki Y, Mizuno S, Suzuki K. Virucidal activity and viral protein modification by myeloperoxidase: A candidate for defense factor of human polymorphonuclear leukocytes against influenza virus infection. J Infect Dis. 1991;164(1):8–14.

190. Hirche TO, Gaut JP, Heinecke JW, Belaaouaj A. Myeloperoxidase Plays Critical Roles in Killing Klebsiella pneumoniae and Inactivating Neutrophil Elastase: Effects on Host Defense . J Immunol. 2005;174(3):1557–65.

191. Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, et al. Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. Proc Natl Acad Sci U S A. 2005;102(2):431–6.

192. El Kebir D, József L, Pan W, Filep JG. Myeloperoxidase delays neutrophil apoptosis through CD11b/CD18 integrins and prolongs inflammation. Circ Res. 2008;103(4):352–9.

193. Kanayama A, Miyamoto Y. Apoptosis triggered by phagocytosis-related oxidative stress through FLIP S down-regulation and JNK activation . J Leukoc Biol. 2007;82(5):1344–52.

194. Tsurubuchi T, Aratani Y, Maeda N, Koyama H. Retardation of early-onset PMA-induced apoptosis in mouse neutrophils deficient in myeloperoxidase. J Leukoc Biol [Internet]. 2001;70(1):52–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11435485

195. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Münzel T, et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation. 2003;108(12):1440–5.

196. Heslop CL, Frohlich JJ, Hill JS. Myeloperoxidase and C-Reactive Protein Have Combined Utility for Long-Term Prediction of Cardiovascular Mortality After Coronary Angiography. J Am Coll Cardiol. 2010;55(11):1102–9.

197. Mocatta TJ, Pilbrow AP, Cameron VA, Senthilmohan R, Frampton CM, Richards AM, et al. Plasma Concentrations of Myeloperoxidase Predict Mortality After Myocardial Infarction. J Am Coll Cardiol. 2007;49(20):1993–2000.

198. Rudolph V, Goldmann BU, Bös C, Rudolph TK, Klinke A, Friedrichs K, et al. Diagnostic value of MPO plasma levels in patients admitted for suspected myocardial infarction. Int J Cardiol. 2011;153(3):267–71.

199. Tang WHW, Wu Y, Nicholls SJ, Hazen SL. Plasma myeloperoxidase predicts incident cardiovascular risks in stable patients undergoing medical management for coronary artery disease. Clin Chem. 2011;57(1):33–9.

200. Ndrepepa G, Braun S, Mehilli J, Von Beckerath N, Schömig A, Kastrati A. Myeloperoxidase level in patients with stable coronary artery disease and acute coronary syndromes. Eur J Clin Invest. 2008;38(2):90–6.

201. Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, et al. Association between myeloperoxidase levels and risk of coronary artery disease. J Am Med Assoc. 2001;286(17):2136–42.

202. Hazell LJ, Arnold L, Flowers D, Waeg G, Malle E, Stocker R. Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J Clin Invest. 1996;97(6):1535–44.

203. Malle E, Waeg G, Schreiber R, Gröne EF, Sattler W, Gröne HJ. Immunohistochemical evidence for the myeloperoxidase/H2O2/halide system in human atherosclerotic lesions. Colocalization of myeloperoxidase and hypochlorite-modified proteins. Eur J Biochem. 2000;267(14):4495–503.

204. Cai H, Chuang CY, Vanichkitrungruang S, Hawkins CL, Davies MJ. Hypochlorous acid-modified extracellular matrix contributes to the behavioral switching of human coronary artery smooth muscle cells. Free Radic Biol Med. 2019;134:516–26.

205. Nybo T, Cai H, Chuang CY, Gamon LF, Rogowska-Wrzesinska A, Davies MJ. Chlorination and oxidation of human plasma fibronectin by myeloperoxidase-derived oxidants, and its consequences for smooth muscle cell function. Redox Biol. 2018;19:388–400.

206. Vanichkitrungruang S, Chuang CY, Hawkins CL, Hammer A, Hoefler G, Malle E, et al.

Oxidation of human plasma fibronectin by inflammatory oxidants perturbs endothelial cell function. Free Radic Biol Med. 2019;136:118–34.

207. Marsche G, Heller R, Fauler G, Kovacevic A, Nuszkowski A, Graier W, et al. 2-Chlorohexadecanal derived from hypochlorite-modified high-density lipoprotein - Associated plasmalogen is a natural inhibitor of endothelial nitric oxide biosynthesis. Arterioscler Thromb Vasc Biol. 2004;24(12):2302–6.

208. Nuszkowski A, Gräbner R, Marsche G, Unbehaun A, Malle E, Heller R. Hypochlorite-modified Low Density Lipoprotein Inhibits Nitric Oxide Synthesis in Endothelial Cells via an Intracellular Dislocalization of Endothelial Nitric-oxide Synthase. J Biol Chem. 2001;276(17):14212–21.

209. Klinke A, Berghausen E, Friedrichs K, Molz S, Lau D, Remane L, et al. Myeloperoxidase aggravates pulmonary arterial hypertension by activation of vascular Rho-kinase. JCI insight. 2018;3(11).

210. Manchanda K, Kolarova H, Kerkenpaß C, Mollenhauer M, Vitecek J, Rudolph V, et al. MPO (myeloperoxidase) reduces endothelial glycocalyx thickness dependent on its cationic charge. Arterioscler Thromb Vasc Biol. 2018;38(8):1859–67.

211. Malle E, Marsche G, Panzenboeck U, Sattler W. Myeloperoxidase-mediated oxidation of high-density lipoproteins: Fingerprints of newly recognized potential proatherogenic lipoproteins. Arch Biochem Biophys. 2006;445(2):245–55.

212. Malle E, Marsche G, Arnhold J, Davies MJ. Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid. Vol. 1761, Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. 2006. p. 392–415.

213. Thomson E, Brennan S, Senthilmohan R, Gangell CL, Chapman AL, Sly PD, et al. Identifying peroxidases and their oxidants in the early pathology of cystic fibrosis. Free Radic Biol Med. 2010;49(9):1354–60.

214. Zhu A, Ge D, Zhang J, Teng Y, Yuan C, Huang M, et al. Sputum myeloperoxidase in chronic obstructive pulmonary disease. Vol. 19, European Journal of Medical Research. 2014.

215. Andelid K, Glader P, Jirholt P, Gjertsson I, Jansson AE, Lindén A. Systemic

myeloperoxidase in COPD. Respir Med. 2013;107(11):S4.

216. Kettle AJ, Turner R, Gangell CL, Harwood DT, Khalilova IS, Chapman AL, et al. Oxidation contributes to low glutathione in the airways of children with cystic fibrosis. Eur Respir J. 2014;44(1):122–9.

217. Garner HP, Phillips JR, Herron JG, Severson SJ, Milla CE, Regelmann WE. Peroxidase activity within circulating neutrophils correlates with pulmonary phenotype in cystic fibrosis. J Lab Clin Med. 2004;144(3):127–33.

218. Magon NJ, Turner R, Gearry RB, Hampton MB, Sly PD, Kettle AJ. Oxidation of calprotectin by hypochlorous acid prevents chelation of essential metal ions and allows bacterial growth: Relevance to infections in cystic fibrosis. Free Radic Biol Med. 2015;86:133–44.

219. Birrer P, Mcelvaney NG, Rüdeberg A, Wirz Sommer C, Liechti-Gallati S, Kraemer R, et al. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. Am J Respir Crit Care Med. 1994;150(1):207–13.

220. Gellhaar S, Sunnemark D, Eriksson H, Olson L, Galter D. Myeloperoxidase-immunoreactive cells are significantly increased in brain areas affected by neurodegeneration in Parkinson's and Alzheimer's disease. Cell Tissue Res. 2017;369(3):445–54.

221. Green PS, Mendez AJ, Jacob JS, Crowley JR, Growdon W, Hyman BT, et al. Neuronal expression of myeloperoxidase is increased in Alzheimer's disease. J Neurochem. 2004;90(3):724–33.

222. Nagra RM, Becher B, Tourtellotte WW, Antel JP, Gold D, Paladino T, et al. Immunohistochemical and genetic evidence of myeloperoxidase involvement in multiple sclerosis. J Neuroimmunol. 1997;78(1-2):97–107.

223. Sacks D, Baxter B, Campbell BCV, Carpenter JS, Cognard C, Dippel D, et al. Multisociety Consensus Quality Improvement Revised Consensus Statement for Endovascular Therapy of Acute Ischemic Stroke. Int J Stroke. 2018;13(6):612–32.

224. Maki RA, Tyurin VA, Lyon RC, Hamilton RL, Dekosky ST, Kagan VE, et al. Aberrant expression of myeloperoxidase in astrocytes promotes phospholipid oxidation and

memory deficits in a mouse model of Alzheimer disease. J Biol Chem. 2009;284(5):3158–69.

225. Yap YW, Whiteman M, Bay BH, Li Y, Sheu FS, Qi RZ, et al. Hypochlorous acid induces apoptosis of cultured cortical neurons through activation of calpains and rupture of lysosomes. J Neurochem. 2006;98(5):1597–609.

226. Forghani R, Wojtkiewicz GR, Zhang Y, Seeburg D, Bautz BRM, Pulli B, et al. Demyelinating diseases: Myeloperoxidase as an imaging biomarker and therapeutic target. Radiology. 2012;263(2):451–60.

227. Üllen A, Singewald E, Konya V, Fauler G, Reicher H, Nusshold C, et al. Myeloperoxidase-Derived Oxidants Induce Blood-Brain Barrier Dysfunction In Vitro and In Vivo. PLoS One. 2013;8(5).

228. Javier Piedrafita F, Molander RB, Vansant G, Orlova EA, Pfahl M, Reynolds WF. An Alu element in the myeloperoxidase promoter contains a composite SP1-thyroid hormone-retinoic acid response element. J Biol Chem. 1996;271(24):14412–20.

229. Kumar AP, Piedrafita FJ, Reynolds WF. Peroxisome Proliferator-activated Receptor γ Ligands Regulate Myeloperoxidase Expression in Macrophages by an Estrogen-dependent Mechanism Involving the -463GA Promoter Polymorphism. J Biol Chem. 2004;279(9):8300–15.

230. Zhou C, Luo Q, Qing Y, Lin X, Zhan Y, Ouyang M. Association between MPO 463G>A polymorphism and risk of lung cancer: A meta-analysis. Tumor Biol. 2013;34(6):3449–55.

231. Yang W jun, Wang M yue, Pan F ze, Shi C, Cen H. Association between MPO-463G > A polymorphism and cancer risk: Evidence from 60 case-control studies. Vol. 15, World Journal of Surgical Oncology. 2017.

232. Vaguliene N, Zemaitis M, Lavinskiene S, Miliauskas S, Sakalauskas R. Local and systemic neutrophilic inflammation in patients with lung cancer and chronic obstructive pulmonary disease. BMC Immunol. 2013;14(1).

233. Däster S, Eppenberger-Castori S, Hirt C, Soysal SD, Delko T, Nebiker CA, et al. Absence of myeloperoxidase and CD8 positive cells in colorectal cancer infiltrates identifies patients with severe prognosis. Oncoimmunology. 2015;4(12).

234. Droeser RA, Hirt C, Eppenberger-Castori S, Zlobec I, Viehl CT, Frey DM, et al. High Myeloperoxidase Positive Cell Infiltration in Colorectal Cancer Is an Independent Favorable Prognostic Factor. PLoS One. 2013;8(5).

235. Kim Y, Yoon S, Kim SJ, Kim JS, Cheong JW, Min YH. Myeloperoxidase expression in acute myeloid leukemia helps identifying patients to benefit from transplant. Yonsei Med J. 2012;53(3):530–6.

236. Roncucci L, Mora E, Mariani F, Bursi S, Pezzi A, Rossi G, et al. Myeloperoxidase-positive cell infiltration in colorectal carcinogenesis as indicator of colorectal cancer risk. Cancer Epidemiol Biomarkers Prev. 2008;17(9):2291–7.

237. Zeindler J, Angehrn F, Droeser R, Däster S, Piscuoglio S, Ng CKY, et al. Infiltration by myeloperoxidase-positive neutrophils is an independent prognostic factor in breast cancer. Breast Cancer Res Treat. 2019;177(3):581–9.

238. Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD. ROS and the DNA damage response in cancer. Vol. 25, Redox Biology. 2019.

239. Valavanidis A, Vlachogianni T, Fiotakis K, Loridas S. Pulmonary oxidative stress, inflammation and cancer: Respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Vol. 10, International Journal of Environmental Research and Public Health. 2013. p. 3886–907.

240. Güngör N, Knaapen AM, Munnia A, Peluso M, Haenen GR, Chiu RK, et al. Genotoxic effects of neutrophils and hypochlorous acid. Mutagenesis. 2010;25(2):149–54.

241. Gomez-Mejiba SE, Zhai Z, Gimenez MS, Ashby MT, Chilakapati J, Kitchin K, et al. Myeloperoxidase-induced genomic DNA-centered radicals. J Biol Chem. 2010;285(26):20062–71.

242. Henderson JP, Byun J, Takeshita J, Heinecke JW. Phagocytes produce 5-chlorouracil and 5-bromouracil, two mutagenic products of myeloperoxidase, in human inflammatory tissue. J Biol Chem. 2003;278(26):23522–8.

243. Whiteman M, Jenner A, Halliwell B. Hypochlorous acid-induced base modifications in isolated calf thymus DNA. Chem Res Toxicol. 1997;10(11):1240–6.

244. Borm PJ, Knaapen AM, Schins RP, Godschalk RW, Schooten FJ. Neutrophils amplify the formation of DNA adducts by benzo[a]pyrene in lung target cells. Environ Health Perspect. 1997;105 Suppl :1089–93.

245. Petruska JM, Mosebrook DR, Jakab GJ, Trush MA. Myeloperoxidase-enhanced formation of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a] pyrene adducts in lung tissue in vitro: A role of pulmonary inflammation in the bioactivation of a procarcinogen. Vol. 13, Carcinogenesis. 1992. p. 1075–81.

246. Tsuruta Y, Subrahmanyam V V., Marshall W, O'brien PJ. Peroxidase-mediated irreversible binding of arylamine carcinogens to DNA in intact polymorphonuclear leukocytes activated by a tumor promoter. Chem Biol Interact. 1985;53(C):25–35.

247. Pero RW, Sheng Y, Olsson A, Bryngelsson C, Lund-Pero M. Hypochlorous acid/N-chloramines are naturally produced DNA repair inhibitors. Carcinogenesis. 1996;17(1):13–8.

248. Van Rensburg CEJ, Van Staden AM, Anderson R. Inactivation of poly(ADP-ribose) polymerase by hypochlorous acid. Free Radic Biol Med. 1991;11(3):285–91.

249. Panagopoulos V, Leach DA, Zinonos I, Ponomarev V, Licari G, Liapis V, et al. Inflammatory peroxidases promote breast cancer progression in mice via regulation of the tumour microenvironment. Int J Oncol. 2017;50(4):1191–200.

250. Rymaszewski AL, Tate E, Yimbesalu JP, Gelman AE, Jarzembowski JA, Zhang H, et al. The role of neutrophil myeloperoxidase in models of lung tumor development. Cancers (Basel). 2014;6(2):1111–27.

251. Liu TW, Gammon ST, Yang P, Fuentes D, Worms DP. Myeloid cell derived HOCl is a paracrine effector that trans-inhibits IKK/NF-kB in melanoma cells and limits early tumor progression. Sci Signal. 2021;14(677).

252. Saed GM, Ali-Fehmi R, Jiang ZL, Fletcher NM, Diamond MP, Abu-Soud HM, et al. Myeloperoxidase serves as a redox switch that regulates apoptosis in epithelial ovarian cancer. Gynecol Oncol. 2010;116(2):276–81.

253. Clark RA, Klebanoff SJ, Einstein AB, Fefer A. Peroxidase H2O2 halide system: cytotoxic effect on mammalian tumor cells. Blood. 1975;45(2):161–70.

254. Odajima T, Onishi M, Hayama E, Motoji N, Momose Y, Shigematsu A. Cytolysis of B-16 melanoma tumor cells mediated by the myeloperoxidase and lactoperoxidase systems. Biol Chem. 1996;377(11):689–93.

255. Pottgiesser SJ, Heinzelmann S, Bauer G. Intercellular HOCl-mediated apoptosis induction in malignant cells: Interplay between NOX1-dependent superoxide anion generation and DUOX-related HOCl-generating peroxidase activity. Anticancer Res. 2015;35(11):5927–43.

256. Bechtel W, Bauer G. Catalase protects tumor cells from apoptosis induction by intercellular ROS signaling. Anticancer Res. 2009;29(11):4541–57.

257. Heinzelmanna S, Bauer G. Multiple protective functions of catalase against intercellular apoptosis-inducing ROS signaling of human tumor cells. Biol Chem. 2010;391(6):675–93.

258. Nakazato T, Sagawa M, Yamato K, Xian M, Yamamoto T, Suematsu M, et al. Myeloperoxidase is a key regulator of oxidative stress-mediated apoptosis in myeloid leukemic cells. Clin Cancer Res. 2007;13(18):5436–45.

259. Metzler KD, Goosmann C, Lubojemska A, Zychlinsky A, Papayannopoulos V. Myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep. 2014;8(3):883–96.

260. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 2010;191(3):677–91.

261. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. Science (80-). 2018;361(6409).

262. Berger-Achituv S, Brinkmann V, Abed UA, Kühn LI, Ben-Ezra J, Elhasid R, et al. A proposed role for neutrophil extracellular traps in cancer immunoediting. Front Immunol. 2013;4(MAR).

263. Jung HS, Gu JY, Kim JE, Nam Y, Song JW, Kim HK. Cancer cell–induced neutrophil extracellular traps promote both hypercoagulability and cancer progression. PLoS One. 2019;14(4).

264. Biedroń R, Konopiński MK, Marcinkiewicz J, Józefowski S. Oxidation by neutrophils-derived HOCl increases immunogenicity of proteins by converting them into ligands of several endocytic receptors involved in antigen uptake by dendritic cells and macrophages. PLoS One. 2015;10(4).

265. Prokopowicz ZM, Arce F, Biedron R, Chiang CL-L, Ciszek M, Katz DR, et al. Hypochlorous Acid: A Natural Adjuvant That Facilitates Antigen Processing, Cross-Priming, and the Induction of Adaptive Immunity. J Immunol. 2010;184(2):824–35.

266. Chiang CLL, Ledermann JA, Rad AN, Katz DR, Chain BM. Hypochlorous acid enhances immunogenicity and uptake of allogeneic ovarian tumor cells by dendritic cells to cross-prime tumor-specific T cells. Cancer Immunol Immunother. 2006;55(11):1384–95.

267. Chiang CLL, Ledermann JA, Aitkens E, Benjamin E, Katz DR, Chain BM. Oxidation of ovarian epithelial cancer cells by hypochlorous acid enhances immunogenicity and stimulates T cells that recognize autologous primary tumor. Clin Cancer Res. 2008;14(15):4898.

268. Liu TW, Gammon ST, Yang P, Ma W, Wang J, Piwnica-Worms D. Inhibition of myeloperoxidase enhances immune checkpoint therapy for melanoma. J Immunother Cancer. 2023;11(2).

269. Becht E, Tolstrup D, Dutertre CA, Ginhoux F, Newell EW, Gottardo R, et al. Infinity Flow: High-throughput single-cell quantification of 100s of proteins using conventional flow cytometry and machine learning. bioRxiv. 2020.

270. Weinhage T, Kölsche T, Rieger-Fackeldey E, Schmitz R, Antoni A-C, Ahlmann M, et al. Cord Blood Low-Density Granulocytes Correspond to an Immature Granulocytic Subset with Low Expression of S100A12. J Immunol. 2020;205(1):56–66.

271. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature CD10+ and immature CD10- neutrophils present in G-CSF-treated donors display opposite effects on T cells. Blood. 2017;129(10):1343–56.

272. Singhal S, Bhojnagarwala PS, O'Brien S, Moon EK, Garfall AL, Rao AS, et al. Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen-Presenting Cell Features in Early-Stage Human Lung Cancer. Cancer Cell. 2016;30(1):120–35.

273. Condamine T, Gabrilovich DI, Dominguez GA, Youn J-I, Kossenkov A V., Mony S, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients [Internet]. Vol. 1, Science immunology. 2016. 1-32 p. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5391495/pdf/nihms849112.pdf

274. Baldus S, Eiserich JP, Mani A, Castro L, Figueroa M, Chumley P, et al. Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. J Clin Invest. 2001;108(12):1759–70.

275. Haegens A, Vernooy JHJ, Heeringa P, Mossman BT, Wouters EFM. Myeloperoxidase modulates lung epithelial responses to pro-inflammatory agents. Eur Respir J. 2008;31(2):252–60.

276. Yamauchi Y, Safi S, Blattner C, Rathinasamy A, Umansky L, Juenger S, et al. Circulating and tumor myeloid-derived suppressor cells in resectable non-small cell lung cancer. Am J Respir Crit Care Med. 2018;198(6):777–87.

277. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Vol. 7, Nature Communications. 2016.

278. Lang S, Bruderek K, Kaspar C, Höing B, Kanaan O, Dominas N, et al. Clinical relevance and suppressive capacity of human myeloid-derived suppressor cell subsets. Clin Cancer Res. 2018;24(19):4834–44.

279. Silverstein RL, Febbraio M. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Vol. 2, Science Signaling. 2009.

280. Gekas C, Graf T. CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. Blood. 2013;121(22):4463–72.

281. Parcina M, Schiller M, Gierschke A, Heeg K, Bekeredjian-Ding I. PDC expressing CD36, CD61 and IL-10 may contribute to propagation of immune tolerance. Autoimmunity. 2009;42(4):353–5.

282. Dejima H, Nakanishi H, Kuroda H, Yoshimura M, Sakakura N, Ueda N, et al. Detection of abundant megakaryocytes in pulmonary artery blood in lung cancer patients using a

microfluidic platform. Lung Cancer. 2018;125:128–35.

283. Wang J, Li Y. CD36 tango in cancer: Signaling pathways and functions. Vol. 9, Theranostics. 2019. p. 4893–908.

284. Drissen R, Buza-Vidas N, Woll P, Thongjuea S, Gambardella A, Giustacchini A, et al. Distinct myeloid progenitor-differentiation pathways identified through single-cell RNA sequencing. Nat Immunol. 2016;17(6):666–76.

285. Mitjavila-Garcia MT, Cailleret M, Godin I, Nogueira MM, Cohen-Solal K, Schiavon V, et al. Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells. Development. 2002;129(8):2003–13.

286. Huang Y, Didonato JA, Levison BS, Schmitt D, Li L, Wu Y, et al. An abundant dysfunctional apolipoprotein A1 in human atheroma. Nat Med. 2014;20(2):193–203.

287. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A Distinct Subset of Proinflammatory Neutrophils Isolated from Patients with Systemic Lupus Erythematosus Induces Vascular Damage and Synthesizes Type I IFNs. J Immunol. 2010;184(6):3284–97.

288. Marini O, Spina C, Mimiola E, Cassaro A, Malerba G, Todeschini G, et al. Identification of granulocytic myeloid-derived suppressor cells (G-MDSCs) in the peripheral blood of Hodgkin and non-Hodgkin lymphoma patients. Oncotarget. 2016;7(19):27676–88.

289. Drifte G, Dunn-Siegrist I, Tissières P, Pugin J. Innate immune functions of immature neutrophils in patients with sepsis and severe systemic inflammatory response syndrome. Crit Care Med. 2013;41(3):820–32.

290. Dominiak A, Chełstowska B, Olejarz W, Nowicka G. Communication in the cancer microenvironment as a target for therapeutic interventions. Vol. 12, Cancers. 2020.

291. Maiocchi SL, Ku J, Thai T, Chan E, Rees MD, Thomas SR. Myeloperoxidase: A versatile mediator of endothelial dysfunction and therapeutic target during cardiovascular disease. Vol. 221, Pharmacology and Therapeutics. 2021.

292. Marsche G, Stadler JT, Kargl J, Holzer M. Understanding Myeloperoxidase-Induced Damage to HDL Structure and Function in the Vessel Wall: Implications for HDL-Based

Therapies. Vol. 11, Antioxidants. 2022.

293. Zou S, Pan X, Hua C, Wu M, He B, Chen Z. Myeloperoxidase -463 G/A polymorphism is associated with lung cancer risk: A meta-analysis with 7420 cases and 9132 controls. J Cancer Res Ther. 2018;14(9):S282–7.

294. Castillo-Tong DC, Pils D, Heinze G, Braicu I, Sehouli J, Reinthaller A, et al. Association of myeloperoxidase with ovarian cancer. Tumor Biol. 2014;35(1):141–8.

295. Qin X, Deng Y, Zeng ZY, Peng QL, Huang XL, Mo CJ, et al. Myeloperoxidase Polymorphism, Menopausal Status, and Breast Cancer Risk: An Update Meta-Analysis. PLoS One. 2013;8(8).

296. Hiraoka K, Miyamoto M, Cho Y, Suzuoki M, Oshikiri T, Nakakubo Y, et al. Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. Br J Cancer. 2006;94(2):275–80.

297. Prado-Garcia H, Romero-Garcia S, Aguilar-Cazares D, Meneses-Flores M, Lopez-Gonzalez JS. Tumor-induced CD8+ T-cell dysfunction in lung cancer patients. Vol. 2012, Clinical and Developmental Immunology. 2012.

298. Sheng SY, Gu Y, Lu CG, Zou JY, Hong H, Wang RF. The distribution and function of human memory T cell subsets in lung cancer. Immunol Res. 2017;65(3):639–50.

299. Takanami I, Takeuchi K, M G. The prognostic value of natural killer cell infiltration in resected pulmonary adenocarcinoma. J Thorac Cardiovasc Surg. 2001;121(6):1058–63.

300. Fernandez-Poma SM, Salas-Benito D, Lozano T, Casares N, Riezu-Boj JI, Mancheño U, et al. Expansion of tumor-infiltrating CD8+ T cells expressing PD-1 improves the efficacy of adoptive T-cell therapy. Cancer Res. 2017;77(13):3672–84.

301. Stankovic B, Bjørhovde HAK, Skarshaug R, Aamodt H, Frafjord A, Müller E, et al. Immune Cell Composition in Human Non-small Cell Lung Cancer. Front Immunol. 2018;9:3101.

302. Ugolini A, Tyurin VA, Tyurina YY, Tcyganov EN, Donthireddy L, Kagan VE, et al. Polymorphonuclear myeloid-derived suppressor cells limit antigen crosspresentation by dendritic cells in cancer. JCI Insight. 2020;5(15):e138581.

303. Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, et al. Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. Proc Natl Acad Sci. 2005;102(2):431–6.

304. Yang JJ, Preston GA, Pendergraft WF, Segelmark M, Heeringa P, Hogan SL, et al. Internalization of proteinase 3 is concomitant with endothelial cell apoptosis and internalization of myeloperoxidase with generation of intracellular oxidants. Am J Pathol. 2001;158(2):581–92.

6 APPENDIX

Table 7. Flow cytometric antibody panels used in the LDN project.

Panel	Antibody	Dilution	Clone	Company	Catalog number
Screening panel	CD45-AF700	1:200	30-F11	BioLegend	103128
	CD3-BUV395	1:40	145-2C11	BD Biosciences	563565
	CD8-PerCPCy5.5	1:80	53-6.7	BioLegend	100734
	CD4-BUV496	1:80	GK1.5	BD Biosciences	564667
	gdTCR-PECF594	1:40	GL3	BD Biosciences	563532
	PD-1-APC	1:40	29F.1A12	BioLegend	135210
	CD62L-BV605	1:50	MEL-14	BioLegend	104438
	CD44-BUV737	1:160	IM7	BD Biosciences	612799
	NKp46-BV510	1:20	29A1.4	BioLegend	137623
	CD19-FITC	1:160	6D5	BioLegend	115506
	CD25-BV785	1:80	PC61	BioLegend	102051
	FoxP3-PE	1:40	FJK-16s	eBio	12-5773-82
Validation panel	CD45-BV785	1:160	30-F11	BioLegend	103149
	Ly6C-APC	1:79	HK1.4	BioLegend	128015
	Ly6G-PE/Dazzle	1:166	1A8	BioLegend	127648
	CD11c-BV605	1:20	N418	BioLegend	117334
	PD-L1-PeCy7	1:79	10F.9G2	BioLegend	124313
	CD206-FITC	1:160	C068C2	BioLegend	141703
	MHCII-PerCP-Cy5.5	1:160	M5/114.15.2	BioLegend	107625

	CD103-BV510	1:40	2E7	BioLegend	121423
	CD11b-BUV737	1:80	M1/70	BD Biosciences	612801
	F4/80-BUV395	1:40	T45-2342	BD Biosciences	565614
	Siglec-F-PE	1:40	E50-2440	BD Biosciences	562068
LDN staining	CD45-FITC	1:100	30-F11	BioLegend	103108
	CD3-BV421	1:25	145-2C11	BioLegend	100336
	CD4-PE-Cy7	1:50	RM4-5	Biolegend	100528
	CD8-PerCP-Cy5.5	1:80	53-6.7	BioLegend	100734
	IFN-γ-PE	1:50	XMG1.2	BioLegend	505808
Screen	CD4- PE	1:100	RPA-T4	BioLegend	300508
	CD8a-FITC	1:100	RPA-T8	BioLegend	301006

Table 8. Flow cytometric antibody panels used in the MPO project.

Panel	Antibody	Dilution	Clone	Company	Catalog number
Tumor-infiltrating lymphoid immune cells (mouse)	CD45-AF700	1:200	30-F11	BioLegend	103128
	CD3-BUV395	1:40	145-2C11	BD Biosciences	563565
	CD8-PerCPCy5.5	1:80	53-6.7	BioLegend	100734
	CD4-BUV496	1:80	GK1.5	BD Biosciences	564667
	gdTCR-PECF594	1:40	GL3	BD Biosciences	563532
	PD-1-APC	1:40	29F.1A12	BioLegend	135210
	CD62L-BV605	1:50	MEL-14	BioLegend	104438
	CD44-BUV737	1:160	IM7	BD Biosciences	612799
	NKp46-BV510	1:20	29A1.4	BioLegend	137623
	CD19-FITC	1:160	6D5	BioLegend	115506
	CD25-BV785	1:80	PC61	BioLegend	102051
	FoxP3-PE	1:40	FJK-16s	eBio	12-5773-82
Tumor-infiltrating myeloid immune cells (mouse)	CD45-BV785	1:160	30-F11	BioLegend	103149
	Ly6C-APC	1:79	HK1.4	BioLegend	128015
	Ly6G-PE/Dazzle	1:166	1A8	BioLegend	127648
	CD11c-BV605	1:20	N418	BioLegend	117334
	PD-L1-PeCy7	1:79	10F.9G2	BioLegend	124313
	CD206-FITC	1:160	C068C2	BioLegend	141703
	MHCII-PerCP-Cy5.5	1:160	M5/114.15.2	BioLegend	107625
	CD103-BV510	1:40	2E7	BioLegend	121423
	CD11b-	1:80	M1/70	BD	612801

	BUV737			Biosciences	
	F4/80-BUV395	1:40	T45-2342	BD Biosciences	565614
	Siglec-F-PE	1:40	E50-2440	BD Biosciences	562068
IFN-γ expression (mouse)	CD45-FITC	1:100	30-F11	BioLegend	103108
	CD3-BV421	1:25	145-2C11	BioLegend	100336
	CD4-PE-Cy7	1:50	RM4-5	Biolegend	100528
	CD8-PerCP-Cy5.5	1:80	53-6.7	BioLegend	100734
	IFN-γ-PE	1:50	XMG1.2	BioLegend	505808
T-cell proliferation (human)	CD4- PE	1:100	RPA-T4	BioLegend	300508
	CD8a-FITC	1:100	RPA-T8	BioLegend	301006
IFN-γ expression (human)	CD4- PE-Cy7	1:25	RPA-T4	BioLegend	300512
	CD8-APC	1:25	RPA-T8	BioLegend	301014
	IFN-γ-BUV395	1:20	B27	BD Biosciences	563563
MPO binding/internalization (human)	CD4-PE-Cy7	1:100	RPA-T4	BioLegend	300512
	CD8-BV510	1:50	RPA-T8	BioLegend	301006
	MPO-FITC	1:2.5	5B8	BD Biosciences	340580
MPO in tumors from patients with NSCLC (human)	CD45-BV510	1:100	H130	BioLegend	304035
	CD66b-APC	1:50	G10F5	BioLegend	305118
	CD163-BV605	1:25	GH1/61	BioLegend	333616
	MPO-FITC	1:2,5	5B8	BD Biosciences	340580

Table 9. GeoMean, MFI and percentage of expression (data expressed as % of CD66b+, % of CD45+ and % of live) of hits identified in screen. Hits were defined by fold change marker expression (GeoMean) with fold change > 2 and < 0.5 (ratio LDNs/HDNs)

Marker	**PBMC**					**PMNL**				
	Geo Mean	MFI	Fq of CD66b (%)	Fq of CD45 (%)	Fq of live (%)	Geo Mean	MFI	Fq of CD66b (%)	Fq of CD45 (%)	Fq of live (%)
CD41	4992	6836	94.7	56.4	55.8	136	102	5.69	2.89	2.80
CD36	2679	4407	78.8	25.6	24.7	86,1	82,2	0.42	0.40	0.40
CD226	2123	2095	98.1	8.95	6.98	76,5	71,9	0.28	0.24	0.23
CD61	2427	2854	96.5	61.4	60.9	121	104	1.97	1.11	1.07
CD102	1192	1275	71.2	17.6	16.9	79,9	75,8	0.23	0.22	0.22
CD42b	904	728	59.2	35.6	35.2	94,3	89,9	0.36	0.19	0.18
CD49b	741	622	67.0	41.9	41.6	106	95,1	1.61	0.83	0.81
CD323	521	429	61.6	16.3	15.7	75,8	73,2	0.13	0.12	0.12
CD194	587	437	69.2	6.24	6.77	92,9	62,9	3.05	2.71	2.69
CD84	680	535	63.0	16.7	16.1	110	105	1.90	1.81	1.81
CD9	1980	3406	70.6	7.83	7.66	357	246	32.4	20.2	19.3
CD62P	660	488	69.6	41.8	41.3	122	111	1.77	0.95	0.92
CD99	873	760	86.6	9.42	9.18	164	133	11.1	6.17	5.95
CD71	404	183	34.8	9.23	8.85	81,4	79,6	0.36	0.35	0.35
CD105	391	278	55.2	1.19	1.19	80,9	73,2	0.32	0.31	0.30
CD29	3322	3497	100.0	62.0	61.5	850	752	83.3	0.44	0.15
C3aR	458	409	68.8	6.90	8.92	129	96,4	8.13	7.23	7.14
CD230	452	351	58.4	15.5	15.0	135	117	8.44	8.02	8.02
CD64	792	424	60.4	6.27	6.07	247	203	21.4	11.2	10.7
LAP	214	139	17.3	1.92	1.85	69,9	61,6	0	0	0
CD45	664	593	98.5	60.2	59.2	222	188	13.5	7.14	6.87
Ig light chain λ	339	249	35.6	3.60	9.25	121	107	2.17	1.89	1.83
CD69	216	161	18.0	1.75	1.70	77,5	69,4	0	0	0
CD112	234	151	28.3	2.67	2.67	93	79,6	2.29	2.12	2.06
CD49e	780	650	92.3	8.92	7.28	354	326	59.5	53.3	52.9
CD51	162	122	12.6	3.10	2.99	77,8	75,8	0.27	0.25	0.25

CD31	3598	3469	100.0	61.0	60.4	1742	1788	99.9	51.9	50.9
CD63	1530	1384	99.9	62.2	61.7	745	561	88.9	49.4	47.9
CD126	421	420	85.0	8.40	8.40	209	199	9.03	8.45	8.28
CD184	524	465	81.8	19.9	19.2	261	225	45.5	43.3	43.3
Notch 2	150	143	3.10	0.32	9.77	76,3	69,4	0.66	0.56	0.51
CD109	178	122	19.4	1.93	1.93	91,3	75,8	2.40	2.28	2.22
CD191	564	455	81.3	8.61	7.59	305	266	42.1	37.5	37.3
CD107a	472	337	67.9	6.76	6.76	263	183	16.0	15.3	15.1
CD92	1185	1163	99.2	9.18	7.65	708	699	96.5	82.7	77.4
HLA-A,B,C	1067	988	99.3	25.6	24.9	660	644	99.7	95.0	94.9
CD47	7586	7365	100	51.5	37.9	4699	4903	100	54.3	51.9
CD93	908	853	85.8	9.18	8.88	575	507	67.3	40.3	38.8
CD66a/c/e	9686	10675	97.6	8.64	6.30	6136	6015	100.0	88.1	85.5
CD97	2244	2270	100	10.8	10.4	1467	1464	100	58.5	56.5
CD32	2792	2992	99.3	10.1	9.11	1840	1906	100.0	86.5	82.2
CD262	73,9	70,6	0.60	0.39	0.39	128	118	2.03	1.10	1.07
CD28	68,8	61,6	1.07	0.68	0.68	125	123	0	0	0
CD278	47,9	42,4	0.75	0.072	0.072	88,1	83,5	0.21	0.20	0.20
CD1a	56,3	52,6	0.76	0.071	0.071	105	92,5	2.71	2.55	2.51
CD30	64,9	62,9	0.12	0.078	0.077	122	113	0	0	0
CD70	56	50,1	0.50	0.049	0.049	113	99	2.58	2.45	2.41
CD3	64,3	60,4	0.64	0.40	0.40	135	121	5.88	0.073	0.025
CD290	75,3	71,9	0.52	0.33	0.32	167	152	0	0	0 %
IFN-γ R b chain	78,1	60,4	3.44	0.33	0.33	186	147	8.39	7.94	7.79
CD261	82	70,6	1.61	1.03	1.02	200	116	11.1	6.16	5.94
CD202b	77	65,5	1.54	0.16	8.09	266	257	30.9	27.0	26.1
Cadherin 11	51,4	46,2	0.40	0.040	0.040	295	278	32.7	31.3	30.7

Table 10. Genes derived from the CPTAC-LUAD dataset.

MPO signature		Control signature			
ADGRG3	HIST1H4L	ACOXL	CLK4	LRRC31	TLR5
ADM	IL1R2	ADGRF5	CNKSR2	MAP3K15	TMEM243
AQP9	LEP	ADHFE1	CREBRF	MAPK10	TNN
C11orf24	LIF	ANK3	CRYM	MR1	WWC1
CDH2	LRFN4	ANKDD1B	CYP4V2	MS4A2	ZDHHC15
CEACAM3	MRPL12	ANKRD44	CYSLTR1	MYOZ1	ZMAT1
CEMIP	MTHFD2	AR	ETFBKMT	N4BP2L1	ZNF441
CHD7	NAMPT	ARHGAP31	FAM161B	NFIX	ZNF540
CTD-3088G3.8	NFXL1	ARHGDIB	FAM184A	NIPAL3	ZNF554
CXCL2	NOP56	ATP13A4	FMO4	NPC2	ZNF563
CXCL3	P3H4	ATP13A5	FMO5	NRN1L	ZNF763
CXCL8	PCBP1	B3GAT1	GAB1	PIGR	ZNF846
CYP24A1	PI15	C16orf89	GANC	POU2F3	ZRSR2
ECT2	PI3	C17orf50	GPR160	PPP2R3A	
EIF4A1	PLAUR	C1orf116	GSAP	PTPN13	
EIF5A	PLEKHG2	CA1	HAS3	RBL2	
ENTPD7	PNO1	CA13	HLA-DPB1	REPS2	
FAM64A	PPIF	CACNA1F	HLF	RNASE1	
FAM83D	PPM1G	CAMK2D	HNMT	RP11-490B18.9	
FCAR	PVR	CAPN3	HPGDS	SCTR	
FCGR3B	RCOR2	CARF	HSD17B6	SGF29	
FKBP10	SLC2A1	CD1B	IL11RA	SLC15A2	
FPR2	SNRPA1	CD1E	IL6R	SLC9A5	
HCAR3	SNRPD1	CD302	KAT2B	SLFN14	
	TDG	CD40LG	KCNK5	SMCO3	
	TGM3	CD74	KIF13A	SOGA3	
	TTL	CEBPA	KLHDC1	TCEANC	
	UBE2S	CFAP221	LANCL3	TEF	
	UCK2	CIRBP	LRP2BP	TLR3	

MPO Signature: genes that were significantly upregulated in the MPO high group. Control Signature: genes that were significantly downregulated in the MPO low group.