UNIVERSITY OF NAPLES FEDERICO II

PH.D. PROGRAM IN

CLINICAL AND EXPERIMENTAL MEDICINE

CURRICULUM IN TRANSLATIONAL MEDICAL SCIENCES

XXXIV Cycle

(Years 2018-2021)

Chairman: Prof. Francesco Beguinot

PH.D. THESIS

NEUTROPHILS AND NEUTROPHIL EXTRACELLULAR TRAPS IN THYROID CANCER

TUTOR

Prof. Maria Rosaria Galdiero

PH.D. STUDENT

Dr. Leonardo Cristinziano

MATR: DR994044

TABLE OF CONTENTS

ABSTRACT

Neutrophils are key effector cells that orchestrate inflammatory responses in the tumor microenvironment. Although neutrophil extracellular DNA traps (NETs) entrap and kill pathogens, they also contribute to chronic inflammation and cancer progression. Thyroid cancer (TC) is the most frequently occurring cancer of the endocrine system, accounting for 70% of deaths due to endocrine tumors. Although anaplastic TC (ATC) is rare among TCs, it is highly lethal. We recently demonstrated that tumor-infiltrating neutrophil density correlated with TC size. Moreover, TC-derived soluble mediators modulate the human neutrophil phenotype. Our study aimed to investigate the involvement of NETs in human TC. Highly purified neutrophils from healthy donors were primed *in vitro* with a papillary TC or ATC cell line conditioned medium (CM) or with a normal thyroid CM as control. NET release was quantified using a High-Content Imaging System. Neutrophil viability was assessed by flow cytometry. Fluorescence microscopy, flow cytometry, and PCR were performed to determine the mitochondrial origin of ATC-induced NETs. ATC CM-primed neutrophils were cocultured with ATC cells to determine the effects exerted by NETs on cell proliferation. ATC CM induce NET release, whereas papillary TC or normal thyroid CM did not. ATC CM-induced NET production occurred in a reactive oxygen species-dependent and cell death-independent manner and was associated with mitochondrial reactive oxygen species production; the NETs contained mitochondrial DNA. ATC CM-primed neutrophils promoted ATC cell proliferation in a NET-dependent manner.

INTRODUCTION

1. Cancer development: an overview

Cancer consists an abnormal growth of cells which lost their normal control mechanisms. Indeed, cancer cells are able to proliferate continuously, invade adjacent tissues, migrate to distant sites, and promote the growth of new blood vessels from which they draw nutrients. The term "tumor" is used when malignant cells grow and multiply, so they form a mass of cancerous tissue that invades and destroys normal adjacent tissues. However, in a more recent point of view, tumors are not merely considered as masses composed by proliferating cancer cells but a complex microenvironment characterized by diverse cell types which interact each other. In 2000, in their seminal paper, Hanahan and Weinberg first described the six hallmarks of cancer (Hanahan and Weinberg, 2000). First of all, cancer cells are capable to sustain proliferative signaling because of autocrine proliferative signals produced by cancer cells themselves. In most cases, activation of oncogenes and/or deactivation of tumor suppressor genes lead to uncontrolled cell cycle progression and inactivation of apoptotic mechanisms. The *RB* (retinoblastoma) gene and *TP53* gene, are examples of tumor suppressor genes which regulate the decision of cells to proliferate or to activate senescence or apoptotic pathways (Burkhart and Sage, 2010; Sherr and McCormick, 2002). Apoptosis is a highly regulated process of cell death important in the development and maintenance of a normal cell population in mature organisms. Deregulation of apoptosis pathways is a key feature of carcinogenesis. Some studies reveal how apoptosis is attenuated in the most cases of tumors (Adams and Cory, 2007). Thus, tumors resists to cell death and retain replicative immortality. Indeed, in normal cells, repeated cycles of cell division induce senescence and cell death; by contrast, cancer cells exhibit an unlimited replicative potential (Blasco, 2005). This transition is called "immortalization". Finally, cancer retains the ability to promote angiogenesis,

invasion and metastasis. Angiogenesis and lymphangiogenesis are processes whereby new blood and lymphatic vessels form, respectively. Both play essential roles to supply nutrients. Angiogenesis and lymphangiogenesis are important processes in the progression of cancer. In fact, tumor growth and metastasis depend on these processes. In the last decade, an intense experimental effort led to the identification of two emerging hallmarks: the capability of cancer cells to reprogram the cellular metabolism and to evade immunological destruction. Otto Warburg first observed an anomalous characteristic of cancer cell energy metabolism: even in the presence of oxygen, cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed "aerobic glycolysis" (Warburg, 1956). Mechanisms resulting in evasion of immune attack include the selection of tumor variants resistant to immune effectors (designated as "immunoediting") and progressive formation of an immune suppressive environment within the tumor (Vinay et al., 2015). The acquisition of all these hallmarks is possible by two enabling characteristics: the genomic instability of cancer cells and cancer related inflammation (Hanahan and Weinberg, 2011).

2. Cancer related inflammation.

Rudolf Virchow in 1863 first described a link between cancer and inflammation. Indeed, he first observed that tumors arose at sites of chronic inflammation and some classes of irritants, together with tissue injury and inflammation enhanced cell proliferation (Balkwill and Mantovani, 2001). Cancers are not just masses of malignant cells but complex organs, in which many other cells are recruited and can be corrupted by the transformed cells. Interactions between malignant and non-transformed cells give rise to the tumor microenvironment (TME). These interactions are made possible by a network of inflammatory mediators (cytokines, chemokines, prostaglandins). Beyond malignant cells,

TME is composed by blood and lymphatic vessels, pericytes, fibroblast and immune cells (Quail and Joyce, 2013). It is widely accepted that stromal and tumor cells produce chemokines and other molecules that contribute to activation and recruitment of tumor-associated macrophages (TAM). Indeed, these chemoattractants were found in supernatant fluids of cultured human and mouse tumor cells (Bottazzi et al., 1983). Vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β) and macrophage colony stimulating factor (M-CSF/CSF1) are key factors promoting macrophage survival and polarization (Bottazzi et al., 1983) while CCL2/MCP1 is the main chemotactic factor for TAMs (Li et al., 2013). In response to signals derived from microbes, damaged tissues or activated lymphocytes, macrophages undergo reprogramming which leads to two different polarization states M1 or M2. M1 macrophages present an IL-12 high, IL-23 high, IL-10 low phenotype, while M2 exhibit an IL-12 low, IL-23 low, IL-10 high phenotype. According to the diverse phenotype, these two population show different functions. Indeed, M1 cells promote resistance to intracellular parasites and tumors, while M2 cell play a crucial role in angiogenesis, wound healing, tissue remodeling and tumor progression (Biswas and Mantovani, 2010; Galdiero et al., 2013; Mantovani et al., 2004). TAMs have been found in several tumor types, their activities are usually pro-tumorigenic. Indeed, clinical evidence showed that TAMs were associated with poor prognosis (Bingle et al., 2002). However, they can exert an anti-tumoral function.

TAMs are not the only one cell population that influence tumor growth and progression. Recent evidence reveals that neutrophils also infiltrate tumors (TANs) and play important roles in tumor progression (Fridlender et al., 2009).

3. The Neutrophils

The existence of polymorphonuclear granulocytes was first described by Paul Ehrlich in 1878 on the basis of their distinct nuclear morphology. He called these cells "cells with polymorphous nucleus" and in 1905 Elie Metchnikoff renamed the cells "polymorphonuclear leukocytes". Paul Ehrlich also observed that granules from different cells displayed specific staining properties. He called "basophils" for their affinity for basic aniline dyes, "eosinophils" for their affinity for acid aniline dyes and "neutrophils" for their low affinity for both basic and acid aniline dyes (Borregaard and Cowland, 1997; Kaufmann, 2008). In the same period, Elie Metchnikoff elaborated the phagocytic theory, whereby circulating cells contributed to defense against infectious agents by killing invading pathogens. Metchnikoff described two types of phagocytes, namely *microphages* (smaller phagocytes with highly polymorphic and fragmented nuclei) and *macrophages* (large phagocytic cells with a non-polymorphic nucleus). He proposed that microphages exerted their phagocytic role essentially in septicaemia and anthrax and macrophages in tuberculosis. Therefore, he attributed a major role to granulocytes in acute infections by extracellular bacteria and a pivotal role to macrophages in chronic infections by intracellular bacteria (Kaufmann, 2008).

Since their discovery and characterization, neutrophils have been considered as the first line of defense against pathogens and their role in immunology and immunopathology limited to the early phases of inflammation and resistance against extracellular pathogens (Amulic et al., 2012; Borregaard, 2010; Jaillon et al., 2013). Indeed, neutrophils were characterized by limited lifespan, easy activation and proper role of pathogen elimination. Therefore, their role in orchestrating the various phases of inflammation and the immune response has long been neglected. However, in the last two decades several evidence have challenged this view, suggesting a number of new fascinating roles and novel functional aspects for these underestimated phagocytes, revealing highly versatile and sophisticated cells, whose functions

go far beyond the elimination of microbes. The finding of a prolonged lifespan under some circumstances (such as inflammatory conditions) and the introduction of new techniques useful to obtain highly pure neutrophil preparations and to perform *in vivo* studies of neutrophils in tissues have shed new light on neutrophil functions (Jaillon et al., 2013). The improvement in purification techniques has significantly contributed to the characterization of cytokine expression pattern (Davey et al., 2011; Tecchio et al., 2013).

3.1. Granulocytopoiesis

Neutrophil production is the main activity of the bone marrow, with 10^9 neutrophils per kg of body weight produced every day (Mary, 1985). Under the influence of cytokines and growth factors, hematopoietic stem cells differentiate into myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells and finally granulocytes (Borregaard, 2010). In physiological conditions, only mature neutrophils leave the bone marrow, with CXCR4 playing a crucial role in modulating neutrophil homing or egression (Lapidot and Kollet, 2002). Bone marrow hematopoietic stem cells express CXCR4, which binds CXCL12/SDF-1 expressed by stromal cells, particularly osteoblasts. CXCR4 mediates bone marrow retention of myeloid cells and its expression decreases progressively during myeloid maturation. In parallel, CXCR2 expression increases and mediates mature neutrophil bone marrow egression (Lapidot and Kollet, 2002). Indeed, CXCR2 deficiency results in a myelokathexis-like phenotype with a cell-intrinsic retention of neutrophils in the bone marrow. Under inflammatory conditions, however, such as murine models of inflammation-driven colon cancer, Granulocytic Myeloid Derived Suppressor Cells (G-MDSC) accumulated in peripheral blood of $CXCR2^{-/-}$ mice, even though the recruitment in the tumor and adjacent inflammatory mucosa was significantly impaired (Katoh et al., 2013). CXCR4 and CXCR2 antagonistically regulate neutrophil release from the bone marrow, but CXCR4 plays a

dominant role. Indeed, in absence of CXCR4, CXCR2 signals are not required for neutrophil mobilization. Osteoblasts and endothelial cells both express CXCL12 and CXCR2 ligands (CXCL1 and CXCL2). However, osteoblasts display a higher expression of CXCL12, whereas endothelial cells display a higher expression of CXCR2 ligands (CXCL1 and CXCL2). Thus, while endosteal osteoblasts produce CXCL12 for neutrophil retention, endothelial-derived chemokines (CXCR2 ligands) induce neutrophil chemotaxis to the circulation.

Granulocyte colony stimulating factor (G-CSF) is the main regulator of neutrophil production and differentiation. Bone marrow stromal cells of monocyte/macrophage lineage as well as vascular endothelial cells, fibroblasts and mesothelial cells, produce G-CSF. Upon binding to its receptor, G-CSF induces proliferation, differentiation and activation of granulocyte precursors (Demetri and Griffin, 1991). G-CSF is crucial during infections, but it is not absolutely required for granulocytopoiesis because G-CSF$^{-/-}$ mice, even though neutropenic, still display residual granulocytopoiesis and produce fully mature neutrophils (Lieschke et al., 1994). In stress conditions, maximal blood neutrophil responses to G-CSF require CXCR2 signaling. Indeed, G-CSF administration was associated with marked suppression of endosteal osteoblast functions and increased CXCL2 expression by bone marrow endothelial cells. Thus, a shift in the bone marrow balance from pro-retention (CXCL12) to mobilizing (CXCL1 and CXCL2) chemokines contributes to neutrophil mobilization by G-CSF (Eash et al., 2010).

The production of neutrophils in the bone marrow is a tightly regulated process, resumed in a so-called “neutrostat regulatory loop” (Ley et al., 2006; Stark et al., 2005). The rate of apoptosis of peripheral neutrophils is an important regulator of myelopoiesis. Under homeostatic conditions, bone marrow neutrophil production strictly balances neutrophil peripheral elimination, thus keeping the stable level of circulating neutrophils (Stark et al., 2005). Neutrophil turnover can be delayed or accelerated during inflammatory response,

suggesting that modulation of neutrophil lifespan is important in determining whether an inflammatory response needs to be extended or terminated (Colotta et al., 1992). Under inflammatory conditions, in gut-associated lymphatic tissue, macrophages and DC produce IL-23. IL-23 stimulates IL-17A production by Th17 cells, which in turn stimulates G-CSF production in BM stromal cells, thus leading to enhanced granulocytopoiesis (Stark et al., 2005). When efficient neutrophils carry out their functions in target tissues, they undergo apoptosis and are phagocytosed by macrophages and DC. This process reduces IL-23 production by myeloid cells, completing the finely tuned loop feedback that regulates granulocytopoiesis (Stark et al., 2005).

Elegant *in vivo* studies also describe that a circadian rhythm was involved in the patrol of circulating neutrophil number. This mechanism was related to a feedback circuit that involves selectins, chemokine receptors and β_2-integrins. As circulating neutrophils age, they undergo down-regulation of L-selectin (CD62L), up-regulation of CXCR4 and CD11b and nuclear hypersegmentation. $CD62L^{low}CXCR4^{high}$ neutrophils return to the bone marrow, where local macrophages ingest them. Phagocytosis of aged neutrophils by BM-associated macrophages decreases the production of CXCL12 in macrophages, favoring the release of $CD62^{high}CXCR4^{low}$ neutrophils from the BM to circulation (Casanova-Acebes et al., 2013). This circadian fluctuation of neutrophil trafficking is likely due to signals derived from light cycle and transmitted through the nervous system. During the period of activity, sympathetic nerves induce expression of ICAM-1 and CCL2 in peripheral tissues endothelial cell together with the expression of V-CAM and CXCL12 in bone marrow (Scheiermann et al., 2012). These signals give rise to the peak of the peripheral tissue and bone marrow recruitment, with the lowest leukocyte counts in the peripheral blood (Lucas et al., 2008).

3.2. Neutrophil granules and related proteins

Neutrophil granules were first named “specific” to be distinguished from artifacts with a granular aspect. Subsequently two types of granules were distinguished on the basis of their affinity for dyes: azurophil granules, named for their affinity for the basic dye azure A, and specific granules, without affinity for this dye. The peroxidase staining method for electron microscopy revealed that myeloperoxidase was expressed only in azurophil granules. This technique allowed the distinction between the two subsets of granules: specific granules were considered as secretory granules, with important functions in modulating the inflammatory response, whereas azurophil granules were often considered as lysosomes, with a prevalent role in phagocytosis (Baggiolini, 1972; Gallin et al., 1984). This simplistic view was challenged when a tertiary type of granule formed in the late stages of granulopoiesis was revealed by electron microscopy (Spicer and Hardin, 1969).

Granulopoiesis occurs during neutrophil maturation and granule appearance defines the transition from myeloblast to promyelocyte. The formation of granules continues until the segmented stage of maturation. During granulopoiesis, distinct proteins are synthesized and stored in distinct granules, which are actually classified in three subsets: primary (azurophil), secondary (specific) and tertiary (gelatinase) granules. Primary granules are formed during the promyelocyte stage and are characterized by the presence of myeloperoxidase (MPO). Secondary granules are formed during the myeloblast stage and are characterized by the presence of lactoferrin and the pattern recognition molecule pentraxin-3 (PTX3). Tertiary granules are formed during the metamyelocyte/band cell stage and are characterized by the presence of gelatinase. Based on the “targeting by timing hypothesis”, granule proteins that are synthesized during the formation of particular granules are stored in these granules. Thus, granules formed at different stages during maturation display a different set of proteins (Borregaard, 2010). This distinction is operative because it not only reflects differences in

content of granules but also differences in their mobilization with the granules formed latest being the first ones to be released (Borregaard and Cowland, 1997). During the last 25 years, high-resolution subcellular fractionation techniques, immune-electron microscopy and flow cytometry have shown a wide heterogeneity of neutrophil granules, allowing to identify an additional regulated exocytotic storage organelle, the secretory vesicles (Borregaard et al., 1990; Borregaard et al., 1992). Secretory vesicles consist in cytosolic organelles of membrane proteins containing a set of plasma proteins (Borregaard et al., 1992). They are generated by endocytosis during the last stage of granulopoiesis and upon neutrophil activation fuse their membrane with cell plasma membrane (Borregaard et al., 1987)·(Sengelov et al., 1995). Granules and secretory vesicles do not have to be considered just storage organelles of proteolytic or bactericidal proteins retained until released. Indeed, they also contain membrane proteins, which are expressed on neutrophil surface upon the fusion of the secretory vesicle membrane to the neutrophil cellular membrane, during degranulation. In this way, neutrophil granules and secretory vesicles modify the ability of neutrophils to respond to the signals derived from the microenvironment (Borregaard and Cowland, 1997).

3.3. Neutrophil activation at sites of inflammation

Neutrophil behavior following transendothelial migration is dictated by the microenvironment, in particular by local chemokines and inflammatory factors, such as CXCL8 and TNF-α. These molecules activate a number of downstream signals that finally lead to neutrophil activation and respiratory burst. It is important to take in mind that activation of Pattern Recognition Receptors (PRRs), such as Toll-Like Receptors, can also directly activate the oxidative burst (Hayashi et al., 2003; Trinchieri and Sher, 2007). Indeed, human neutrophils express all the known TLR, with the exception of TLR3. The activation of TLRs induced cytokines release, superoxide generation, L-selectin shedding while inhibited

chemotaxis to CXCL8 and increased phagocytosis (Hayashi et al., 2003). A number of mechanisms are used by neutrophils to kill pathogens, including phagocytosis, antimicrobial peptides and the release of Reactive Oxygen Species (ROS) during respiratory burst (Borregaard et al., 2007).

However, during the last 10 years, neutrophils were found to display an antimicrobial armamentarium far beyond ROS and antimicrobial peptides. For instance, neutrophils extrude an extracellular fibrillary network, called Neutrophil Extracellular Traps (NETs) composed of nuclear elements (DNA and histones) together with proteins from primary (e.g. MPO and elastase (Brinkmann et al., 2004), secondary (e.g. lactoferrin and PTX3) (Jaillon et al., 2007) and tertiary granules (e.g. MMP-9) (Brinkmann et al., 2004). A study has identified by mass spectrometry 24 neutrophil NET-associated proteins (Urban et al., 2009). Among them are a number of antimicrobial proteins, such as histones, defensins, elastase, cathepsin G and MPO (Urban et al., 2009) and also the pattern recognition molecule PTX3 (Jaillon et al., 2007). Recently, mitochondrial DNA was also found in NETs suggesting a role for mitochondria in NETs formation (Yousefi et al., 2009). These web-like structures trap different microbes (Amini et al., 2018; Brinkmann et al., 2004; Pilsczek et al., 2010; Robledo-Avila et al., 2018; Yousefi et al., 2008), fungi (Muniz et al., 2018; Silva et al., 2020), as well as the human immunodeficiency virus-1 (HIV-1) (Sivanandham et al., 2018; Yazdani et al., 2019), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zuo et al., 2020). For instance, the antimicrobial heterodimer calprotectin released in NETs plays a key role in defense against *Candida albicans* and *Aspergillus fumigatus* (McCormick et al., 2010).

3.4. Neutrophil-derived cytokines

Neutrophils have also been reconsidered as a source of stored and newly synthesized cytokines. For instance, recent evidence demonstrated that murine neutrophils produce IL-1β following LPS stimulation, in a NLRP3 inflammosome dependent manner (Mankan et al., 2012). Previous results indicated that human neutrophils were not able to activate the MyD88-independent/TRIF-dependent pathway upon TLR4 engagement and thereby failed to produce IFN-β following LPS stimulation (Tamassia et al., 2007). The same group subsequently found that DNA-transfected human neutrophils become able to produce IFN-β following IRF3 activation because of the triggering of intracellular DNA sensors, highlighting the role of neutrophils in recognizing microbial cytosolic DNA (Tamassia et al., 2012). Moreover, neutrophils have been described to produce a number of other cytokines and chemokines, such as TNF-Related Apoptosis Inducing Ligand (TRAIL), CCL20, CXCL8, IL-1 Receptor Antagonist (IL-1Ra) and B cell Activating Factor (BAFF). More in details, the two B-cell related cytokines BAFF and A Proliferation Inducing Ligand (APRIL) have been recently described as part of the neutrophil-derived cytokines, suggesting a central role for neutrophils in B cell mediated autoimmune or neoplastic diseases (Mantovani et al., 2011; Puga et al., 2012). In addition, human and murine neutrophils have been described to express IL-17A in psoriasis and rheumatoid arthritis (Lin et al., 2011; Moran et al., 2011). However it is important to take in mind that murine and human neutrophils significantly differ each other. For instance, the production of IL-10 from human neutrophils has been a matter of debate. Indeed, murine neutrophils have been shown to produce IL-10 in several pathologic conditions, such as *Candida* or *Staphylococcus aureus* infections (Greenblatt et al., 2010; Zhang et al., 2009). In contrast, the finding of IL-10 production by human neutrophils following serum Amyloid A stimulation (De Santo et al., 2010) has not been confirmed by other groups, thus pointing to the need for a stringent neutrophil purification, devoid of any

minimal monocyte contamination (Davey et al., 2011; Tamassia et al., 2013). Furthermore, Tamassia and colleagues clarified this aspect showing that, unlike monocytes or the murine counterpart, human neutrophils could not switch on the IL-10 gene because of the inactive state of the locus (Tamassia et al., 2013).

3.5. Neutrophil Extracellular Traps (NETs)

During infections, neutrophils migrate from peripheral blood to tissues in response to several chemotactic stimuli released within the inflammatory site (Pittman and Kubes, 2013). They can rapidly kill pathogens following phagocytosis, but also through the release of their potent antimicrobial arsenal, which includes granular enzymes and proteins, oxidants (Reactive Oxygen Species: ROS) (Carnevale et al., 2020; Lehman and Segal, 2020) as well as NETs (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012; Yousefi et al., 2009). NETs are structures composed by a DNA scaffold with associated granule proteins that are extruded by neutrophils in a process sometimes termed NETosis (Brinkmann et al., 2004; Martinelli et al., 2004b), although cell death is not necessarily required for NET formation (Yousefi et al., 2009; Yousefi et al., 2019).

The process of NET formation is an alternative type of death, distinct from necrosis or apoptosis and is called *NETosis* (Fuchs et al., 2007). NETs formation is a complex mechanism involving specific molecular pathways and morphological changes and it is not completely understood. NETosis requires chromatin decondensation, which involves the generation of ROS, the translocation of neutrophil elastase (NE) from granules to nucleus, the citrullination of histones by Peptidylarginine Deaminase 4 (PAD4) and the induction of neutrophil autophagy (Wang and Wang, 2013). Accordingly, PAD4 deficient mice are not able to form NETs and display a significantly impaired antimicrobial activity (Loges et al., 2010). NET formation is also related to oxygen peroxide produced by NADPH oxidase,

further converted by MPO. Indeed, neutrophils deficient in MPO or NAPDH (such as in Chronic Granulomatous Disease - CGD - patients) display reduced NET production, likely responsible for the higher susceptibility to infections observed in CGD (Bianchi et al., 2009; Metzler et al., 2011).

The NETosis process is tightly regulated. Indeed, the activation of this process relies on the competition of two cellular processes for neutrophil elastase (NE): phagolysosome homing or nuclear translocation. Small microbes are taken up in the phagosome and activate phagocytosis. Phagocytosis involves the fusion of azurophilic granules to the phagosome, and therefore the sequestration of NE within the phagolysosome away from the nucleus. In contrast, when microbes are too large to be phagocytosed, NE is released from the azurophilic granules to the nucleus (Branzk et al., 2014). Once in the nucleus, NE cleaves histones, decondenses chromatin and drives NET formation (Papayannopoulos et al., 2010). According to these findings, the size of the microbes is the factor that determines the need of NET formation. Since a number of pathogens have evolved virulence strategies to circumvent phagocytosis, NET formation can also be viewed as an immune response to overcome these resistance strategies (Branzk et al., 2014).

Microbes have developed a number of tools to escape NET trapping too and, then, to enhance their virulence. For example, *Streptococcus pyogenes* and *Streptococcus pneumoniae* express DNAse-1 which disrupt NETs (Beiter et al., 2006; Buchanan et al., 2006). In addition, some strains of *Pseudomonas aeruginosa* have developed resistance to NET-mediated killing in fibrosis cystic patients airways (Young et al., 2011). Moreover, *Leishmania donovani* presents some cell wall structures (lipophosphoglycan) which confers resistance to NETs-mediated killing, even though NETs still contribute to the containment of the parasites (Gabriel et al., 2010).

Similarly to other neutrophil related molecules, NETs can also be dangerous for the host. Excessive and deregulated NET formation is responsible for various pathologic conditions,

such as autoimmunity, sepsis and thrombosis. For example, a subset of SLE patients has been identified with an impaired DNAse-1 activity. This defect was due to the presence of DNAse-1 inhibitors or to antibodies that bind to NETs and protect them from DNAse-1. In these patients, persistence of NETs, anti-NETs antibodies, anti-dsDNA titers and lupus nephritis were strongly correlated. It was proposed that anti-NETs antibodies and persistent NETs could form "NET immunocomplexes", which are relevant in the exacerbation of SLE and could be pathogenic in the development of lupus nephritis (Hakkim et al., 2010). Indeed, clinically, flares of autoimmune diseases are often associated with bacterial infections and may reflect the consequences of acute activation of neutrophils and the generation of more NETs and antigens (Zandman-Goddard and Shoenfeld, 2005). In addition, NETs were recently shown to activate platelets and induce thrombosis. Indeed, NETs provide a scaffold for platelet and red blood cell adhesion and aggregation (Fuchs et al., 2010). In a murine model of deep venous thrombosis, neutrophils were necessaries for activation of factor XII and propagation of the thrombosis cascade (von Bruhl et al., 2012). In addition, NET components such as DNA, histones and proteases retain intrinsic pro-coagulant activities. For instance, genomic DNA potentiated proteolytic activity of coagulation factors (Kannemeier et al., 2007). Moreover, histones contained in NETs are cytotoxic for endothelium and can be responsible for thrombosis *in vivo*, as observed in animal model of sepsis (Xu et al., 2009).

During the last years, several experimental and clinical studies have reported the association between tumor initiation and progression, cancer-associated thrombosis and NETs (Al-Haidari et al., 2019; Arelaki et al., 2016; Boone et al., 2018; Cools-Lartigue et al., 2013; Demers et al., 2012; Hisada et al., 2020; Pieterse et al., 2017; Tohme et al., 2016).

In 1996 a pivotal study described a novel form of neutrophil death, different from apoptosis and necrosis, which was induced by the phorbol 12-myristate 13-acetate (PMA) (Takei et al., 1996). PMA caused a peculiar sequence of neutrophil morphological changes, including the loss of chromatin compactness and the fusion of the nuclear envelope with the cytoplasmic

organelles. After 3 hours of incubation with PMA, DNA degradation products were released from PMNs and the process, leading to cell death, was dependent on ROS production (Takei et al., 1996). Beyond PMA, additional agonists responsible for NET release were found such as IL-8/CXCL8, IFN-γ/IFN- γ/C5a, GM-CSF/C5a, and LPS (Amini et al., 2018; Brinkmann et al., 2004; Caielli et al., 2016; Martinelli et al., 2004a; Yousefi et al., 2009). A variety of stimuli can additionally induce NET formation: several bacteria (Brinkmann et al., 2004), fungi (Urban et al., 2006), viruses (Raftery et al., 2014), anti-neutrophil cytoplasmatic antibodies (Ben-Smith et al., 2001), activated platelets (Clark et al., 2007) and calcium ionophores (Fuchs et al., 2007; Parker et al., 2012). NETs are classically detected within 2–4 h after neutrophil activation because this phenomenon is believed to require cell death (Douda et al., 2014; Fuchs et al., 2007; Keshari et al., 2013; Remijsen et al., 2011; Yoo et al., 2014), although earlier time points were often not analyzed. This form of NET release was defined as "suicidal" NETosis, which describes the release of NETs in association with a special type of cell death that is different from apoptosis and necrosis. NETosis was initially considered to be NADPH-oxidase (Nox)-dependent (Brinkmann et al., 2004; Remijsen et al., 2011). Recently, a Nox-independent NET formation was also described, associated with calcium influx and mitochondrial ROS production (Ravindran et al., 2019). During suicidal NETosis, PMA (Brinkmann et al., 2004; Takei et al., 1996) binds to protein kinase C (PKC), which induces the release of calcium from intracellular stores leading to Raf-MEK-ERK pathway activation (Hakkim et al., 2011), as well as to the ROS-dependent activation of p38 MAPK (Keshari et al., 2013). Myeloperoxidase (MPO) and neutrophil elastase (NE) are released from the azurophil granules (Metzler et al., 2014) and translocate into the nucleus (Papayannopoulos et al., 2010). The increase of intracellular calcium levels activates the PAD4. Once translocated in the nucleus, PAD4 catalyzes the deamination of histones H2A, H3 and H4, converting the arginine residues into citrullines. As a result, histones lose their positive charge and chromatin loses its compaction (Neeli et al., 2009; Wang et al., 2009). As a consequence, nuclear and

mitochondrial membranes lose their integrity, the cytoplasm and karyoplasm content are mixed together and the cellular membrane disintegrates and the intracellular content is released in the extracellular space (Brinkmann, 2018). As a final result of this activation pathway, the neutrophil dies.

Different groups of investigators have described a "vital" form of NET formation, in which the intracellular content is released in the extracellular space but the neutrophil remains alive (Amini et al., 2018; Cristinziano et al., 2020; Jorch and Kubes, 2017; Pilsczek et al., 2010; Stojkov et al., 2017; Yipp and Kubes, 2013; Yousefi et al., 2009). Vital NET release was first described under the granulocyte-macrophage colony stimulating factor (GM-CSF) priming with subsequent stimulation with C5a or LPS. NET formation occurred within few minutes of stimulation and was associated with the release of mitochondrial DNA (Yousefi et al., 2009). The vital NET release occurred also within few minutes of bacterial stimulation with *Staphylococcus aureus* (Pilsczek et al., 2010).

In vital NET formation, the multilobed nucleus was rapidly rounded and condensed. The separation of the inner and outer nuclear membranes and budding of vesicles occur, with the vesicles filled with nuclear DNA. The vesicles are extruded intact into the extracellular space where they are rupture, and the chromatin is released. This entire process occurs *via* a unique, very rapid (5–60 min), oxidant-independent mechanism (Pilsczek et al., 2010). In an elegant *in vivo* model of *Staphylococcus aureus* skin infection, unique patterns of crawling and cell morphology were observed during NET formation. In this model, neutrophils release NETs during crawling and develop diffuse decondensed nuclei becoming devoid of DNA. A combined requirement of Toll like receptor 2 (TLR2)- and complement-mediated opsonization tightly regulated NET release. Anucleated neutrophils were still alive and capable of phagocytosis (Sendo et al., 1996; Yipp and Kubes, 2013; Yipp et al., 2012). Vital NET formation is independent of oxidant production (Jorch and Kubes, 2017). In contrast, vital NET formation as a NADPH oxidase, cytoskeleton, and glycolysis-dependent

mechanism was described (Amini et al., 2018; Stojkov et al., 2017; Yousefi et al., 2009). Importantly, in the context of cancer, we found that soluble mediators derived from anaplastic thyroid cancer (ATC) cell lines induced mitochondrial ROS activation and vital release of NETs containing mitochondrial DNA (Cristinziano et al., 2020). We also demonstrated that ATC-induced NETs promoted *in vitro* the viability of cancer cells, suggesting an important tumor-promoting role of NETs in ATC.

3.6. NETs and neutrophils in tumor progression

NETs have been described in several experimental and human cancer types. NETs were reported to drive endothelial-to-mesenchymal transition (EMT) (Pieterse et al., 2017), which plays an important role in tumorigenesis (Visciano et al., 2015). NETs facilitate experimental ovarian (Lee et al., 2019) and pancreatic cancer (Boone et al., 2015). In murine models of 4T1-induced breast cancer and Lewis lung carcinoma, increased plasma NET levels were found in tumor bearing mice compared to control mice. In tumor-bearing mice, neutrophils displayed an increased susceptibility to spontaneously produce NETs, which correlated with thrombi formation and a pro-coagulation state (Demers et al., 2012; Park et al., 2016). In an *in vivo* model of orthotopic breast cancer, neoplastic cells stimulated NET formation, which increased breast cancer cells motility and promoted lung metastasis (Park et al., 2016). Low-density neutrophils (LDNs) released NETs and promoted intestinal tumorigenesis (Guglietta et al., 2016). In a murine model of orthotopic pancreatic adenocarcinoma, genetic deletion of PAD4 reduced circulating NET levels, decreased tumor growth and improved survival in tumor-bearing mice. NETs activated pancreatic stellate cells, which promoted tumor proliferation (Miller-Ocuin et al., 2019). NET dismantling reduced stromal activation and tumor growth (Miller-Ocuin et al., 2019). In human diffuse large B cell lymphomas, intratumoral and circulating NETs correlated with a worse patient prognosis. Moreover, NETs

promoted tumor cell proliferation and migration *in vivo* and *in vitro* (Nie et al., 2019). In patients with metastatic colorectal cancer undergoing major liver resection, high circulating NET markers were associated with a high risk of recurrence (Tohme et al., 2016). Recently, it has been shown that IL-8/CXCL8 mediates a positive loop connecting NET formation and colorectal cancer liver metastasis (Yang et al., 2020a).

Tumor cells can prime neutrophils to form NETs (Erpenbeck and Schon, 2017). IL-8/CXCL8, autocrinously produced by several human cancer cell lines (Bates et al., 2018; Idorn et al., 2018; Niu et al., 2018), can induce NET formation from neutrophils and granulocytic myeloid derived suppressor cells (PMN-MDSCs) (Alfaro et al., 2016; An et al., 2019; Cristinziano et al., 2020; Najmeh et al., 2015; Nie et al., 2019). Beyond IL-8/CXCL8, several cancer-related stimuli (CXCR1/CXCR2-ligands, G-CSF, TGF-β) can induce the release of NETs from human and murine neutrophils (Alfaro et al., 2016; Azevedo et al., 2018; Bertini et al., 2004; Teijeira et al., 2020; Weiss and Kretschmer, 2018), thus linking cancer and NET release.

Moreover, NETs predict improved survival in patients with head and neck squamous cell carcinoma (Millrud et al., 2017) and exert cytotoxic effects on melanoma cells (Schedel et al., 2020). Whether changes in quality and quantity of NETs play pro- or anti-tumorigenic effects in different types and in different stages of cancers requires further investigations.

The vast majority of cancer-related death is due to metastatic disease. Metastatic cancer cells can remain as dormant tumor cells for prolonged periods of time, likely due to the balance between survival/proliferation and cell death caused by insufficient angiogenic capacity or by protective effects of immune cells (Malladi et al., 2016; Sosa et al., 2014). The stimuli that alter the balance between dormant and awaken cancer cells are largely unknown. There is some evidence that low-grade inflammation may play a role in the switch between dormancy and proliferation of metastatic cells (Barkan et al., 2010; Pierce et al., 2009). Albrengues and collaborators have demonstrated that repeated nasal instillation of LPS into

mice bearing dormant cancer cells in the lung caused marked neutrophil recruitment and activation (Albrengues et al., 2018). Neutrophil depletion blocked LPS-induced awakening of dormant cancer cells. LPS instillation caused rapid (4 hours) and persistent (24 hours) NET presence in the lung. Blockade (PAD4 inhibitor) o digestion of DNA prevented or decreased LPS-induced NET formation and awakening of dormant cancer cells. These findings were extended and confirmed in a prostate cancer model and when tobacco smoke was used as a pro-inflammatory stimulus. These results indicate that NETs formed under different inflammatory stimuli induced awakening of dormant cancer cells in multiple mouse models. The authors also found that NET-associated NE and matrix metallopeptidase 9 (MMP9) were required for awakening from dormancy through ECM remodeling. In particular, both NET-associated proteases (NE and MPM9) were necessary to cleave laminin and to induce cancer cell awakening. These findings indicate that the proteolytical remodeling of laminin leads to the emergence of new epitope in laminin that is sensed by $\alpha 3\beta 1$ integrin to promote cancer cell proliferation. An antibody (Ab28) that recognized the epitope on NET-remodeled laminin prevented the awakening of dormant cancer cells following LPS administration or tobacco smoke exposure. The results of this study have translational implications for the prevention/treatment of the metastatic cascade. Inhibition of awakening of cancer cells by NE and MMP9 suggested that they could represent therapeutic targets. PAD4 inhibitors (GSK484) and DNase I also inhibited cancer cell awakening. Importantly, Ab28 represented a novel and innovative strategy to prevent the awakening of dormant cancer cells.

A series of elegant experiments demonstrated that CXCR1 and CXCR2 chemokine receptor agonists produced by tumor cells induced NET formation which shielded tumor cells against NK- and T cell-mediated cytotoxicity (Teijeira et al., 2020). The authors first demonstrated that supernatants from different primary melanoma cell lines and the colon carcinoma cell line HT29 induced NET formation in neutrophils from healthy volunteers and in polymorphonuclear MDSC (PMN-MDSC) from cancer patients. Reparixin, a CXCR1 and

CXCR2 antagonist, or a CXCR1 blocking mAb, inhibited conditioned medium-induced NET release. Similarly, spleen PMN-MDSC from 4T1-bearing mice also underwent NET formation in response to CXCR1/CXCR2 chemokines. To demonstrate the presence of NETs in 4T1 tumors, H3 histone co-localized with dsDNA in neutrophil-positive areas within cancers. The *in vivo* presence of NETs was reduced by treatment with reparixin. Similar results were confirmed in the Lewis lung carcinoma experimental model. The authors also found that co-coltures of activated neutrophils with cytotoxic lymphocytes (CTL) or NK cells induced NET release, which impaired cytotoxicity by shielding tumor cells. Time-lapse confocal microscopy demonstrated that NETs-coating of tumor cells prevented cancer cells from entering in contact with cytotoxic immune cells. These findings were extended *in vivo* by intravital microscopy showing that NETs impaired cytotoxic cell contact with tumor cells in subcutaneous cancers. This study highlighted a novel mechanism by which NETs can coat and shield tumor cells, thus preventing cytotoxicity mediated by $CD8^+$T cells and NK cells. The mechanism of action of NETs could extend beyond physical shielding of cancer cells and include the effect of neutrophil-derived mediators detrimental to CTL and/or NK cells. The results of this study have also translational implications. In fact, inhibitors of NET formation (GSK484), DNase I or reparixin (CXCR1/CXCR2 antagonist) all inhibited tumor growth and the formation of metastasis. Interestingly, pharmacologic inhibition of NET formation synergized with combined immunotherapy (anti-PD-1 and anti-CTLA-4 mAbs) of tumors.

NETs display strong adhesive properties, which enable them to bind pathogens as well as platelets. It has been speculated that they provide intravascular support favoring tumor cell adhesion and leading to hematogenous metastasis (Erpenbeck and Schon, 2017). Due to their web-like structure and their stickiness, NETs can capture circulating tumor cells (CTCs) and carry them abroad in the circulation, thus favoring the metastatic process. Systemic inflammation significantly increased the adhesion of CTCs to hepatic sinusoids, thus favoring distant micrometastases (McDonald et al., 2009). In a murine model of orthotopic breast

cancer, even in absence of systemic inflammation, tumors induced by metastatic cell lines recruited higher proportions of neutrophils at the tumor site, compared to tumors induced by non-metastatic cell lines (Park et al., 2016). In this model, intravenous administration of the metastatic cell line gave rise to NET deposition in the lungs, favoring the metastatic niche formation (Park et al., 2016).

Yang and collaborators have highlighted a novel mechanism by which neutrophil DNA and NETs promoted cancer metastasis (Yang et al., 2020b). First, they found that MPO and H3Cit were present in primary tumors and liver metastatic lesions of a large cohort of breast cancer patients. The DNA component of NETs in the liver was chemotactic for cancer cells by interacting with the coiled-coil domain containing protein 25 (CCDC25), a transmembrane protein expressed on cancer cells. DNA-CCDC25 interactions triggered an intracellular signaling cascade promoting directional migration of cancer cells and the formation of metastasis. In mouse models, the *in vivo* administration of a neutralizing antibody anti-CCDC25 reduced the formation of NET-mediated metastases, suggesting that targeting CCDC25 could be a novel therapeutic strategy for the prevention of metastasis.

Beyond these contact-dependent mechanisms, NETs can favor metastasis through additional mechanisms, such as endothelial damage (Snoderly et al., 2019). Circulating NETs rapidly altered endothelial cells (ECs) contacts and induced vascular leakage. Indeed, NET-associated NE induced the proteolysis of the intercellular junction protein VE-cadherin thus increasing EC permeability (Pieterse et al., 2017).

Angiogenesis, the growth of new blood vessels from pre-existing ones, occurs physiologically during embryonic and postnatal development, and during inflammatory diseases (Cristinziano et al., 2021; Marone et al., 2020; Varricchi et al., 2018) and tumor growth (Cristinziano et al., 2020; Sammarco et al., 2019). Angiogenesis relies on the production of vascular endothelial growth factors (VEGFs), the most potent pro-angiogenic molecules (Staiano et al., 2016). The angiopoietin (ANGPT) family is another group of

important angiogenic factors, whose functions are mediated through two tyrosine kinase receptors, Tie1 and Tie2 on ECs (Varricchi et al., 2020). The first evidence that NETs can promote angiogenesis was provided by Aldabbous and collaborators (Aldabbous et al., 2016). They found that NETs induced by prolonged incubation of human PMNs with PMA provoked angiogenesis in a 2-dimensional matrigel tube formation assay and in a 3-dimensional spheroid sprouting assay.

Both ANGPT1 and ANGPT2 induced the synthesis of PAF from human neutrophils (Maliba et al., 2006). Only ANGPT1 induced the release of cytokines (e.g., IL-1β, IL-8/CXCL8) from human neutrophils (Haddad and Sirois, 2014; Neagoe et al., 2012). Incubation (3 hours) of human PMNs with both ANGPT1/2 increased NET formation (Lavoie et al., 2018). In this study, PAF promoted NET formation, which was inhibited by a PAF receptor antagonist. NET induced by ANGPT1/2 or PMA increased capillary-like tube length, the number of loops and tubule area. Collectively, these studies indicate that both ANGPTs can promote NET formation, which exert pro-angiogenic activities.

4. Thyroid cancer

Thyroid cancer (TC) is the most common endocrine malignancy and in 50-70% of the cases thyroid cancer derives from a thyroid nodule. Thyroid neoplasms are usually classified in three types according to their histology: the well differentiated follicular and papillary (PTC), poorly differentiated (PDTC) and undifferentiated thyroid carcinomas or anaplastic thyroid cancer (ATC). Risk factors for TC include ionizing radiations (the most common and consolidate responsible for DTC), chronic inflammatory conditions such as obesity and ingestion of some nutrients such as iodine (Ward, 2014), (Cunha et al., 2014).

TC initiation has been linked to mutations in the mitogen-activated protein kinase (MAPK) pathway, which lead to a constitutive activation of MAPK effectors, resulting in a non-

controlled and abnormal TC cells proliferation (Riesco-Eizaguirre and Santisteban, 2007). BRAF mutations are the most frequent genetic alterations in PTC, occurring in 45% of cases. In the majority of cases, BRAF mutations consists in the substitution of valine for glutamic acid at position 600 of the protein (V600E) (Riesco-Eizaguirre and Santisteban, 2007). In other cases, we can have transversion at gene position 601 (K601E), fusion with the A-kinase anchor protein 9 (AKAP9) gene (Ciampi et al., 2005) and small in – frame insertions and deletions around codon 600. RET/PTC rearrangement has been considered a genetic hallmark of PTC, even if its presence was found in few cases. Only in a subset of PTCs RAS mutations were found (Kimura et al., 2003). Point mutations of the RAS gene frequently occur in FTC. These mutations interest three RAS genes: H-RAS, K-RAS and N-RAS and have been found in 12, 13, or 61 codons. A specific molecular marker of follicular carcinomas is the PAX8-PPARγ gene fusion, which has been found in a high percentage of cases(Nikiforova et al., 2003). In FTC, mutations in PTEN and PIK3CA have also been described.

ATC is the most aggressive type of thyroid cancer. Mutations of p53 are commonly found in ATC while BRAF mutations interest only 10% of cases. In addition, β-catenin mutations have also been detected (Garcia-Rostan et al., 1999).

5. The immune system in thyroid cancer

The involvement of immune response in differentiated thyroid carcinomas (DTC) has long been demonstrated. Thyroid tumor consists of thyroid follicular cells and immune cells which interact each others in TME. They both release cytokines and chemokines necessary to tumor progression. For example, IL-1 promotes tumor growth and angiogenesis through the induction of VEGF, CXCL8/IL-8. IL-10 increases the expression of anti-apoptotic proteins (Todaro et al., 2006), IL-4 or IL-13 promote macrophage M2 polarization and TGF-β promotes regulatory T cell (Treg) expansion. Chemokines (e.g. CXCL1, CXCL8, CXCL9,

CXCL10) are shown to be involved in inducing chemotaxis, angiogenesis and lymphangiogenesis in TC (Bosisio et al., 2014). The TME is strongly supported by M2 macrophages. In fact, their presence correlates with lymph-node metastasis, larger tumor size and reduced survival (Jung et al., 2015). Several studies demonstrated that TAM depletion reduce tumor growth. Dendritic cells (DCs) in TC TME display an immature phenotype with an impaired antigen presentation capability. Mast cells (MC) are present in FTC at high density, their chemotaxis is mediated by VEGF-A and they promote TC cell proliferation through CXCL1/GROα and CXCL10 release. TC development is sustained also by NK and Treg cells. Usually, NK cells play important roles in tumor immunosurveillance but in TC they contribute to tumor development because of their reduced number in tumor tissues probably due to Treg immune suppressive activities. Indeed, Tregs control NK cell activation and suppress their immune response (Ghiringhelli et al., 2006). Indeed, there is an inverse correlation of NK cells infiltration with tumor stage (Gogali et al., 2012). About TANs, it has been proposed that the ratio between peripheral blood neutrophil and lymphocyte count (Neutrophil to lymphocyte ratio-NLR) is associated with tumor progression. Indeed, in TC patients a correlation between higher NLR and a larger tumor size was found (Galdiero et al., 2016b). PMNs infiltrated human TC and correlated with tumor size, supporting the potential tumor-promoting role of TANs in TC (Galdiero et al., 2018a).

MATERIALS AND METHODS

Cell cultures and preparation of tumor-conditioned media

Human thyroid tumor cell lines TPC1 (PTC) and 8505c (ATC) were purchased from American Type Culture Collection, cultured and maintained in DMEM supplemented with 10% heat-inactivated FCS (endotoxin level < 0.1 endotoxin units/ml), 50 U/ml penicillin/streptomycin, and 2 mM L-glutamine (EuroClone, Milan, Italy) at 37˚C in a humidified atmosphere (5% CO2 and 95% air). Normal thyroid (NT) cells provided by Prof. Alessandro Antonelli from University of Pisa (Antonelli et al., 2010) were cultured and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (endotoxin level , 0.1 endotoxin units/ml), 50 U/ml penicillin/streptomycin, and 2 mM L-glutamine (EuroClone) at 37˚C in a humidified atmosphere (5% CO2 and 95% air) (Antonelli et al., 2010). CM were prepared as previously described (Galdiero et al., 2018a). Cells were seeded at 10–20% confluence in tissue culture plates. Once the cells reached a confluence of 85–90%, the cell culture medium was replaced with a fresh serum-free medium. After 24 h, this CM was harvested, filtered (0.20-mm-pore-size filter), and stored at -20˚C. All cell lines were routinely examined for Mycoplasma contamination.

Neutrophil purification and culture

The study protocol involving the use of human blood cells was approved by the Ethics Committee of the University of Naples Federico II (n. 301/12), and written informed consent was obtained from blood donors according to the principles expressed in the Declaration of Helsinki. Granulocytes were isolated from buffy coats of healthy donors ($HBsAg^{-}$, HCV^{-}, and HIV^{-}) obtained from a leukapheresis unit. Leukocytes were separated from erythrocytes by dextran sedimentation. Neutrophils were purified by Ficoll-Paque Histopaque®-1077

(Sigma Aldrich, Milan, Italy) density gradient centrifugation (400 × *g* for 30 minutes at 22°C), followed by Percoll (Sigma Aldrich, Milan, Italy) (65%) density gradient centrifugation (660 × *g* for 20 minutes at 22°C), as previously described (Muzio et al., 1994). Finally, neutrophils were isolated from granulocytes (to reach >99% purity) by positive elimination of all contaminating cells using the EasySep Neutrophil Enrichment Kit (StemCell Technologies, Vancouver, Canada)(Calzetti et al., 2017).

These cells were >99% neutrophils as evaluated by flow-cytometric analysis with the following antibodies: anti-CD3, anti-CD14, anti-CD15, anti-CD11b, anti-CD193 (Miltenyi Biotec, Germany), anti-CD62L (L-Selectin) (BD Biosciences, USA), and anti-CD66b (Biolegend, CA, USA). Samples were analyzed on the MACSQuant Analyzer 10 (Miltenyi Biotec, Germany) and in the FlowJo software, v.10. Doublets and debris were excluded from the analysis. Data were expressed as a percentage of positive cells or median fluorescence intensity. Spontaneous activation of neutrophils was evaluated by analyzing CD11b and L-selectin expression by flow-cytometric analysis before and after neutrophil purification; only L-selectin^{+}CD11b^{low} (non activated) neutrophils were chosen for the study (data not shown).

Fluorescence, time-lapse, and high-content microscopy

Microscopy experiments were conducted using the Operetta High-Content Imaging System (PerkinElmer), as previously described (Borriello et al., 2016; Gobert et al., 2009; Gupta et al., 2018). Neutrophils were cultured in 96-well black CellCarrier plates (PerkinElmer) in an 8505c, TPC1, or NT CM, in the presence or absence of 0.5 μM of the cell impermeant SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific) at a controlled temperature (37˚C) and CO_2 concentration (5%). Nuclei were stained with the bisbenzimide DNA dye Hoechst 33342 (Thermo Fisher Scientific). For time-lapse experiments, neutrophils were cultured for up to 60 min. Within this time period, fluorescence microscopy images of three

fields per well were captured every 10 min through a 10x objective. PhenoLOGIC (PerkinElmer) was used for image segmentation and for calculating single-cell results using the dedicated analysis sequence. Cells were identified based on nuclei staining, and NETing cells were identified by their green cloudy appearance. In selected experiments, neutrophils were pretreated with 10 μM diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, or 10 mM N-acetylcysteine (NAC), an ROS scavenger, for 30 min. In specific additional experiments, neutrophils were incubated with 8505c CM with 10 μg/ml the mouse monoclonal anti-CXCL8/IL-8 and/or anti–GM-CSF blocking Ab (clone 6217 and clone 3209, respectively; R&D Systems) or the corresponding control isotype (R&D Systems) at 37˚C and 5% CO2 for 60 min. Following this, the percentage of cells (a) that had produced NETs (i.e., cells surrounded by the green cloudy emission detected in the FITC channel) over total cells (b) was calculated using the following formula: (a/b) x 100.

Apoptosis assay

Purified neutrophils (2×10^6 cells/ml) were cultured in a 8505c, TPC1 or NT CM for 60 minutes. Neutrophils were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) according to the protocol provided by the manufacturer (Miltenyi Biotec, Germany). Quantification was performed on a MACS Quant flow cytometer (Miltenyi Biotec, Germany). Live cells were assumed to be double-negative annexin V^-PI^- cells. Analysis was performed by means of FlowJo v.10.

Detection of mitochondrial ROS production using flow cytometry

Highly purifed (>99%) neutrophils were kept in RPMI with 10% of FCS for 30 min at+37 C. For the detection of mitochondrial ROS, highly purified primary neutrophils were kept in

RPMI 1640 with 10% of FCS for 60 minutes, washed in phosphate buffered solution (PBS) and stimulated with a 8505c, TPC1 or NT CM for 120 minutes. For each time point, $5x10^5$ cells per sample were incubated with 5 μM MitoSOX Red (Thermo Fisher Scientific, USA) for 30 min at 37°C, according to the manufacturer's instructions. Finally, cells were washed with PBS, acquired on the MACS Quant Analyzer 10 (Miltenyi Biotec) and analyzed in FlowJo v.10. Doublets and debris (identifed based on forward and side scatter properties), dead cells (identifed with Zombie Violet Fixable Viability Kit; BioLegend) and eosinophils (identifed based on the $CCR3^+$ exclusion gate) were excluded from the analysis. Flow cytometric analysis with the following antibodies: FITC-conjugated anti-human CD14 (clone Tuk4, dilution 1:50, from Miltenyi Biotec), VioBlue-conjugated anti-human CD15 (clone VIMC6, dilution 1:10; from Miltenyi Biotec), PE-conjugated anti-human CCR3 (clone 5E8, dilution 1:10; from BioLegend), allophycocyanin-conjugated anti-human CD66b (clone G10F5, dilution 1:20; from BioLegend), allophycocyaninconjugated anti-human CD11b (clone ICRF44, dilution 1:50; from eBioscience), and FITC-conjugated anti-human CD62L (clone DREG-56, dilution 1:10; from BD Biosciences).

Mitochondrial DNA detection using fluorescence microscopy

Neutrophils were seeded on 12-mm glass coverslips (Sigma-Aldrich) and primed with 8505c, TPC1, or NT CM or a control medium (CTRL) at 37°C for 60 min. For mitochondrial DNA detection, the slides were treated with 5 μM MitoSOX Red (Thermo Fisher Scientific), following the manufacturer instructions and previously described methods (Amini et al., 2018; Morshed et al., 2014). Cells were fixed with 4% paraformaldehyde, washed with PBS, and mounted in a drop of fluorescent mounting medium with DAPI for DNA staining (Thermo Fisher Scientific). Images of three randomly selected and noncontiguous fields were captured for each coverslip using fluorescence microscopy to accurately represent the effect

exerted by the stimuli. Axio Observer 7 with ApoTome 2.0 (Carl Zeiss MicroImaging, Jena, Germany), which allows creation of optical sections free of scattered light, was used for fluorescence microscopy. Images were digitized at 63x objective magnification. The image files were analyzed using ImageJ 1.49v software (National Institutes of Health). Extracellular MitoSOX Red fluorescence was calculated for each field, and the mean values obtained for three distinct regions were used for data analysis.

PCR

PCR DNA released from neutrophils cultured in TC CM or CTRL was purified using classical phenol, phenol/chloroform, and chloroform extraction. The source of the extracellular DNA was determined by amplifying two nuclear (GAPDH and FAS) and two mitochondrial genes (ND1 and CYTOB). Specific PCR conditions, including primers used, have previously been described (Yousefi et al., 2008).

Coculture of TC cells with neutrophils

Coculture experiments were conducted using the Operetta High-Content Imaging System (PerkinElmer). The 8505c cells (2 x 10^3 cells) cultured in DMEM supplemented with 10% FCS were plated in 96-well black CellCarrier plates (PerkinElmer) and maintained at 37˚C in a humidified atmosphere with 5% CO_2 for 24 h. The cells were then stained with CFSE according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After washing, the cells were cultured for 4 h in DMEM supplemented with 10% FBS. Neutrophils isolated from peripheral blood of healthy donors were preincubated with 8505c CM or CTRL for 60 min and then added to the 8505c culture at a 5:1 or at a 20:1 neutrophil to tumor cell ratio for 24 h. Because DNAse I completely dissolved NETs *in vitro* (Brinkmann et al., 2004), 60,000 IU/ml

DNAse I (Calbiochem, San Diego, CA) was added to the cocultures in selected experiments to evaluate whether the effect exerted by the CM-primed neutrophils was due to the release of NETs. After 24 h of coculture, fluorescence microscopic images of four fields per well were captured through a 10x objective. The PhenoLOGIC software was used for image segmentation and for calculating the single-cell results using the dedicated analysis sequence. The 8505c cells were identified based on their FITC-emitting fluorescence. The percentage of CFSE-positive area (emitted in the FITC channel) over total field area was calculated. The results were expressed as percentage compared with the control (i.e., 8505c cells alone) for each experimental condition. To evaluate the effect of NETs on tumor cell viability, neutrophils and CFSE-labeled tumor cells were stained with allophycocyanin-conjugated annexin V and PI after 24 h of coculture, according to the protocol provided by the manufacturer (BioLegend, San Diego, CA). The 8505c cells were distinguished from neutrophils and gated based on FITC fluorescence emission. Quantification was performed using the MACSQuant flow cytometer (Miltenyi Biotec). Live cells were assumed to be double-negative annexin V2PI2 cells. Analysis was performed using FlowJo v.10.

Statistical analysis

The data are expressed as mean ± SEM of the indicated number of experiments. Statistical analysis was performed in Prism 7 (GraphPad Software). Values from groups were compared by Student's t test or Mann–Whitney U test based on the parametric or nonparametric distribution of the continuous variables. Repeated measures one-way or two-way ANOVA was used were appropriated and described in the figure legends. Differences were assumed to be statistically significant when the p value was < 0.05.

RESULTS

ATC-derived CM induced NET release from highly purified human neutrophils

In the first group of experiments, we used the Operetta HighContent Imaging Screening System to evaluate the ability of TC cell lines to induce NET release from human PMNs. Highly purified PMNs (≥ 99%) from peripheral blood of healthy donors were cultured in TC CM derived from the PTC cell line TPC1 (TPC1 CM), the ATC cell line 8505c (8505c CM), NT CM cells, or a CTRL. NETting cells were analyzed using the PhenoLOGIC software with a dedicated analysis sequence up to 60 min of incubation. **Fig. 1** depicts the kinetics of NET release. The ATC CM–induced release of NETs occurred rapidly after ~40 min of stimulation TPC1 CM induced a marginal, yet insignificant, increase in NET release **(Fig. 1,** NT CM and CTRL did not induce NET release from PMNs **(Fig. 1)**. These results suggest that CM from the ATC cell line 8505c induced rapid and selective NET release from PMNs

ATC CM–induced NET release was dependent on CXCL8/IL-8 and ROS production

We investigated the mechanism underlying NET release by ATC CM induction. NET formation and release requires the production of ROS (Stojkov et al., 2017; Varricchi et al., 2017), and we have previously demonstrated that TC CM induces ROS production by PMNs (Galdiero et al., 2018a). Therefore, to evaluate whether the effect exerted by the ATC CM on NET release could be associated with the production of ROS, the PMNs were pretreated with two different ROS inhibitors, DPI (10 μM) or NAC (10 mM), for 30 min (Sim et al., 2005; Van Avondt et al., 2016). Following this, the PMNs were cultured *in vitro* in an 8505c, TPC1, or NT CM or in a CTRL medium (the latter not shown). NETting cells were analyzed for up to 60 min of incubation. Notably, both ROS inhibitors DPI and NAC completely abrogated the release of NETs induced by 8505c CM **(Fig. 2A)**, and the marginal, yet insignificant, NET

release induced by TPC1 CM was abrogated as well by ROS inhibition **(Fig. 2B)**. As expected, ROS inhibition did not exert any effect on NET production induced by NT CM **(Fig. 2C)**. These results suggest that 8505c CM induces NET release from human neutrophils in an ROS-dependent manner. In addition, to evaluate whether ROS inhibition could modulate additional features of neutrophil activation, we stained the neutrophils for detection of CD66b, CD11b, or CD62L following DPI or NAC pretreatment and TC CM priming. As we previously demonstrated (Galdiero et al., 2018a), 8505c CM significantly up-regulated CD66b and CD11b expression and down-regulated CD62L expression, confirming that soluble mediators released by 8505c induced neutrophil activation. Notably, ROS inhibition did not modify CD66b, CD11b, or CD62L expression, suggesting that ROS are not involved in these aspects of neutrophil activation. TC cell lines autocrinously produce a significant quantity of CXCL chemokines (Melillo et al., 2010; Rotondi et al., 2013) and GM-CSF (Galdiero et al., 2018a). Both CXCL8/IL-8 and GM-CSF were able to induce NET release from neutrophils and play central roles in the maintenance of the TME (Alfaro et al., 2016; Liu et al., 2016; Yousefi et al., 2009). Neutralizing Abs against CXCL8/IL-8 and/or GM-CSF were used to investigate the mechanism underlying NET release. Notably, anti-CXCL8/IL-8 blocking Ab completely inhibited the NET release induced by 8505c CM **(Fig. 3)**. When used alone, the anti–GM-CSF blocking Ab did not modify the release of NETs, and its addition did not further enhance the inhibitory effect exerted by the anti-CXCL8 blocking Ab **(Fig. 3)**. These results are compatible with the hypothesis that CXCL-8/IL-8 produced by ATC plays a major role in inducing NET release from ATC CM–stimulated neutrophils.

ATC CM–induced NET release was independent of neutrophil death

To investigate whether ATC CM–induced NET release was associated with cell death, PMN viability was determined in all the experimental conditions. PMNs were pretreated with DPI or NAC or left untreated and cultured in 8505c, TPC1, or NT CM or in a CTRL for 60

min. PMN viability was evaluated at the end of the incubation period using flow cytometry. The results illustrated in **Fig. 4A** demonstrate that the viability of PMNs was maintained in all experimental conditions. **Fig. 4B** depicts representative flow cytometry panels of one of four independent experiments. One-hour incubation did not modify PMN viability in TC CM or in NT CM culture. Moreover, pretreatment with ROS inhibitors did not influence PMN viability. These results suggest that TC CM–induced NET release is an active phenomenon independent of PMN death.

ATC CM–induced NET release was associated with mitochondrial ROS production

Because NETs released by viable neutrophils were observed to possess mitochondrial DNA (Yousefi et al., 2009), we investigated whether an 8505c, TPC1, or NT CM induced the generation of mitochondrial ROS. We observed mitochondrial ROS generation induced selectively by 8505c CM using MitoSOX Red, a triphenylphosphonium-linked dihydroethidium compound that accumulates within mitochondria and fluoresces red when oxidized by ROS. NT CM and the CTRL did not induce mitochondrial ROS generation, whereas TPC1 CM induced a marginal, yet insignificant, ROS generation **(Fig. 5A, 5D)**. Notably, 8505c CM–induced ROS generation was evident after 30 min of incubation **(Fig. 5A, 5D)** and was restored to control levels within 60 min **(Fig. 5B, 5C, 5E, 5F)**, suggesting that ATC CM–induced mitochondrial ROS generation occurs early on and precedes NET formation and release (evident from 40 min of incubation, as indicated in Fig. 1).

ATC CM induced oxidized mitochondrial DNA release

To evaluate the release of oxidized mitochondrial DNA from PMNs under TC CM priming, the cells were incubated with MitoSOX Red, fixed and mounted with DAPI, and then

visualized using an inverted fluorescence microscope. **Fig. 6** demonstrates that 8505c CM induced the release of oxidized mitochondrial DNA from PMNs **(Fig. 6A, 6B)**. TPC1 CM induced a marginal, yet insignificant, release of extracellular mitochondrial DNA, whereas NT CM did not induce extracellular mitochondrial DNA release from PMNs, similar to the effect exerted by the CTRL **(Fig. 6A, 6B)**. To improve the characterization of the origin of the extracellular DNA released by neutrophils under 8505c CM stimulation, we used PCR to investigate whether the extracellular DNA contained sequences of the mitochondrial or nuclear genome. In support of the microscopic observations, we were able to amplify two mitochondrial genes (ND1 and CYTOB) from the DNA released by 8505c CM–primed neutrophils. Compared with the effect exerted by control neutrophils, mitochondrial genes (ND1 and CYTOB) were amplified, and their expression was significantly increased by 8505c CM–primed PMNs **(Fig. 6C, lower panels)**. These results support the microscopic findings presented in **Fig. 6A, 6B**. Nuclear genes (GAPDH and FAS) were amplified in control PMNs **(Fig. 6C, upper panels)**. Compared with control PMNs, no further increase in GAPDH expression was induced by 8505c CM– stimulated PMNs, and FAS expression was suppressed in 8505c CM–stimulated PMNs.

Neutrophils promoted *in vitro* growth of ATC cells in a NET-dependent manner

Based on the findings stated above, we investigated the functional role of NETs in TC progression through *in vitro* cocultures using two different experimental approaches. PMNs were incubated (primed) for 60 min with 8505c CM or CTRL to allow their activation and subsequent NET release. PMNs were then added to CFSE-labeled 8505c cells at a 5:1 or at a 20:1 neutrophil to tumor cell ratio and maintained for 24 h. Taking into account the high number of circulating PMNs, the coculture ratios used in these experiments are possibly an underestimation of the actual *in vivo* ratios (Granot et al., 2011). In the first group of

experiments, 8505c cell proliferation was evaluated by live-cell imaging. Compared with 8505c cells without neutrophils (used as controls), the addition of PMNs primed with 8505c CM (PMN 8505c CM) to the cell culture at a 20:1 ratio increased tumor cell confluency; the addition of PMNs primed with CTRL (PMN CTRL) did not significantly increase the tumor cell confluency either **(Fig. 7A)**. Dissolving NETs with DNAse I completely reverted this effect **(Fig. 7A)**, suggesting that the enhancement of 8505c cell growth induced by CM-primed PMNs is dependent on NET production. Notably, the addition of PMNs to the 8505c cell culture at a 5:1 ratio did not influence 8505c cell growth **(Fig. 7A)**. These results suggest a dose-dependent effect exerted by neutrophils on ATC cell growth. In the second group of experiments, we investigated the mechanisms underlying the NET-dependent increase in tumor cell confluency by evaluating tumor cell apoptosis. We observed inhibited tumor cell apoptosis compared with 8505c cells alone (set as control), or to PMN CTRL **(Fig. 7B)**. Notably, the addition of DNAse I completely reverted this effect **(Fig. 7B)**. Cocultures of PMNs with TPC1 cells, with TPC1-CM priming or with CTRL priming, did not affect TPC1 cell growth in vitro (data not shown). Collectively, these results are compatible with the hypothesis that NET release by ATC CM–stimulated neutrophils promotes ATC cell growth by enhancing their viability.

DISCUSSION

To the best of our knowledge, this is the first study to provide evidence of rapid and selective induction of non-cytotoxic release of NETs from highly purified human PMNs by ATC CM. We also observed that ROS inhibition blocked ATC CM–induced NET release, suggesting that ATC-induced NET release occurred in an ROS-dependent manner. Although NET release can be associated with neutrophil death (Brinkmann et al., 2004) we observed that NET release induced by ATC cell line was independent of neutrophil death. ATC CM–induced NET contained mitochondrial DNA, and its release was associated with mitochondrial ROS production. Additionally, we provided evidence in support of the potential role of NETs in sustenance of *in vitro* cancer cell viability in ATC cultures. PMNs perform numerous functions in cancer-related inflammation (Cassatella et al., 2009; Galdiero et al., 2018b; Ley et al., 2018) . Under the influence of distinct stimuli derived from the TME, PMNs can be polarized toward specific phenotypes. On one hand, PMNs are able to kill tumor cells, activate antitumor T cell immunity, and inhibit angiogenesis and metastasis (Eruslanov et al., 2014; Galdiero et al., 2016a; Granot et al., 2011; Loffredo et al., 2017), whereas, on the other hand, PMNs sustain genetic instability, tumor growth, and metastatic spread (Houghton et al., 2010; Jablonska et al., 2010; Queen et al., 2005).

TC is the most frequent cancer of the endocrine system (Nikiforov and Nikiforova, 2011), and the association between chronic inflammation and TC has long been recognized (Cunha et al., 2014; Fallahi et al., 2018; Galdiero et al., 2016b). PMNs were detected in human TC samples and had positive correlation with tumor size (Galdiero et al., 2018a). PMN biology was significantly modified by soluble factors derived from human TC cells. TC CM induced PMN chemotaxis through the release of CXCL8/IL-8 and improved PMN survival through the release of GM-CSF. In addition, TC CM induced PMN activation (up-regulation of

CD11b and CD66b and shedding of CD62L) and modified PMN morphology and kinetic properties. Furthermore, TC CM induced the production of ROS, expression of proinflammatory and angiogenic stimuli, and release of MMP-9 (Galdiero et al., 2018a).

In this study, we demonstrated that release of NETs by PMNs can also be induced by TC-derived CM. Notably, compared with NT CM and CTRL, the CM derived from the PTC cell line TPC1 induced a marginal, yet insignificant, increase in the release of NETs, suggesting that ATC and PTC influence PMN biology differently. The differential effect of the two CM can be linked to the different mediators secreted by the two cell lines. The TPC1 cell line is derived from a PTC subtype and contains the RET/PTC1 gene rearrangement. The 8505c cell line is derived from an ATC subtype and contains both BRAF and TP53 mutations (Saiselet et al., 2012). The two cell lines possess several distinct biological behaviors; in an orthotopic TC model in nude mice, the 8505c cell line demonstrated a take rate of 100% and formed lung metastases (Bellelli et al., 2012; Morrison et al., 2015). The metastasis of the tumor to the lungs is particularly significant, as ATC in humans has a strong propensity for lung metastasis. As observed in an intracardiac injection metastasis model, the 8505c cell line also formed distant metastases with take rates of 70% (Morrison et al., 2015). In contrast, TPC1 cells failed to form significant tumors in the orthotopic model in immunocompromised mice (Morrison et al., 2015). TPC1 and 8505c cell lines produce different quantities of a wide spectrum of mediators. Although both cell lines can produce CXCL8/ IL-8 and TNF-α, the mediators are produced in different quantities, with production by 8505c being greater than that by TPC1 (data not shown). Therefore, it is not surprising that CM derived from ATC and PTC exert different effects on NET release from human neutrophils. To better support our observations, we evaluated NET release from human neutrophils stimulated with CM derived from primary thyroid cell cultures of papillary or anaplastic cancers (Antonelli et al., 2008; Antonelli et al., 2009) and observed that the CM derived from anaplastic primary cell cultures induced the release of NETs by human primary neutrophils. In contrast, CM derived from

PTC primary cell culture did not induce NET release (data not shown). Although rare, ATC is the most lethal TC subtype; it represents 1–2% of all TC subtypes, yet accounts for 38–50% of TC mortality (Salehian et al., 2019). ATC patients display a poor median survival (3–10 mo), possibly due to rapid growth and radio- and chemoresistance of the tumor (Sun et al., 2013). A treatment paradigm that significantly alters the course of this disease or improves outcomes remains unavailable to date (Salehian et al., 2019). To evaluate and quantitatively analyze NET release, we used a High-Content Image System, a high-throughput system that allows the uniform standardization of cell image acquisition and analyses with a dedicated software (Cortjens et al., 2016; Gupta et al., 2018). Although manual methods are employed for quantification of fluorescence analysis, they possess several limitations, as they are error prone and operator dependent. Although the majority of methods published involve use of microscopy-based automated NET quantification (Brinkmann et al., 2012; Kraaij et al., 2016; Mohanty et al., 2017), they are not fully automated and typically rely on an increase in the staining area, as the single decisive factor defining the extent of NET formation without information at the single-cell level. Automated image quantification offers significant advantages, as multiple images can be processed rapidly using standardized NET-defining parameters (Gupta et al., 2018). Therefore, an automated software based on single-cell analysis offers great advantages to researchers working in this field. In our experimental setup, we demonstrated that ATC-derived CM induced NET release from PMNs in an ROS-dependent manner. Pharmacological inhibition of ROS production significantly abrogated NET release. In a seminal paper, NETosis was described as a novel cell death process, distinct from apoptosis and necrosis and depending on ROS generation by NADPH oxidase (Fuchs et al., 2007). Notably, we observed that, despite ROS production, PMNs remain viable after NETosis. These results are consistent with increasing evidences that support the existence of an alternative pathway of NET release. In addition to the first identified cell death pathway (Fuchs et al., 2007), the alternative pathway is a vital process occurring independent of cell

death and within minutes from activation. In the latter case, the PMNs remain viable to perform further functions (e.g., phagocytosis) (Yousefi and Simon, 2016). Our findings are in line with this alternative evidence supporting vital NETosis characterized by extracellular release of NETs in absence of plasma membrane rupture (Yousefi and Simon, 2016). The last form of NETosis seems to be better suited to the role of PMNs in cancer progression, because it is important for PMNs to remain viable to perform their middle-/long-term functions in TME. GM-CSF is considered to be one of the primary mediators responsible for vital NETosis (Yousefi et al., 2009) as well as one of the supporters of neutrophil survival (Barreda et al., 2004; Hofman, 2004). CXCL8/IL-8 is also described as a chemokine that is able to induce NET release and portrays a pivotal role in cancer settings (Alfaro et al., 2016; Liu et al., 2016). Both CXCL-8/IL-8 and GM-CSF are present in the TC CM, supporting PMN chemotaxis and survival (Galdiero et al., 2018a). Notably, we observed that CXCL8/IL-8 inhibition, rather than GM-CSF blockage, significantly inhibited NET release from TC CM–primed human neutrophils. In addition, ROS inhibition did not modulate alternative features of neutrophil activation, suggesting a specific effect exerted by the ROS pathway on NET release.

Because NETs released by viable PMNs were described to contain mitochondrial DNA (Yousefi et al., 2009), we investigated this finding and observed that the 8505c CM induced mitochondrial ROS activation after 30 min of incubation and that the 8505c CM–induced NETs contained mitochondrial DNA. Based on these findings and on the time-course of NET release, one could infer that ATC CM–induced mitochondrial ROS generation is an early event that precedes NET formation and release of both nucleic and mitochondrial DNA. To better characterize the origin of the extracellular DNA released by neutrophils under 8505c CM stimulation, we amplified two mitochondrial genes (ND1 and CYTOB) from the DNA released by 8505c CM–primed neutrophils, and we observed the expression of the two mitochondrial genes was significantly up-regulated in the extracellular DNA that, in the

DNA, released by control neutrophils. Nuclear genes were also observed to be amplified; however, no differences were detected in GAPDH expression when compared with control neutrophils, and a significant down-regulation of FAS expression was evident in the extracellular DNA released by 8505c CM–primed neutrophils compared with that released by control neutrophils. These results support the hypothesis that mitochondrial DNA is released by 8505c CM–stimulated human neutrophils. Moreover, these findings suggest that, in our experimental conditions, TC CM induced the release of both mitochondrial and nucleic DNA from human neutrophils. However, it appears that mitochondrial DNA content was higher in 8505c CM–stimulated compared with control neutrophils.

In our experiments, ATC CM–induced NETs sustained the viability of the anaplastic cell line. This effect was NET specific and dependent on the integrity of the NET chromatin scaffold, because it was abrogated by DNAse I treatment. The possible role of NETs in cancer was first reported in Ewing sarcoma (Berger-Achituv et al., 2013). NETs were required for resuscitation of dormant lung cancer cells (Kargl et al., 2017), B cell lymphoma progression (Di Caro et al., 2016), and *in vitro* and *in vivo* metastatic dissemination (Al-Haidari et al., 2019; Cools-Lartigue et al., 2013; Park et al., 2016; Tohme et al., 2016). Our results are in agreement with previous findings and support the direct role of NETs in sustaining TC growth.

We previously demonstrated that density of tumor-infiltrating neutrophils within human TC samples was positively correlated with tumor size, suggesting a tumor-promoting role of neutrophils in TC (Galdiero et al., 2018a). We observed that ATC released soluble factors that could recruit neutrophils, sustain their survival, promote their activation, and enable the release of ROS and MMP-9. Additionally, we observed that ATC induced vital NETosis, and that ATC-primed neutrophils directly sustain tumor cell viability in a NET-dependent manner. Further experiments are required to provide evidence on this aspect and to elucidate the mechanisms by which ATC-induced NETs eventually sustain *in vivo* tumor cell growth.

Notably, there exist different populations of PMNs that tend to produce NETs. For example, low-density neutrophils in patients with systemic lupus erythematosus demonstrate enhanced NET formation (Villanueva et al., 2011), whereas only a fraction of PMNs from peripheral blood of healthy individuals form NETs, even when stimulated with strong agonists (Ng et al., 2019). Therefore, because our *in vitro* experiments were performed using PMNs from healthy donors, a further enhanced NET formation at the tumor site could be observed using tumor-associated neutrophils. Further experiments should be performed on a cohort of ATC and PTC specimens to detect local NET formation and correlation with patient clinic-pathological features and outcomes.

In conclusion, we demonstrated the ROS-dependent and cell death–independent vital release of NETs from human PMNs exposed to ATC-derived CM. Although NETs formed in our in vitro experiments sustained the viability of ATC cells, further studies are required to elucidate the mechanisms by which NETs contribute to TC growth and metastasis. These findings shed light on the innate immune cell population and call for a re-evaluation of their roles in context of cancer.

Figures

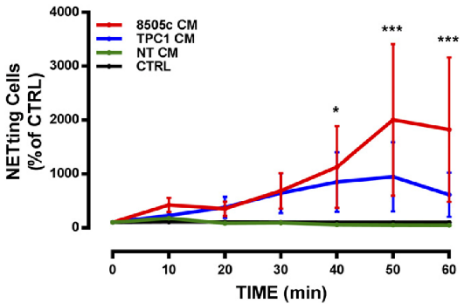

Figure 1 ATC-derived CM induced NET release from highly purified human neutrophils. Neutrophils (1 x 10^6 cells/ml) were seeded in a 96- well CellCarrier plate and cultured in an 8505c, TPC1, or NT CM or in a CTRL (37°C, up to 60 min) in the presence of the cell-impermeant SYTOX Green Nucleic Acid Stain (0.5 µM). The percentage of cells that produced NETs over total cells was calculated. Data are expressed as percentage of NETting cells relative to the CTRL (mean ± SEM of six independent experiments using six different donor samples). The control was set at 100%. *p < 0.05, ***p < 0.005.

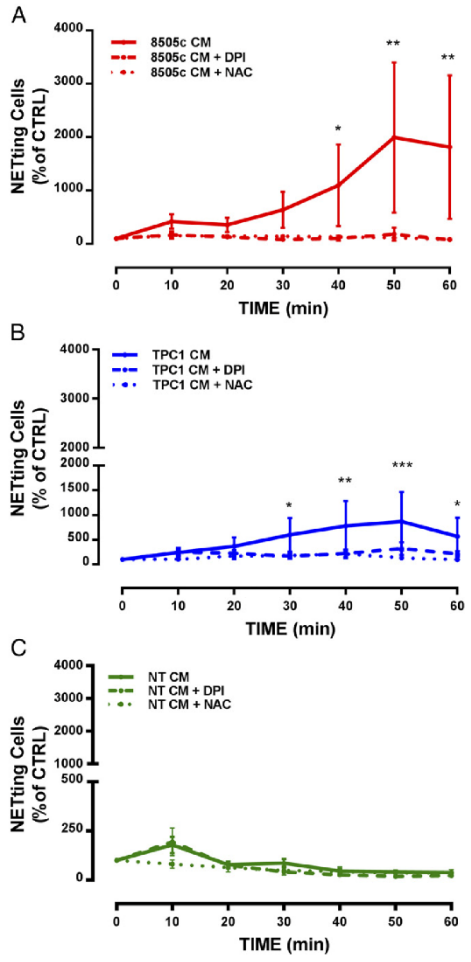

Figure 2 ATC CM–induced NET release was dependent on ROS production. Neutrophils (1 x 10^6 cells/ml) were pretreated with ROS inhibitors DPI (10 μM) (dashed line) or NAC (10 mM) (dotted line) for 30 min, or left untreated, and cultured in an 8505c **(A)**, TPC1 **(B)**, or NT **(C)** CM or in the CTRL (data not shown) in the presence of the cell impermeant SYTOX Green Nucleic Acid Stain (0.5 μM) for up to 60 min at 37°C. Data are expressed as percentage of NETting cells relative to the CTRL (mean ± SEM of six independent experiments using six different donor samples). The CTRL was set at 100%. *p < 0.05, **p<0.01, ***p<0.005.

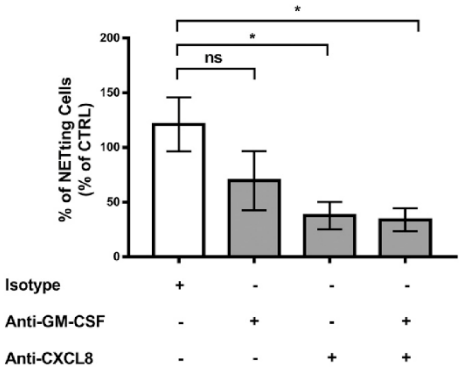

Figure 3 ATC CM–induced NET release was dependent on CXCL8/IL-8. Neutrophils (1 x 10^6 cells/ml) were cultured in an 8505c-derived CM in the presence of an anti-CXCL8/IL-8 and/or anti–GM-CSF blocking Ab or the relative isotype control (10 mg/ml) in the presence of the cell impermeant SYTOX Green Nucleic Acid Stain (0.5 μM) at 37°C. Data are expressed as percentage of NETting cells relative to the isotype CTRL (mean 6 SEM of five independent experiments using five different donor samples). *p < 0.05. ns, not significant.

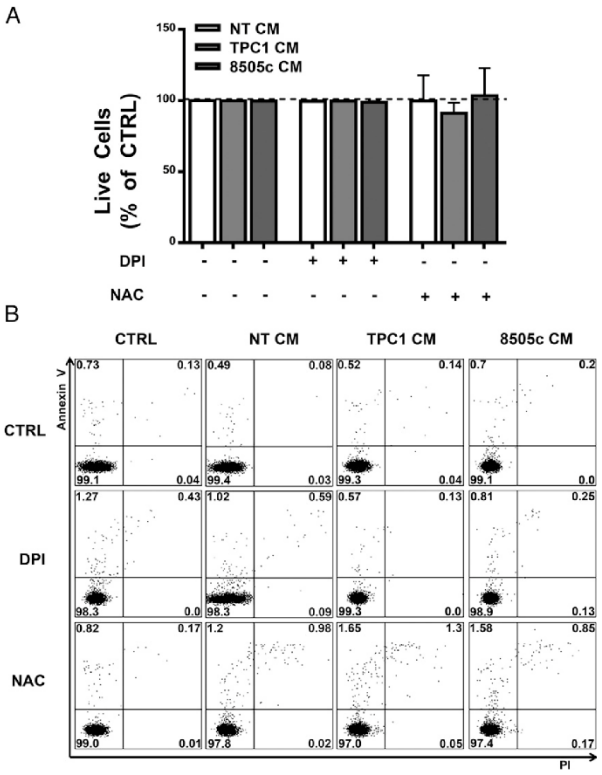

Figure 4 ATC CM–induced NET release was independent of cell death. **(A)** Live cells were evaluated by flow cytometry with FITC-conjugated annexin V and PI at 60 min of incubation. Results are expressed as percentage of live cells compared with the CTRL (the CTRL was set at 100% and indicated by the dotted line) (mean ± SEM of four independent experiments using four different donor samples). **(B)** Representative flow cytometry panels of dot plots of PMNs pretreated with CTRL (upper panels) or the ROS inhibitor DPI (middle panels) or NAC (lower panels) and cultured in an 8505c, TPC1, NT CM, or CTRL.

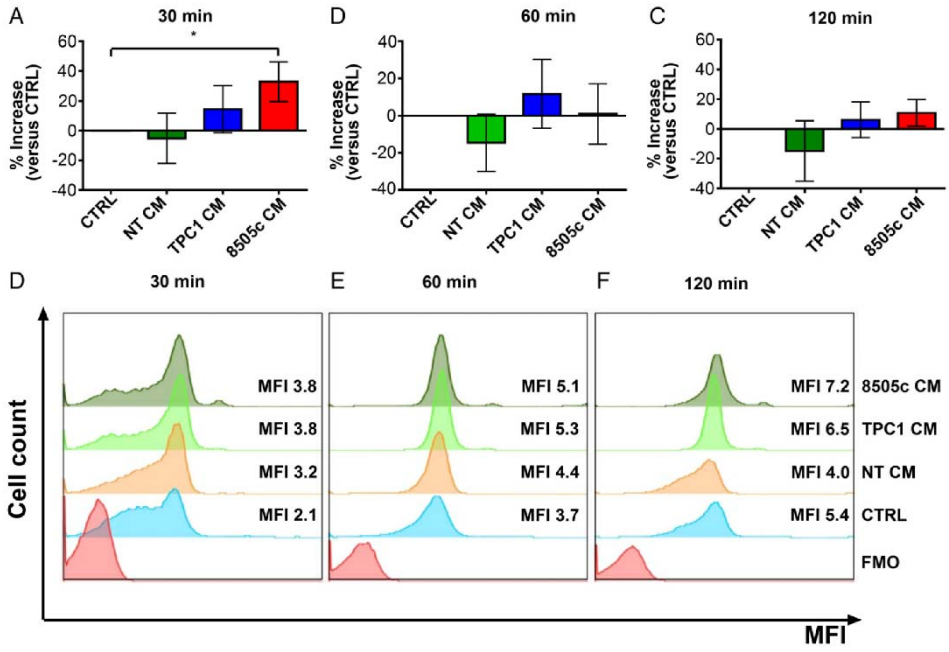

Figure 5 ATC CM–induced NET release was associated with mitochondrial ROS production. **(A–C).** PMNs (1 x 10^6 cells/ml) were primed with an 8505c, TPC1, or NT CM or with a CTRL for up to 120 min at 37°C. At each time point [30, 60, and 120 min for **(A)–(C)**, respectively], the cells were incubated with MitoSOX 5 μM for 30 min and subjected to flow cytometry analysis. The MFI was calculated. The results were expressed as percentage of the CTRL (mean ± SEM of five independent experiments using five different donor samples). **(D–F)** Representative histograms illustrating MFI and cell counts for MitoSOX Red at 30 **(D)**, 60 **(E)**, and 120 **(F)** min intervals for one of five independent experiments using five different donor samples. *p < 0.05. FMO, fluorescence minus one.

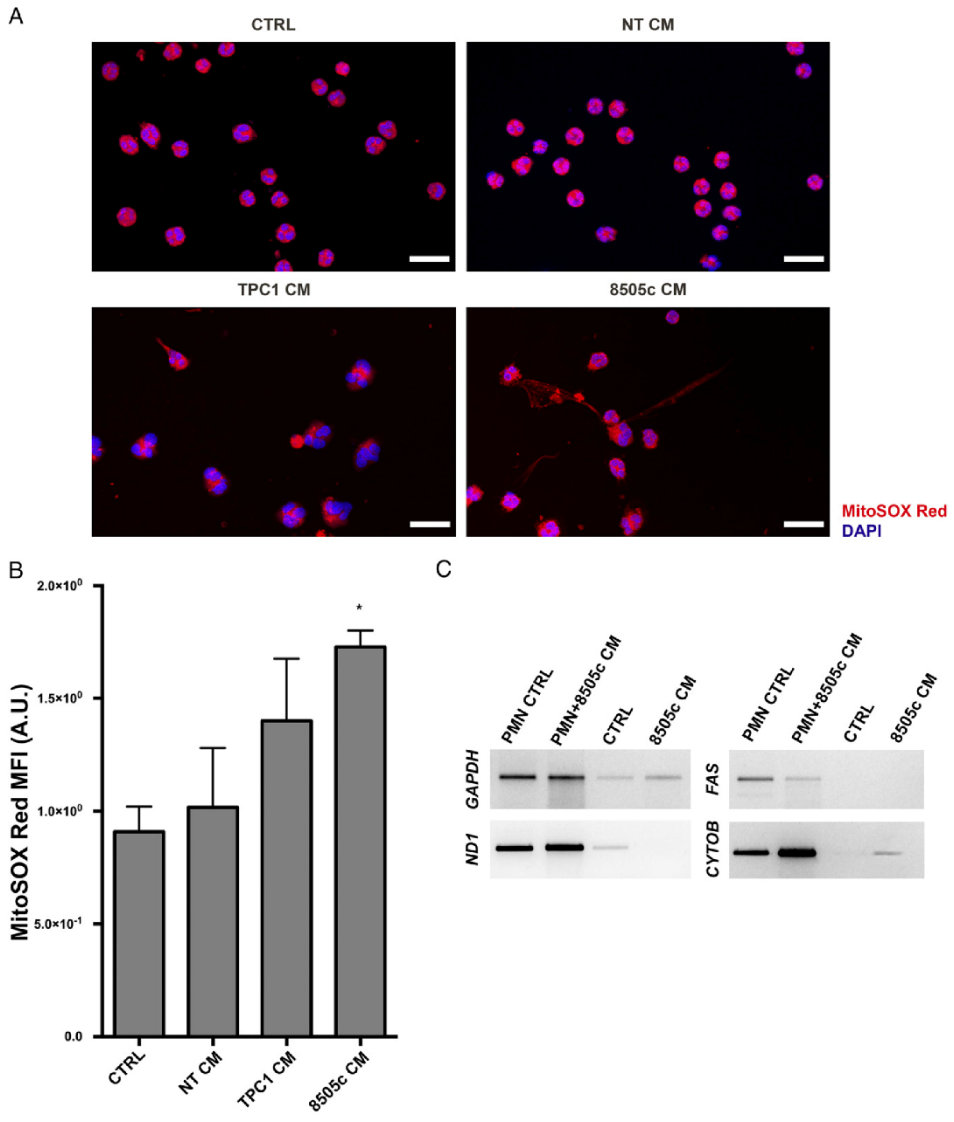

Figure 6 ATC CM–induced oxidized mitochondrial DNA release. **(A)** Representative immunofluorescence images of highly purified peripheral blood PMNs primed with TC CM, NT CM, or CTRL. PMNs (1 x 10^6 cells/ml) were seeded on glass coverslips and stimulated with 8505c, TPC1, or NT CM or with CTRL for 60 min at 37°C. The cells were incubated with MitoSOX Red for 30 min, fixed with 4% paraformaldehyde, and mounted with mounting medium with DAPI. The cells were visualized using an original magnification x63 oil immersion objective with an inverted fluorescence microscope with ApoTome; DNAwas stained with DAPI

(blue). Mitochondrial oxidized DNA was stained with MitoSOX Red (red). Original magnification x63, immersion oil. Scale bar, 20 mm. The images are representative of three independent experiments using three different donor samples. **(B)** Quantification of the extracellular MitoSOX Red MFI for each experimental condition (PMNs cultured in TC CM, NT CM, or in CTRL). Values are expressed as mean ± SEM of three randomly selected and noncontiguous fields of three independent experiments using three different donor samples. **(C)** PCR: two mitochondrial (lower panels) and two nuclear genes (upper panels) were amplified from different DNA templates. Compared with the DNA released by control neutrophils (PMN CTRL), mitochondrial genes were upregulated in the DNA released from ATC CM–primed neutrophils (PMN+8505c CM). Nuclear genes were also observed to be amplified (upper panels); however, no differences in GAPDH expression were observed when compared with control neutrophils, and a significant downregulation in FAS expression was evident in the extracellular DNA released by 8505c CM–primed neutrophils compared with control neutrophils. The 8505c CM and CTRL were also tested as controls. The results presented in all panels are representative of at least three independent experiments. *p<0.01. A.U., arbitrary units.

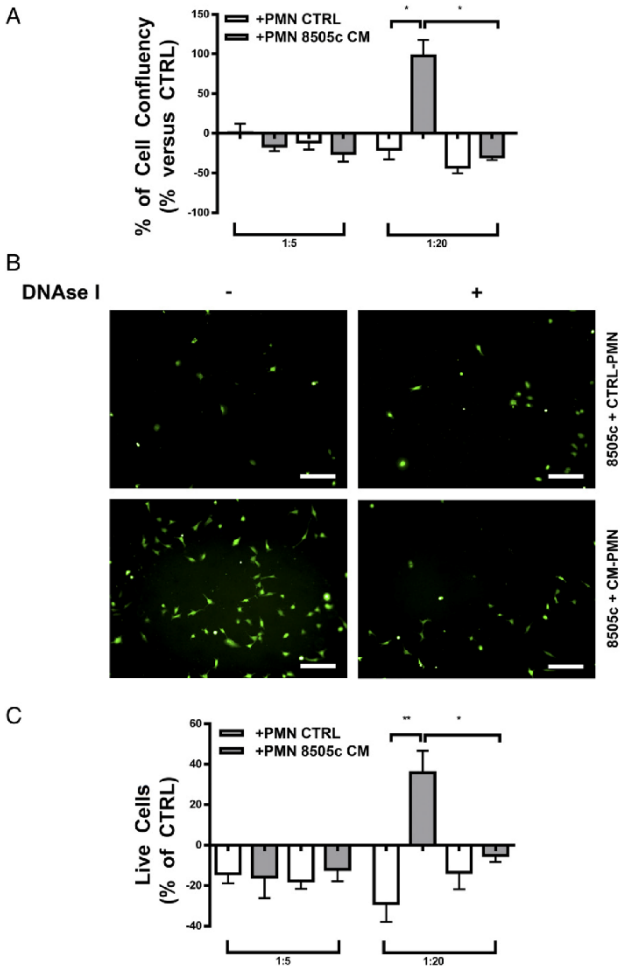

Figure 7 NETs sustained the viability of ATC cells **(A)** PMNs (1 x 10^6 cells/ml) were primed with 8505c CM (gray bars) or CTRL (white bars) for 60 min and then added to CFSE labeled 8505c cells for 24 h at a 5:1 or at a 20:1 neutrophil to tumor cell ratio (37°C, 5% CO_2). In selected experiments, DNAse I (60,000 UI/ ml) was added to the cocultures. Data are expressed as percentage of cell confluency after 24 h,

based on the CFSE+ area (mean ± SEM of seven independent experiments), and normalized for the control (CFSE+ area of 8505c cells without neutrophils). **(B)** Representative live imaging fluorescence images of CFSE-labeled 8505c cells after 24 h of cocultures with CTRL primed PMNs (upper panels) or 8505c CM– primed PMNs (lower panels), in absence (left panels) or presence (right panels) of DNAse I. Scale bar, 200 μm. The images are representative of seven independent experiments using seven different donor samples. **(C)** After 24 h incubation, CFSE+ 8505c live cells were evaluated by flow cytometry using allophycocyanin conjugated annexin V and PI. Results are expressed as percentages of live cells compared with the control (CFSE+ cells without neutrophils) (mean ± SEM of seven independent experiments using seven different donor samples). *$p<0.05$, **$p<0.01$.

REFERENCES

Adams, J.M., and Cory, S. (2007). Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol *19*, 488-496.

Al-Haidari, A.A., Algethami, N., Lepsenyi, M., Rahman, M., Syk, I., and Thorlacius, H. (2019). Neutrophil extracellular traps promote peritoneal metastasis of colon cancer cells. Oncotarget *10*, 1238-1249.

Albrengues, J., Shields, M.A., Ng, D., Park, C.G., Ambrico, A., Poindexter, M.E., Upadhyay, P., Uyeminami, D.L., Pommier, A., Kuttner, V.*, et al.* (2018). Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. Science *361*.

Aldabbous, L., Abdul-Salam, V., McKinnon, T., Duluc, L., Pepke-Zaba, J., Southwood, M., Ainscough, A.J., Hadinnapola, C., Wilkins, M.R., Toshner, M.*, et al.* (2016). Neutrophil Extracellular Traps Promote Angiogenesis: Evidence From Vascular Pathology in Pulmonary Hypertension. Arteriosclerosis, thrombosis, and vascular biology *36*, 2078-2087.

Alfaro, C., Teijeira, A., Onate, C., Perez, G., Sanmamed, M.F., Andueza, M.P., Alignani, D., Labiano, S., Azpilikueta, A., Rodriguez-Paulete, A.*, et al.* (2016). Tumor-Produced Interleukin-8 Attracts Human Myeloid-Derived Suppressor Cells and Elicits Extrusion of Neutrophil Extracellular Traps (NETs). Clin Cancer Res *22*, 3924-3936.

Amini, P., Stojkov, D., Felser, A., Jackson, C.B., Courage, C., Schaller, A., Gelman, L., Soriano, M.E., Nuoffer, J.M., Scorrano, L.*, et al.* (2018). Neutrophil extracellular trap formation requires OPA1-dependent glycolytic ATP production. Nature communications *9*, 2958.

Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D., and Zychlinsky, A. (2012). Neutrophil function: from mechanisms to disease. Annual review of immunology *30*, 459-489.

An, Z., Li, J., Yu, J., Wang, X., Gao, H., Zhang, W., Wei, Z., Zhang, J., Zhang, Y., Zhao, J.*, et al.* (2019). Neutrophil extracellular traps induced by IL-8 aggravate atherosclerosis via activation NF-kappaB signaling in macrophages. Cell Cycle *18*, 2928-2938.

Antonelli, A., Ferrari, S.M., Fallahi, P., Berti, P., Materazzi, G., Barani, L., Marchetti, I., Ferrannini, E., and Miccoli, P. (2008). Primary cell cultures from anaplastic thyroid cancer obtained by fine-needle aspiration used for chemosensitivity tests. Clin Endocrinol (Oxf) *69*, 148-152.

Antonelli, A., Ferrari, S.M., Fallahi, P., Frascerra, S., Piaggi, S., Gelmini, S., Lupi, C., Minuto, M., Berti, P., Benvenga, S.*, et al.* (2009). Dysregulation of secretion of CXC alpha-chemokine CXCL10 in papillary thyroid cancer: modulation by peroxisome proliferator-activated receptor-gamma agonists. Endocr Relat Cancer *16*, 1299-1311.

Antonelli, A., Ferrari, S.M., Frascerra, S., Pupilli, C., Mancusi, C., Metelli, M.R., Orlando, C., Ferrannini, E., and Fallahi, P. (2010). CXCL9 and CXCL11 chemokines modulation by peroxisome proliferator-activated receptor-alpha agonists secretion in Graves' and normal thyrocytes. J Clin Endocrinol Metab *95*, E413-420.

Arelaki, S., Arampatzioglou, A., Kambas, K., Papagoras, C., Miltiades, P., Angelidou, I., Mitsios, A., Kotsianidis, I., Skendros, P., Sivridis, E.*, et al.* (2016). Gradient Infiltration of Neutrophil Extracellular Traps in Colon Cancer and Evidence for Their Involvement in Tumour Growth. PloS one *11*, e0154484.

Azevedo, P.O., Paiva, A.E., Santos, G.S.P., Lousado, L., Andreotti, J.P., Sena, I.F.G., Tagliati, C.A., Mintz, A., and Birbrair, A. (2018). Cross-talk between lung cancer and bones results in neutrophils that promote tumor progression. Cancer Metastasis Rev *37*, 779-790.

Baggiolini, M. (1972). The enzymes of the granules of polymorphonuclear leukocytes and their functions. Enzyme *13*, 132-160.

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539-545.

Barkan, D., El Touny, L.H., Michalowski, A.M., Smith, J.A., Chu, I., Davis, A.S., Webster, J.D., Hoover, S., Simpson, R.M., Gauldie, J.*, et al.* (2010). Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. Cancer research *70*, 5706-5716.

Barreda, D.R., Hanington, P.C., and Belosevic, M. (2004). Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol *28*, 509-554.

Bates, A.M., Gomez Hernandez, M.P., Lanzel, E.A., Qian, F., and Brogden, K.A. (2018). Matrix metalloproteinase (MMP) and immunosuppressive biomarker profiles of seven head and neck squamous cell carcinoma (HNSCC) cell lines. Transl Cancer Res *7*, 533-542.
Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A., and Henriques-Normark, B. (2006). An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. Current biology : CB *16*, 401-407.
Bellelli, R., Castellone, M.D., Garcia-Rostan, G., Ugolini, C., Nucera, C., Sadow, P.M., Nappi, T.C., Salerno, P., Cantisani, M.C., Basolo, F*., et al.* (2012). FOXM1 is a molecular determinant of the mitogenic and invasive phenotype of anaplastic thyroid carcinoma. Endocr Relat Cancer *19*, 695-710.
Ben-Smith, A., Dove, S.K., Martin, A., Wakelam, M.J., and Savage, C.O. (2001). Antineutrophil cytoplasm autoantibodies from patients with systemic vasculitis activate neutrophils through distinct signaling cascades: comparison with conventional Fcgamma receptor ligation. Blood *98*, 1448-1455.
Berger-Achituv, S., Brinkmann, V., Abed, U.A., Kuhn, L.I., Ben-Ezra, J., Elhasid, R., and Zychlinsky, A. (2013). A proposed role for neutrophil extracellular traps in cancer immunoediting. Frontiers in immunology *4*, 48.
Bertini, R., Allegretti, M., Bizzarri, C., Moriconi, A., Locati, M., Zampella, G., Cervellera, M.N., Di Cioccio, V., Cesta, M.C., Galliera, E*., et al.* (2004). Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. Proc Natl Acad Sci U S A *101*, 11791-11796.
Bianchi, M., Hakkim, A., Brinkmann, V., Siler, U., Seger, R.A., Zychlinsky, A., and Reichenbach, J. (2009). Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood *114*, 2619-2622.
Bingle, L., Brown, N.J., and Lewis, C.E. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol *196*, 254-265.
Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol *11*, 889-896.
Blasco, M.A. (2005). Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet *6*, 611-622.
Boone, B.A., Murthy, P., Miller-Ocuin, J., Doerfler, W.R., Ellis, J.T., Liang, X., Ross, M.A., Wallace, C.T., Sperry, J.L., Lotze, M.T*., et al.* (2018). Chloroquine reduces hypercoagulability in pancreatic cancer through inhibition of neutrophil extracellular traps. BMC Cancer *18*, 678.
Boone, B.A., Orlichenko, L., Schapiro, N.E., Loughran, P., Gianfrate, G.C., Ellis, J.T., Singhi, A.D., Kang, R., Tang, D., Lotze, M.T*., et al.* (2015). The receptor for advanced glycation end products (RAGE) enhances autophagy and neutrophil extracellular traps in pancreatic cancer. Cancer Gene Ther *22*, 326-334.
Borregaard, N. (2010). Neutrophils, from marrow to microbes. Immunity *33*, 657-670.
Borregaard, N., Christensen, L., Bejerrum, O.W., Birgens, H.S., and Clemmensen, I. (1990). Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. The Journal of clinical investigation *85*, 408-416.
Borregaard, N., and Cowland, J.B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. Blood *89*, 3503-3521.
Borregaard, N., Kjeldsen, L., Rygaard, K., Bastholm, L., Nielsen, M.H., Sengelov, H., Bjerrum, O.W., and Johnsen, A.H. (1992). Stimulus-dependent secretion of plasma proteins from human neutrophils. The Journal of clinical investigation *90*, 86-96.
Borregaard, N., Miller, L.J., and Springer, T.A. (1987). Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. Science *237*, 1204-1206.
Borregaard, N., Sorensen, O.E., and Theilgaard-Monch, K. (2007). Neutrophil granules: a library of innate immunity proteins. Trends Immunol *28*, 340-345.
Borriello, F., Iannone, R., Di Somma, S., Loffredo, S., Scamardella, E., Galdiero, M.R., Varricchi, G., Granata, F., Portella, G., and Marone, G. (2016). GM-CSF and IL-3 Modulate Human Monocyte TNF-alpha Production and Renewal in In Vitro Models of Trained Immunity. Frontiers in immunology *7*, 680.
Bosisio, D., Salvi, V., Gagliostro, V., and Sozzani, S. (2014). Angiogenic and antiangiogenic chemokines. Chem Immunol Allergy *99*, 89-104.

Bottazzi, B., Polentarutti, N., Acero, R., Balsari, A., Boraschi, D., Ghezzi, P., Salmona, M., and Mantovani, A. (1983). Regulation of the macrophage content of neoplasms by chemoattractants. Science *220*, 210-212.
Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., and Papayannopoulos, V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol *15*, 1017-1025.
Brinkmann, V. (2018). Neutrophil Extracellular Traps in the Second Decade. Journal of innate immunity *10*, 414-421.
Brinkmann, V., Goosmann, C., Kuhn, L.I., and Zychlinsky, A. (2012). Automatic quantification of in vitro NET formation. Frontiers in immunology *3*, 413.
Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science *303*, 1532-1535.
Brinkmann, V., and Zychlinsky, A. (2012). Neutrophil extracellular traps: is immunity the second function of chromatin? The Journal of cell biology *198*, 773-783.
Buchanan, J.T., Simpson, A.J., Aziz, R.K., Liu, G.Y., Kristian, S.A., Kotb, M., Feramisco, J., and Nizet, V. (2006). DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Current biology : CB *16*, 396-400.
Burkhart, D.L., and Sage, J. (2010). Complex transcriptional regulatory networks around the retinoblastoma tumor suppressor gene. Cell Cycle *9*, 1663-1664.
Caielli, S., Athale, S., Domic, B., Murat, E., Chandra, M., Banchereau, R., Baisch, J., Phelps, K., Clayton, S., Gong, M.*, et al.* (2016). Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. J Exp Med *213*, 697-713.
Calzetti, F., Tamassia, N., Arruda-Silva, F., Gasperini, S., and Cassatella, M.A. (2017). The importance of being "pure" neutrophils. The Journal of allergy and clinical immunology *139*, 352-355 e356.
Carnevale, S., Ghasemi, S., Rigatelli, A., and Jaillon, S. (2020). The complexity of neutrophils in health and disease: Focus on cancer. Seminars in immunology *48*, 101409.
Casanova-Acebes, M., Pitaval, C., Weiss, L.A., Nombela-Arrieta, C., Chevre, R., N, A.G., Kunisaki, Y., Zhang, D., van Rooijen, N., Silberstein, L.E.*, et al.* (2013). Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell *153*, 1025-1035.
Cassatella, M.A., Locati, M., and Mantovani, A. (2009). Never underestimate the power of a neutrophil. Immunity *31*, 698-700.
Ciampi, R., Knauf, J.A., Kerler, R., Gandhi, M., Zhu, Z., Nikiforova, M.N., Rabes, H.M., Fagin, J.A., and Nikiforov, Y.E. (2005). Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. The Journal of clinical investigation *115*, 94-101.
Clark, S.R., Ma, A.C., Tavener, S.A., McDonald, B., Goodarzi, Z., Kelly, M.M., Patel, K.D., Chakrabarti, S., McAvoy, E., Sinclair, G.D.*, et al.* (2007). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med *13*, 463-469.
Colotta, F., Re, F., Polentarutti, N., Sozzani, S., and Mantovani, A. (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood *80*, 2012-2020.
Cools-Lartigue, J., Spicer, J., McDonald, B., Gowing, S., Chow, S., Giannias, B., Bourdeau, F., Kubes, P., and Ferri, L. (2013). Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. The Journal of clinical investigation.
Cortjens, B., de Boer, O.J., de Jong, R., Antonis, A.F., Sabogal Pineros, Y.S., Lutter, R., van Woensel, J.B., and Bem, R.A. (2016). Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. J Pathol *238*, 401-411.
Cristinziano, L., Modestino, L., Loffredo, S., Varricchi, G., Braile, M., Ferrara, A.L., de Paulis, A., Antonelli, A., Marone, G., and Galdiero, M.R. (2020). Anaplastic Thyroid Cancer Cells Induce the Release of Mitochondrial Extracellular DNA Traps by Viable Neutrophils. J Immunol *204*, 1362-1372.
Cristinziano, L., Poto, R., Criscuolo, G., Ferrara, A.L., Galdiero, M.R., Modestino, L., Loffredo, S., de Paulis, A., Marone, G., Spadaro, G.*, et al.* (2021). IL-33 and Superantigenic Activation of Human Lung Mast Cells Induce the Release of Angiogenic and Lymphangiogenic Factors. Cells *10*.
Cunha, L.L., Marcello, M.A., and Ward, L.S. (2014). The role of the inflammatory microenvironment in thyroid carcinogenesis. Endocr Relat Cancer *21*, R85-R103.

Davey, M.S., Tamassia, N., Rossato, M., Bazzoni, F., Calzetti, F., Bruderek, K., Sironi, M., Zimmer, L., Bottazzi, B., Mantovani, A.*, et al.* (2011). Failure to detect production of IL-10 by activated human neutrophils. Nat Immunol *12*, 1017-1018; author reply 1018-1020.
De Santo, C., Arscott, R., Booth, S., Karydis, I., Jones, M., Asher, R., Salio, M., Middleton, M., and Cerundolo, V. (2010). Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. Nat Immunol *11*, 1039-1046.
Demers, M., Krause, D.S., Schatzberg, D., Martinod, K., Voorhees, J.R., Fuchs, T.A., Scadden, D.T., and Wagner, D.D. (2012). Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. Proc Natl Acad Sci U S A *109*, 13076-13081.
Demetri, G.D., and Griffin, J.D. (1991). Granulocyte colony-stimulating factor and its receptor. Blood *78*, 2791-2808.
Di Caro, G., Carvello, M., Pesce, S., Erreni, M., Marchesi, F., Todoric, J., Sacchi, M., Montorsi, M., Allavena, P., and Spinelli, A. (2016). Circulating Inflammatory Mediators as Potential Prognostic Markers of Human Colorectal Cancer. PloS one *11*, e0148186.
Douda, D.N., Yip, L., Khan, M.A., Grasemann, H., and Palaniyar, N. (2014). Akt is essential to induce NADPH-dependent NETosis and to switch the neutrophil death to apoptosis. Blood *123*, 597-600.
Eash, K.J., Greenbaum, A.M., Gopalan, P.K., and Link, D.C. (2010). CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. The Journal of clinical investigation *120*, 2423-2431.
Erpenbeck, L., and Schon, M.P. (2017). Neutrophil extracellular traps: protagonists of cancer progression? Oncogene *36*, 2483-2490.
Eruslanov, E.B., Bhojnagarwala, P.S., Quatromoni, J.G., Stephen, T.L., Ranganathan, A., Deshpande, C., Akimova, T., Vachani, A., Litzky, L., Hancock, W.W.*, et al.* (2014). Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. The Journal of clinical investigation *124*, 5466-5480.
Fallahi, P., Ferrari, S.M., Piaggi, S., Luconi, M., Cantini, G., Gelmini, S., Elia, G., Ruffilli, I., and Antonelli, A. (2018). The paramount role of cytokines and chemokines in papillary thyroid cancer: a review and experimental results. Immunologic research *66*, 710-722.
Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., Worthen, G.S., and Albelda, S.M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell *16*, 183-194.
Fuchs, T.A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., and Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. The Journal of cell biology *176*, 231-241.
Fuchs, T.A., Brill, A., Duerschmied, D., Schatzberg, D., Monestier, M., Myers, D.D., Jr., Wrobleski, S.K., Wakefield, T.W., Hartwig, J.H., and Wagner, D.D. (2010). Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A *107*, 15880-15885.
Gabriel, C., McMaster, W.R., Girard, D., and Descoteaux, A. (2010). Leishmania donovani promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol *185*, 4319-4327.
Galdiero, M.R., Bianchi, P., Grizzi, F., Di Caro, G., Basso, G., Ponzetta, A., Bonavita, E., Barbagallo, M., Tartari, S., Polentarutti, N.*, et al.* (2016a). Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer. Int J Cancer *139*, 446-456.
Galdiero, M.R., Garlanda, C., Jaillon, S., Marone, G., and Mantovani, A. (2013). Tumor associated macrophages and neutrophils in tumor progression. J Cell Physiol *228*, 1404-1412.
Galdiero, M.R., Varricchi, G., Loffredo, S., Bellevicine, C., Lansione, T., Ferrara, A.L., Iannone, R., di Somma, S., Borriello, F., Clery, E.*, et al.* (2018a). Potential involvement of neutrophils in human thyroid cancer. PloS one *13*, e0199740.
Galdiero, M.R., Varricchi, G., Loffredo, S., Mantovani, A., and Marone, G. (2018b). Roles of neutrophils in cancer growth and progression. Journal of leukocyte biology *103*, 457-464.
Galdiero, M.R., Varricchi, G., and Marone, G. (2016b). The immune network in thyroid cancer. Oncoimmunology *5*, e1168556.

Gallin, J.I., Metcalf, J.A., Roos, D., Seligmann, B., and Friedman, M.M. (1984). Organelle-depleted human neutrophil cytoplasts used to study fmet-leu-phe receptor modulation and cell function. J Immunol *133*, 415-421.
Garcia-Rostan, G., Tallini, G., Herrero, A., D'Aquila, T.G., Carcangiu, M.L., and Rimm, D.L. (1999). Frequent mutation and nuclear localization of beta-catenin in anaplastic thyroid carcinoma. Cancer research *59*, 1811-1815.
Ghiringhelli, F., Menard, C., Martin, F., and Zitvogel, L. (2006). The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. Immunol Rev *214*, 229-238.
Gobert, M., Treilleux, I., Bendriss-Vermare, N., Bachelot, T., Goddard-Leon, S., Arfi, V., Biota, C., Doffin, A.C., Durand, I., Olive, D.*, et al.* (2009). Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. Cancer research *69*, 2000-2009.
Gogali, F., Paterakis, G., Rassidakis, G.Z., Kaltsas, G., Liakou, C.I., Gousis, P., Neonakis, E., Manoussakis, M.N., and Liapi, C. (2012). Phenotypical analysis of lymphocytes with suppressive and regulatory properties (Tregs) and NK cells in the papillary carcinoma of thyroid. J Clin Endocrinol Metab *97*, 1474-1482.
Granot, Z., Henke, E., Comen, E.A., King, T.A., Norton, L., and Benezra, R. (2011). Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell *20*, 300-314.
Greenblatt, M.B., Aliprantis, A., Hu, B., and Glimcher, L.H. (2010). Calcineurin regulates innate antifungal immunity in neutrophils. J Exp Med *207*, 923-931.
Guglietta, S., Chiavelli, A., Zagato, E., Krieg, C., Gandini, S., Ravenda, P.S., Bazolli, B., Lu, B., Penna, G., and Rescigno, M. (2016). Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. Nature communications *7*, 11037.
Gupta, S., Chan, D.W., Zaal, K.J., and Kaplan, M.J. (2018). A High-Throughput Real-Time Imaging Technique To Quantify NETosis and Distinguish Mechanisms of Cell Death in Human Neutrophils. J Immunol *200*, 869-879.
Haddad, L.E., and Sirois, M.G. (2014). Angiopoietin-1 upregulates de novo expression of IL-1beta and Il1-Ra, and the exclusive release of Il1-Ra from human neutrophils. PloS one *9*, e88980.
Hakkim, A., Fuchs, T.A., Martinez, N.E., Hess, S., Prinz, H., Zychlinsky, A., and Waldmann, H. (2011). Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat Chem Biol *7*, 75-77.
Hakkim, A., Furnrohr, B.G., Amann, K., Laube, B., Abed, U.A., Brinkmann, V., Herrmann, M., Voll, R.E., and Zychlinsky, A. (2010). Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A *107*, 9813-9818.
Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.
Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.
Hayashi, F., Means, T.K., and Luster, A.D. (2003). Toll-like receptors stimulate human neutrophil function. Blood *102*, 2660-2669.
Hisada, Y., Grover, S.P., Maqsood, A., Houston, R., Ay, C., Noubouossie, D.F., Cooley, B.C., Wallen, H., Key, N.S., Thalin, C.*, et al.* (2020). Neutrophils and neutrophil extracellular traps enhance venous thrombosis in mice bearing human pancreatic tumors. Haematologica *105*, 218-225.
Hofman, P. (2004). Molecular regulation of neutrophil apoptosis and potential targets for therapeutic strategy against the inflammatory process. Curr Drug Targets Inflamm Allergy *3*, 1-9.
Houghton, A.M., Rzymkiewicz, D.M., Ji, H., Gregory, A.D., Egea, E.E., Metz, H.E., Stolz, D.B., Land, S.R., Marconcini, L.A., Kliment, C.R.*, et al.* (2010). Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. Nat Med *16*, 219-223.
Idorn, M., Skadborg, S.K., Kellermann, L., Halldorsdottir, H.R., Holmen Olofsson, G., Met, O., and Thor Straten, P. (2018). Chemokine receptor engineering of T cells with CXCR2 improves homing towards subcutaneous human melanomas in xenograft mouse model. Oncoimmunology *7*, e1450715.
Jablonska, J., Leschner, S., Westphal, K., Lienenklaus, S., and Weiss, S. (2010). Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. The Journal of clinical investigation *120*, 1151-1164.
Jaillon, S., Galdiero, M.R., Del Prete, D., Cassatella, M.A., Garlanda, C., and Mantovani, A. (2013). Neutrophils in innate and adaptive immunity. Seminars in immunopathology *35*, 377-394.

Jaillon, S., Peri, G., Delneste, Y., Fremaux, I., Doni, A., Moalli, F., Garlanda, C., Romani, L., Gascan, H., Bellocchio, S*., et al.* (2007). The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. J Exp Med *204*, 793-804.
Jorch, S.K., and Kubes, P. (2017). An emerging role for neutrophil extracellular traps in noninfectious disease. Nat Med *23*, 279-287.
Jung, K.Y., Cho, S.W., Kim, Y.A., Kim, D., Oh, B.C., Park, D.J., and Park, Y.J. (2015). Cancers with Higher Density of Tumor-Associated Macrophages Were Associated with Poor Survival Rates. J Pathol Transl Med *49*, 318-324.
Kannemeier, C., Shibamiya, A., Nakazawa, F., Trusheim, H., Ruppert, C., Markart, P., Song, Y., Tzima, E., Kennerknecht, E., Niepmann, M*., et al.* (2007). Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. Proc Natl Acad Sci U S A *104*, 6388-6393.
Kargl, J., Busch, S.E., Yang, G.H., Kim, K.H., Hanke, M.L., Metz, H.E., Hubbard, J.J., Lee, S.M., Madtes, D.K., McIntosh, M.W*., et al.* (2017). Neutrophils dominate the immune cell composition in non-small cell lung cancer. Nature communications *8*, 14381.
Katoh, H., Wang, D., Daikoku, T., Sun, H., Dey, S.K., and Dubois, R.N. (2013). CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis. Cancer Cell *24*, 631-644.
Kaufmann, S.H. (2008). Elie Metchnikoff's and Paul Ehrlich's impact on infection biology. Microbes and infection / Institut Pasteur *10*, 1417-1419.
Keshari, R.S., Verma, A., Barthwal, M.K., and Dikshit, M. (2013). Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. Journal of cellular biochemistry *114*, 532-540.
Kimura, E.T., Nikiforova, M.N., Zhu, Z., Knauf, J.A., Nikiforov, Y.E., and Fagin, J.A. (2003). High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer research *63*, 1454-1457.
Kraaij, T., Tengstrom, F.C., Kamerling, S.W., Pusey, C.D., Scherer, H.U., Toes, R.E., Rabelink, T.J., van Kooten, C., and Teng, Y.K. (2016). A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes. Autoimmun Rev *15*, 577-584.
Lapidot, T., and Kollet, O. (2002). The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia *16*, 1992-2003.
Lavoie, S.S., Dumas, E., Vulesevic, B., Neagoe, P.E., White, M., and Sirois, M.G. (2018). Synthesis of Human Neutrophil Extracellular Traps Contributes to Angiopoietin-Mediated In Vitro Proinflammatory and Proangiogenic Activities. J Immunol *200*, 3801-3813.
Lee, W., Ko, S.Y., Mohamed, M.S., Kenny, H.A., Lengyel, E., and Naora, H. (2019). Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. J Exp Med *216*, 176-194.
Lehman, H.K., and Segal, B.H. (2020). The role of neutrophils in host defense and disease. The Journal of allergy and clinical immunology *145*, 1535-1544.
Ley, K., Hoffman, H.M., Kubes, P., Cassatella, M.A., Zychlinsky, A., Hedrick, C.C., and Catz, S.D. (2018). Neutrophils: New insights and open questions. Sci Immunol *3*.
Ley, K., Smith, E., and Stark, M.A. (2006). IL-17A-producing neutrophil-regulatory Tn lymphocytes. Immunologic research *34*, 229-242.
Li, M., Knight, D.A., L, A.S., Smyth, M.J., and Stewart, T.J. (2013). A role for CCL2 in both tumor progression and immunosurveillance. Oncoimmunology *2*, e25474.
Lieschke, G.J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., Fowler, K.J., Basu, S., Zhan, Y.F., and Dunn, A.R. (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood *84*, 1737-1746.
Lin, A.M., Rubin, C.J., Khandpur, R., Wang, J.Y., Riblett, M., Yalavarthi, S., Villanueva, E.C., Shah, P., Kaplan, M.J., and Bruce, A.T. (2011). Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunol *187*, 490-500.
Liu, Q., Li, A., Tian, Y., Wu, J.D., Liu, Y., Li, T., Chen, Y., Han, X., and Wu, K. (2016). The CXCL8-CXCR1/2 pathways in cancer. Cytokine & growth factor reviews *31*, 61-71.

Loffredo, S., Borriello, F., Iannone, R., Ferrara, A.L., Galdiero, M.R., Gigantino, V., Esposito, P., Varricchi, G., Lambeau, G., Cassatella, M.A*., et al.* (2017). Group V Secreted Phospholipase A2 Induces the Release of Proangiogenic and Antiangiogenic Factors by Human Neutrophils. Frontiers in immunology *8*, 443.
Loges, S., Schmidt, T., Tjwa, M., van Geyte, K., Lievens, D., Lutgens, E., Vanhoutte, D., Borgel, D., Plaisance, S., Hoylaerts, M*., et al.* (2010). Malignant cells fuel tumor growth by educating infiltrating leukocytes to produce the mitogen Gas6. Blood *115*, 2264-2273.
Lucas, D., Battista, M., Shi, P.A., Isola, L., and Frenette, P.S. (2008). Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. Cell stem cell *3*, 364-366.
Maliba, R., Lapointe, S., Neagoe, P.E., Brkovic, A., and Sirois, M.G. (2006). Angiopoietins-1 and -2 are both capable of mediating endothelial PAF synthesis: intracellular signalling pathways. Cell Signal *18*, 1947-1957.
Malladi, S., Macalinao, D.G., Jin, X., He, L., Basnet, H., Zou, Y., de Stanchina, E., and Massague, J. (2016). Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. Cell *165*, 45-60.
Mankan, A.K., Dau, T., Jenne, D., and Hornung, V. (2012). The NLRP3/ASC/Caspase-1 axis regulates IL-1beta processing in neutrophils. European journal of immunology *42*, 710-715.
Mantovani, A., Cassatella, M.A., Costantini, C., and Jaillon, S. (2011). Neutrophils in the activation and regulation of innate and adaptive immunity. Nature reviews Immunology *11*, 519-531.
Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol *25*, 677-686.
Marone, G., Rossi, F.W., Pecoraro, A., Pucino, V., Criscuolo, G., Paulis, A., Spadaro, G., and Varricchi, G. (2020). HIV gp120 Induces the Release of Proinflammatory, Angiogenic, and Lymphangiogenic Factors from Human Lung Mast Cells. Vaccines (Basel) *8*.
Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P.A., Baumann, C., Fey, M.F., Dummer, R., Simon, H.U., and Yousefi, S. (2004a). Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem *279*, 44123-44132.
Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P.A., Baumann, C., Fey, M.F., Dummer, R., Simon, H.U., and Yousefi, S. (2004b). Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. The Journal of biological chemistry *279*, 44123-44132.
Mary, J.Y. (1985). Normal human granulopoiesis revisited. II. Bone marrow data. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie *39*, 66-77.
McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D., Loeffler, J., Heesemann, J., and Ebel, F. (2010). NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. Microbes and infection / Institut Pasteur *12*, 928-936.
McDonald, B., Spicer, J., Giannais, B., Fallavollita, L., Brodt, P., and Ferri, L.E. (2009). Systemic inflammation increases cancer cell adhesion to hepatic sinusoids by neutrophil mediated mechanisms. Int J Cancer *125*, 1298-1305.
Melillo, R.M., Guarino, V., Avilla, E., Galdiero, M.R., Liotti, F., Prevete, N., Rossi, F.W., Basolo, F., Ugolini, C., de Paulis, A*., et al.* (2010). Mast cells have a protumorigenic role in human thyroid cancer. Oncogene *29*, 6203-6215.
Metzler, K.D., Fuchs, T.A., Nauseef, W.M., Reumaux, D., Roesler, J., Schulze, I., Wahn, V., Papayannopoulos, V., and Zychlinsky, A. (2011). Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood *117*, 953-959.
Metzler, K.D., Goosmann, C., Lubojemska, A., Zychlinsky, A., and Papayannopoulos, V. (2014). A Myeloperoxidase-Containing Complex Regulates Neutrophil Elastase Release and Actin Dynamics during NETosis. Cell Rep *8*, 883-896.
Miller-Ocuin, J.L., Liang, X., Boone, B.A., Doerfler, W.R., Singhi, A.D., Tang, D., Kang, R., Lotze, M.T., and Zeh, H.J., 3rd (2019). DNA released from neutrophil extracellular traps (NETs) activates pancreatic stellate cells and enhances pancreatic tumor growth. Oncoimmunology *8*, e1605822.
Millrud, C.R., Kagedal, A., Kumlien Georen, S., Winqvist, O., Uddman, R., Razavi, R., Munck-Wikland, E., and Cardell, L.O. (2017). NET-producing CD16(high) CD62L(dim) neutrophils migrate to tumor sites and predict improved survival in patients with HNSCC. Int J Cancer *140*, 2557-2567.

Mohanty, T., Sorensen, O.E., and Nordenfelt, P. (2017). NETQUANT: Automated Quantification of Neutrophil Extracellular Traps. Frontiers in immunology *8*, 1999.
Moran, E.M., Heydrich, R., Ng, C.T., Saber, T.P., McCormick, J., Sieper, J., Appel, H., Fearon, U., and Veale, D.J. (2011). IL-17A expression is localised to both mononuclear and polymorphonuclear synovial cell infiltrates. PloS one *6*, e24048.
Morrison, J.A., Pike, L.A., Lund, G., Zhou, Q., Kessler, B.E., Bauerle, K.T., Sams, S.B., Haugen, B.R., and Schweppe, R.E. (2015). Characterization of thyroid cancer cell lines in murine orthotopic and intracardiac metastasis models. Horm Cancer *6*, 87-99.
Morshed, M., Hlushchuk, R., Simon, D., Walls, A.F., Obata-Ninomiya, K., Karasuyama, H., Djonov, V., Eggel, A., Kaufmann, T., Simon, H.U*., et al.* (2014). NADPH oxidase-independent formation of extracellular DNA traps by basophils. J Immunol *192*, 5314-5323.
Muniz, V.S., Silva, J.C., Braga, Y.A.V., Melo, R.C.N., Ueki, S., Takeda, M., Hebisawa, A., Asano, K., Figueiredo, R.T., and Neves, J.S. (2018). Eosinophils release extracellular DNA traps in response to Aspergillus fumigatus. The Journal of allergy and clinical immunology *141*, 571-585 e577.
Muzio, M., Re, F., Sironi, M., Polentarutti, N., Minty, A., Caput, D., Ferrara, P., Mantovani, A., and Colotta, F. (1994). Interleukin-13 induces the production of interleukin-1 receptor antagonist (IL-1ra) and the expression of the mRNA for the intracellular (keratinocyte) form of IL-1ra in human myelomonocytic cells. Blood *83*, 1738-1743.
Najmeh, S., Cools-Lartigue, J., Giannias, B., Spicer, J., and Ferri, L.E. (2015). Simplified Human Neutrophil Extracellular Traps (NETs) Isolation and Handling. J Vis Exp.
Neagoe, P.E., Dumas, E., Hajjar, F., and Sirois, M.G. (2012). Angiopoietin-1 but not angiopoietin-2 induces IL-8 synthesis and release by human neutrophils. J Cell Physiol *227*, 3099-3110.
Neeli, I., Dwivedi, N., Khan, S., and Radic, M. (2009). Regulation of extracellular chromatin release from neutrophils. Journal of innate immunity *1*, 194-201.
Ng, L.G., Ostuni, R., and Hidalgo, A. (2019). Heterogeneity of neutrophils. Nature reviews Immunology *19*, 255-265.
Nie, M., Yang, L., Bi, X., Wang, Y., Sun, P., Yang, H., Liu, P., Li, Z., Xia, Y., and Jiang, W. (2019). Neutrophil Extracellular Traps Induced by IL8 Promote Diffuse Large B-cell Lymphoma Progression via the TLR9 Signaling. Clin Cancer Res *25*, 1867-1879.
Nikiforov, Y.E., and Nikiforova, M.N. (2011). Molecular genetics and diagnosis of thyroid cancer. Nat Rev Endocrinol *7*, 569-580.
Nikiforova, M.N., Lynch, R.A., Biddinger, P.W., Alexander, E.K., Dorn, G.W., 2nd, Tallini, G., Kroll, T.G., and Nikiforov, Y.E. (2003). RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab *88*, 2318-2326.
Niu, Z., Tang, W., Liu, T., Xu, P., Zhu, D., Ji, M., Huang, W., Ren, L., Wei, Y., and Xu, J. (2018). Cell-free DNA derived from cancer cells facilitates tumor malignancy through Toll-like receptor 9 signaling-triggered interleukin-8 secretion in colorectal cancer. Acta Biochim Biophys Sin (Shanghai) *50*, 1007-1017.
Papayannopoulos, V., Metzler, K.D., Hakkim, A., and Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. The Journal of cell biology *191*, 677-691.
Park, J., Wysocki, R.W., Amoozgar, Z., Maiorino, L., Fein, M.R., Jorns, J., Schott, A.F., Kinugasa-Katayama, Y., Lee, Y., Won, N.H*., et al.* (2016). Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med *8*, 361ra138.
Parker, H., Dragunow, M., Hampton, M.B., Kettle, A.J., and Winterbourn, C.C. (2012). Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. Journal of leukocyte biology *92*, 841-849.
Pierce, B.L., Ballard-Barbash, R., Bernstein, L., Baumgartner, R.N., Neuhouser, M.L., Wener, M.H., Baumgartner, K.B., Gilliland, F.D., Sorensen, B.E., McTiernan, A*., et al.* (2009). Elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients. J Clin Oncol *27*, 3437-3444.
Pieterse, E., Rother, N., Garsen, M., Hofstra, J.M., Satchell, S.C., Hoffmann, M., Loeven, M.A., Knaapen, H.K., van der Heijden, O.W.H., Berden, J.H.M*., et al.* (2017). Neutrophil Extracellular Traps

Drive Endothelial-to-Mesenchymal Transition. Arteriosclerosis, thrombosis, and vascular biology *37*, 1371-1379.
Pilsczek, F.H., Salina, D., Poon, K.K., Fahey, C., Yipp, B.G., Sibley, C.D., Robbins, S.M., Green, F.H., Surette, M.G., Sugai, M.*, et al.* (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol *185*, 7413-7425.
Pittman, K., and Kubes, P. (2013). Damage-associated molecular patterns control neutrophil recruitment. Journal of innate immunity *5*, 315-323.
Puga, I., Cols, M., Barra, C.M., He, B., Cassis, L., Gentile, M., Comerma, L., Chorny, A., Shan, M., Xu, W.*, et al.* (2012). B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol *13*, 170-180.
Quail, D.F., and Joyce, J.A. (2013). Microenvironmental regulation of tumor progression and metastasis. Nat Med *19*, 1423-1437.
Queen, M.M., Ryan, R.E., Holzer, R.G., Keller-Peck, C.R., and Jorcyk, C.L. (2005). Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. Cancer research *65*, 8896-8904.
Raftery, M.J., Lalwani, P., Krautkrmer, E., Peters, T., Scharffetter-Kochanek, K., Kruger, R., Hofmann, J., Seeger, K., Kruger, D.H., and Schonrich, G. (2014). beta2 integrin mediates hantavirus-induced release of neutrophil extracellular traps. J Exp Med *211*, 1485-1497.
Ravindran, M., Khan, M.A., and Palaniyar, N. (2019). Neutrophil Extracellular Trap Formation: Physiology, Pathology, and Pharmacology. Biomolecules *9*.
Remijsen, Q., Vanden Berghe, T., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., Noppen, S., Delforge, M., Willems, J., and Vandenabeele, P. (2011). Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. Cell research *21*, 290-304.
Riesco-Eizaguirre, G., and Santisteban, P. (2007). Molecular biology of thyroid cancer initiation. Clin Transl Oncol *9*, 686-693.
Robledo-Avila, F.H., Ruiz-Rosado, J.D., Brockman, K.L., Kopp, B.T., Amer, A.O., McCoy, K., Bakaletz, L.O., and Partida-Sanchez, S. (2018). Dysregulated Calcium Homeostasis in Cystic Fibrosis Neutrophils Leads to Deficient Antimicrobial Responses. J Immunol *201*, 2016-2027.
Rotondi, M., Coperchini, F., and Chiovato, L. (2013). CXCL8 in thyroid disease: from basic notions to potential applications in clinical practice. Cytokine & growth factor reviews *24*, 539-546.
Saiselet, M., Floor, S., Tarabichi, M., Dom, G., Hebrant, A., van Staveren, W.C., and Maenhaut, C. (2012). Thyroid cancer cell lines: an overview. Front Endocrinol (Lausanne) *3*, 133.
Salehian, B., Liem, S.Y., Mojazi Amiri, H., and Maghami, E. (2019). Clinical Trials in Management of Anaplastic Thyroid Carcinoma; Progressions and Set Backs: A Systematic Review. Int J Endocrinol Metab *17*, e67759.
Sammarco, G., Varricchi, G., Ferraro, V., Ammendola, M., De Fazio, M., Altomare, D.F., Luposella, M., Maltese, L., Curro, G., Marone, G.*, et al.* (2019). Mast Cells, Angiogenesis and Lymphangiogenesis in Human Gastric Cancer. Int J Mol Sci *20*.
Schedel, F., Mayer-Hain, S., Pappelbaum, K.I., Metze, D., Stock, M., Goerge, T., Loser, K., Sunderkotter, C., Luger, T.A., and Weishaupt, C. (2020). Evidence and impact of neutrophil extracellular traps in malignant melanoma. Pigment Cell Melanoma Res *33*, 63-73.
Scheiermann, C., Kunisaki, Y., Lucas, D., Chow, A., Jang, J.E., Zhang, D., Hashimoto, D., Merad, M., and Frenette, P.S. (2012). Adrenergic nerves govern circadian leukocyte recruitment to tissues. Immunity *37*, 290-301.
Sendo, F., Tsuchida, H., Takeda, Y., Gon, S., Takei, H., Kato, T., Hachiya, O., and Watanabe, H. (1996). Regulation of neutrophil apoptosis--its biological significance in inflammation and the immune response. Human cell *9*, 215-222.
Sengelov, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C., and Borregaard, N. (1995). Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol *154*, 4157-4165.
Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103-112.
Silva, J.C., Rodrigues, N.C., Thompson-Souza, G.A., Muniz, V.S., Neves, J.S., and Figueiredo, R.T. (2020). Mac-1 triggers neutrophil DNA extracellular trap formation to Aspergillus fumigatus independently of PAD4 histone citrullination. Journal of leukocyte biology *107*, 69-83.

Sim, S., Yong, T.S., Park, S.J., Im, K.I., Kong, Y., Ryu, J.S., Min, D.Y., and Shin, M.H. (2005). NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by Entamoeba histolytica. J Immunol *174*, 4279-4288.
Sivanandham, R., Brocca-Cofano, E., Krampe, N., Falwell, E., Venkatraman, S.M.K., Ribeiro, R.M., Apetrei, C., and Pandrea, I. (2018). Neutrophil extracellular trap production contributes to pathogenesis in SIV-infected nonhuman primates. The Journal of clinical investigation *128*, 5178-5183.
Snoderly, H.T., Boone, B.A., and Bennewitz, M.F. (2019). Neutrophil extracellular traps in breast cancer and beyond: current perspectives on NET stimuli, thrombosis and metastasis, and clinical utility for diagnosis and treatment. Breast Cancer Res *21*, 145.
Sosa, M.S., Bragado, P., and Aguirre-Ghiso, J.A. (2014). Mechanisms of disseminated cancer cell dormancy: an awakening field. Nat Rev Cancer *14*, 611-622.
Spicer, S.S., and Hardin, J.H. (1969). Ultrastructure, cytochemistry, and function of neutrophil leukocyte granules. A review. Laboratory investigation; a journal of technical methods and pathology *20*, 488-497.
Staiano, R.I., Loffredo, S., Borriello, F., Iannotti, F.A., Piscitelli, F., Orlando, P., Secondo, A., Granata, F., Lepore, M.T., Fiorelli, A*., et al.* (2016). Human lung-resident macrophages express CB1 and CB2 receptors whose activation inhibits the release of angiogenic and lymphangiogenic factors. Journal of leukocyte biology *99*, 531-540.
Stark, M.A., Huo, Y., Burcin, T.L., Morris, M.A., Olson, T.S., and Ley, K. (2005). Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. Immunity *22*, 285-294.
Stojkov, D., Amini, P., Oberson, K., Sokollik, C., Duppenthaler, A., Simon, H.U., and Yousefi, S. (2017). ROS and glutathionylation balance cytoskeletal dynamics in neutrophil extracellular trap formation. The Journal of cell biology *216*, 4073-4090.
Sun, X.S., Sun, S.R., Guevara, N., Fakhry, N., Marcy, P.Y., Lassalle, S., Peyrottes, I., Bensadoun, R.J., Lacout, A., Santini, J*., et al.* (2013). Chemoradiation in anaplastic thyroid carcinomas. Crit Rev Oncol Hematol *86*, 290-301.
Takei, H., Araki, A., Watanabe, H., Ichinose, A., and Sendo, F. (1996). Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. Journal of leukocyte biology *59*, 229-240.
Tamassia, N., Bazzoni, F., Le Moigne, V., Calzetti, F., Masala, C., Grisendi, G., Bussmeyer, U., Scutera, S., De Gironcoli, M., Costantini, C*., et al.* (2012). IFN-beta expression is directly activated in human neutrophils transfected with plasmid DNA and is further increased via TLR-4-mediated signaling. J Immunol *189*, 1500-1509.
Tamassia, N., Le Moigne, V., Calzetti, F., Donini, M., Gasperini, S., Ear, T., Cloutier, A., Martinez, F.O., Fabbri, M., Locati, M*., et al.* (2007). The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. J Immunol *178*, 7344-7356.
Tamassia, N., Zimmermann, M., Castellucci, M., Ostuni, R., Bruderek, K., Schilling, B., Brandau, S., Bazzoni, F., Natoli, G., and Cassatella, M.A. (2013). Cutting edge: An inactive chromatin configuration at the IL-10 locus in human neutrophils. J Immunol *190*, 1921-1925.
Tecchio, C., Scapini, P., Pizzolo, G., and Cassatella, M.A. (2013). On the cytokines produced by human neutrophils in tumors. Semin Cancer Biol *23*, 159-170.
Teijeira, A., Garasa, S., Gato, M., Alfaro, C., Migueliz, I., Cirella, A., de Andrea, C., Ochoa, M.C., Otano, I., Etxeberria, I*., et al.* (2020). CXCR1 and CXCR2 Chemokine Receptor Agonists Produced by Tumors Induce Neutrophil Extracellular Traps that Interfere with Immune Cytotoxicity. Immunity *52*, 856-871 e858.
Todaro, M., Zerilli, M., Ricci-Vitiani, L., Bini, M., Perez Alea, M., Maria Florena, A., Miceli, L., Condorelli, G., Bonventre, S., Di Gesu, G*., et al.* (2006). Autocrine production of interleukin-4 and interleukin-10 is required for survival and growth of thyroid cancer cells. Cancer research *66*, 1491-1499.
Tohme, S., Yazdani, H.O., Al-Khafaji, A.B., Chidi, A.P., Loughran, P., Mowen, K., Wang, Y.M., Simmons, R.L., Huang, H., and Tsung, A. (2016). Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress. Cancer research *76*, 1367-1380.

Trinchieri, G., and Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. Nature reviews Immunology *7*, 179-190.
Urban, C.F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C., Nacken, W., Brinkmann, V., Jungblut, P.R., and Zychlinsky, A. (2009). Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS pathogens *5*, e1000639.
Urban, C.F., Reichard, U., Brinkmann, V., and Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol *8*, 668-676.
Van Avondt, K., van der Linden, M., Naccache, P.H., Egan, D.A., and Meyaard, L. (2016). Signal Inhibitory Receptor on Leukocytes-1 Limits the Formation of Neutrophil Extracellular Traps, but Preserves Intracellular Bacterial Killing. J Immunol *196*, 3686-3694.
Varricchi, G., Galdiero, M.R., Loffredo, S., Marone, G., Iannone, R., and Granata, F. (2017). Are Mast Cells MASTers in Cancer? Frontiers in immunology *8*, 424.
Varricchi, G., Loffredo, S., Bencivenga, L., Ferrara, A.L., Gambino, G., Ferrara, N., de Paulis, A., Marone, G., and Rengo, G. (2020). Angiopoietins, Vascular Endothelial Growth Factors and Secretory Phospholipase A2 in Ischemic and Non-Ischemic Heart Failure. J Clin Med *9*.
Varricchi, G., Loffredo, S., Galdiero, M.R., Marone, G., Cristinziano, L., and Granata, F. (2018). Innate effector cells in angiogenesis and lymphangiogenesis. Curr Opin Immunol *53*, 152-160.
Villanueva, E., Yalavarthi, S., Berthier, C.C., Hodgin, J.B., Khandpur, R., Lin, A.M., Rubin, C.J., Zhao, W., Olsen, S.H., Klinker, M.*, et al.* (2011). Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol *187*, 538-552.
Vinay, D.S., Ryan, E.P., Pawelec, G., Talib, W.H., Stagg, J., Elkord, E., Lichtor, T., Decker, W.K., Whelan, R.L., Kumara, H.*, et al.* (2015). Immune evasion in cancer: Mechanistic basis and therapeutic strategies. Semin Cancer Biol *35 Suppl*, S185-S198.
Visciano, C., Liotti, F., Prevete, N., Cali, G., Franco, R., Collina, F., de Paulis, A., Marone, G., Santoro, M., and Melillo, R.M. (2015). Mast cells induce epithelial-to-mesenchymal transition and stem cell features in human thyroid cancer cells through an IL-8-Akt-Slug pathway. Oncogene *34*, 5175-5186.
von Bruhl, M.L., Stark, K., Steinhart, A., Chandraratne, S., Konrad, I., Lorenz, M., Khandoga, A., Tirniceriu, A., Coletti, R., Kollnberger, M.*, et al.* (2012). Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med *209*, 819-835.
Wang, S., and Wang, Y. (2013). Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. Biochimica et biophysica acta *1829*, 1126-1135.
Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., Hayama, R., Leonelli, L., Han, H., Grigoryev, S.A.*, et al.* (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. The Journal of cell biology *184*, 205-213.
Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309-314.
Ward, L.S. (2014). Immune response in thyroid cancer: widening the boundaries. Scientifica (Cairo) *2014*, 125450.
Weiss, E., and Kretschmer, D. (2018). Formyl-Peptide Receptors in Infection, Inflammation, and Cancer. Trends Immunol *39*, 815-829.
Xu, J., Zhang, X., Pelayo, R., Monestier, M., Ammollo, C.T., Semeraro, F., Taylor, F.B., Esmon, N.L., Lupu, F., and Esmon, C.T. (2009). Extracellular histones are major mediators of death in sepsis. Nat Med *15*, 1318-1321.
Yang, L., Liu, L., Zhang, R., Hong, J., Wang, Y., Wang, J., Zuo, J., Zhang, J., Chen, J., and Hao, H. (2020a). IL-8 mediates a positive loop connecting increased neutrophil extracellular traps (NETs) and colorectal cancer liver metastasis. J Cancer *11*, 4384-4396.
Yang, L., Liu, Q., Zhang, X., Liu, X., Zhou, B., Chen, J., Huang, D., Li, J., Li, H., Chen, F.*, et al.* (2020b). DNA of neutrophil extracellular traps promotes cancer metastasis via CCDC25. Nature *583*, 133-138.
Yazdani, H.O., Roy, E., Comerci, A.J., van der Windt, D.J., Zhang, H., Huang, H., Loughran, P., Shiva, S., Geller, D.A., Bartlett, D.L.*, et al.* (2019). Neutrophil Extracellular Traps Drive Mitochondrial Homeostasis in Tumors to Augment Growth. Cancer research *79*, 5626-5639.
Yipp, B.G., and Kubes, P. (2013). NETosis: how vital is it? Blood *122*, 2784-2794.

Yipp, B.G., Petri, B., Salina, D., Jenne, C.N., Scott, B.N., Zbytnuik, L.D., Pittman, K., Asaduzzaman, M., Wu, K., Meijndert, H.C*., et al.* (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med *18*, 1386-1393.
Yoo, D.G., Winn, M., Pang, L., Moskowitz, S.M., Malech, H.L., Leto, T.L., and Rada, B. (2014). Release of cystic fibrosis airway inflammatory markers from Pseudomonas aeruginosa-stimulated human neutrophils involves NADPH oxidase-dependent extracellular DNA trap formation. J Immunol *192*, 4728-4738.
Young, R.L., Malcolm, K.C., Kret, J.E., Caceres, S.M., Poch, K.R., Nichols, D.P., Taylor-Cousar, J.L., Saavedra, M.T., Randell, S.H., Vasil, M.L*., et al.* (2011). Neutrophil extracellular trap (NET)-mediated killing of Pseudomonas aeruginosa: evidence of acquired resistance within the CF airway, independent of CFTR. PloS one *6*, e23637.
Yousefi, S., Gold, J.A., Andina, N., Lee, J.J., Kelly, A.M., Kozlowski, E., Schmid, I., Straumann, A., Reichenbach, J., Gleich, G.J*., et al.* (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med *14*, 949-953.
Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I., and Simon, H.U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell death and differentiation *16*, 1438-1444.
Yousefi, S., and Simon, H.U. (2016). NETosis - Does It Really Represent Nature's "Suicide Bomber"? Frontiers in immunology *7*, 328.
Yousefi, S., Stojkov, D., Germic, N., Simon, D., Wang, X., Benarafa, C., and Simon, H.U. (2019). Untangling "NETosis" from NETs. European journal of immunology *49*, 221-227.
Zandman-Goddard, G., and Shoenfeld, Y. (2005). Infections and SLE. Autoimmunity *38*, 473-485.
Zhang, X., Majlessi, L., Deriaud, E., Leclerc, C., and Lo-Man, R. (2009). Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. Immunity *31*, 761-771.
Zuo, Y., Yalavarthi, S., Shi, H., Gockman, K., Zuo, M., Madison, J.A., Blair, C., Weber, A., Barnes, B.J., Egeblad, M*., et al.* (2020). Neutrophil extracellular traps in COVID-19. JCI insight *5*.