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PH.D. THESIS

MELANOMA DERIVED SOLUBLE MEDIATORS MODULATE NEUTROPHIL PLASTICITY

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ABSTRACT

Polymorphonuclear neutrophils (PMNs) are main effector cells in the inflammatory responses. The association between PMN infiltration in cancer patients remain to be clarified. Metastatic melanoma is the most lethal type of skin cancer with an increasing incidence over the last decades. Few studies investigated the role of PMNs in human melanoma. The aim of this study was to investigate the role of PMNs and their mediators in human melanoma. Highly purified human PMNs from healthy donors were stimulated, in *vitro,* with conditioned media derived from the melanoma cell lines SKMEL28 and A375 (melanoma-CM) as well as from primary melanocytes as control. PMN functions (chemotaxis, survival, activation, cell tracking, morphology and NETs release) were evaluated. We found that the A375 cell line produced soluble factors able to promote PMN chemotaxis, survival, activation, and to modify PMN morphological changes and kinetic properties. Furthermore, melanoma-CM induced NET release from PMNs. Coherently, primary melanocytes CM did not modify any PMN biological behavior. In addition, serum levels of MPO, MMP-9, CXCL8/IL-8, GM-CSF and NETs components were significantly increased in advanced melanoma patients compared to healthy controls. Melanoma cell lines produce soluble factors able to 'educate' PMNs towards an activated functional state. Metastatic melanoma patients display increased circulating levels of soluble neutrophil-related mediators and NETs, suggesting that a neutrophil-related signature exists in metastatic melanoma patients. Further investigations are needed to better understand the role of these "tumor-educated neutrophils" in modifying melanoma cell behavior.

INTRODUCTION

1. Cancer Development: the six hallmarks revised

Cancer is caused by the uncontrolled proliferation of cells that have lost their normal regulatory systems. 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" refers to a mass of cancerous tissue that has invaded and destroyed healthy neighboring tissues as a result of malignant cells growing. 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 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 retinoblastoma (RB) and TP53 genes are examples of tumor suppressor genes which regulate the decision of cells to proliferate or to activate senescence or apoptotic pathways (Sherr and McCormick 2002, Burkhart, Ngai et al. 2010). Apoptosis is a highly regulated cell death process, essential for the growth and maintenance of a healthy cell population in mature organisms. A crucial aspect of carcinogenesis is the deregulation of apoptotic mechanisms (Adams and Cory 2007). As a result, cancers overcome cell death and maintain their capacity for replication (Blasco 2005). This transition is called “immortalization”. Finally, cancer retains the ability to promote angiogenesis, invasion and metastasis. Angiogenesis and lymphangiogenesis are processes by which new blood and lymphatic vessels form, respectively. However, in a more recent point of view, tumors are not merely considered as masses composed of proliferating cells but a complex microenvironment characterized by several cell types which interact each other. Two new hallmarks of cancer have been identified during the past 20 years as a result of rigorous experimental work: the ability to modify cellular metabolism and the resistance to immune system. Otto Warburg was the first to describe a peculiar aspect of cancer cell energy metabolism: cancer cells modify their glucose metabolism, and consequently to produce

energy, even in the presence of oxygen by restricting their metabolism primarily to glycolysis, resulting in a condition known as "aerobic glycolysis" (Warburg 1956). Mechanisms resulting in evasion of the immune system include the selection of tumor variants resistant to immune effectors ("immunoediting") and the progressive formation of an immune suppressive environment within the tumor (Vinay, Ryan et al. 2015). The acquisition of these tumor hallmarks is possible by mean of two enabling factors: cancer cell genetic instability and cancer-related inflammation (Hanahan and Weinberg 2011).

2. Cancer related inflammation

In 1863 Rudolf Virchow first described the link between cancer and inflammation. Indeed, he discovered that tumors raised at sites of chronic inflammation, and that some kinds of irritants, in combination with tissue injury and inflammation, promoted cell proliferation (Balkwill and Mantovani 2001). The tumor microenvironment (TME) results from the interactions between cancerous and unaltered cells, through a network of inflammatory mediators (cytokines, chemokines, prostaglandins). TME consists on immune cells, pericytes, fibroblasts, and blood and lymphatic vessels (Quail and Joyce 2013). Stromal and tumor cells produce chemokines and other molecules that contribute to the activation and recruitment of tumor-associated macrophages (TAMs) (Bottazzi, Polentarutti et al. 1983). The main factors supporting macrophage survival and polarization include vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), and macrophage colony stimulating factor (M-CSF/CSF1) (Bottazzi, Polentarutti et al. 1983, Bottazzi, Polentarutti et al. 1983). CCL2/MCP1 is the main chemotactic factor for monocytes (Li, Knight et al. 2013). In response to signals derived from TME, macrophages undergo polarization, i.e. they are able to finely modulate their phenotype and therefore activate specific functional programs, which leads to two different polarization states (Mantovani, Sica et al. 2004, Biswas and Mantovani 2010, Galdiero, Garlanda et al. 2013). Classical activation in M1 occurs

upon stimulation by microbial components (such as Lipopolysaccharide – LPS) and/or INF-γ and is characterized by antigen presentation capacity, high IL-12 and IL-23 production, activation of type IV hypersensitivity response and high reactive oxygen species (ROS) production (Mantovani, Sica et al. 2004). For these reasons, M1 cells promote resistance to intracellular pathogens and tumors. Alternative signals such as IL-4, IL-13 and/or IL-10 induce polarization towards the M2 phenotype, characterized by poor antigen presenting ability, poor production of pro-inflammatory cytokines, ability to produce immunosuppressive cytokines (such as IL-10) and pro-angiogenic factors (such as VEGF-A) (Mantovani, Sica et al. 2004). For these characteristics, M2 macrophages play an important role in the modulation of the inflammatory response, in the promotion of angiogenesis and tissue remodeling. TAMs were found in several tumor types and were associated with poor prognosis (Bingle, Brown et al. 2002). TAMs are not the only cell population that influence tumor growth and progression. Recent evidence reveals that also neutrophils infiltrate tumors (TANs) and play important roles in tumor progression (Fridlender, Sun et al. 2009).

3. The Neutrophils

Neutrophils were discovered by Paul Ehrlich in the late nineteenth century and are the most abundant white blood cells in the human circulation. Neutrophil granules contain a variety of enzymes, membrane and matrix proteins and are classified in three major groups based on their protein content: primary (azurophilic), secondary and tertiary granules. The primary granules are characterized by the presence of myeloperoxidase, a bactericidal and hydrolytic enzyme; the secondary granulesare characterized by the presence of lactoferrin and lysozyme; tertiary granules are characterized by the presence of metalloprotease 9 (MMP-9, also called Gelatinase B) (Bainton, Ullyot et al. 1971).

Neutrophils are as the first line of defense against pathogens and, since their discovery and characterization, their significance was limited to the early stages of inflammation and resistance to extracellular infections (Borregaard 2010, Amulic, Cazalet et al. 2012, Jaillon, Galdiero et al. 2013).

Indeed, neutrophils were characterized by limited lifespan and terminally differentiated phenotype and their function in coordinating the different phases of inflammation and immune response has been neglected for long time. This point of view has been changed by new evidences suggesting several novel functional aspects for these underestimated cells. The discovery that some conditions, such as chronic inflammation, can extend the lifespan of neutrophils together with the development of new methods for obtaining highly pure neutrophil preparations have provided new insights into the functions of neutrophils (Jaillon, Galdiero et al. 2013).

3.1 Granulocytopoiesis

Under the influence of cytokines and growth factors, in the bone marrow (BM) hematopoietic stem cells differentiate into myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells and finally granulocytes (Borregaard 2010). Only mature neutrophils exit the BM under physiological conditions, and CXCR4 is essential for controlling neutrophil homing or egression (Lapidot and Kollet 2002). BM hematopoietic stem cells express CXCR4, which binds the stromal cell protein CXCL12/SDF-1, which is mostly expressed by osteoblasts. Myeloid cell retention in the BM is mediated by CXCR4, whose expression gradually declines during myeloid maturation. In parallel, CXCR2 expression increases and controls BM egression of the mature neutrophil (Lapidot and Kollet 2002). Indeed, CXCR2 deficiency results in a myelokathexis-like phenotype with a retention of neutrophils in the BM. CXCR2 signals are not needed for neutrophil mobilization if CXCR4 is absent. Neutrophil egression and chemotaxis within the circulation are promoted by endothelial-derived chemokines (CXCR2 ligands), whereas BM osteoblasts release CXCL12 which mediates neutrophil retention.

The primary factor controlling the differentiation of neutrophils is granulocyte colony stimulating factor (G-CSF). G-CSF is produced in the BM by stromal cells of the monocyte/macrophage lineage, vascular endothelial cells, fibroblasts, and mesothelial cells. G-CSF stimulates the proliferation,

differentiation, and activation of granulocyte precursors (Demetri and Griffin 1991). G-CSF is essential during infections, but it is not strictly needed for granulocytopoiesis since G-CSF$^{-/-}$ mice still exhibit residual granulocytopoiesis and release mature neutrophils (Lieschke, Grail et al. 1994).

A process known as the "neutrostat regulatory loop" is responsible for the regulation of neutrophil production in the bone marrow (Stark, Huo et al. 2005, Ley, Smith et al. 2006). The rate of apoptosis of peripheral neutrophils is an important regulator of myelopoiesis. Under homeostatic conditions, BM neutrophil production and peripheral neutrophil removal are balanced (Stark, Huo et al. 2005). Neutrophil turnover can be modulated during an inflammatory response (Colotta, Re et al. 1992). IL-23 is produced by macrophages and dendritic cells (DCs) in inflammatory conditions in gut-associated lymphatic tissue. IL-23 stimulates Th17 cells to produce IL17A, which in turn enhances the production of G-CSF by BM stromal cells (Stark, Huo et al. 2005). When neutrophils complete their functions in target tissues, undergo apoptosis and are phagocytosed by macrophages. This process, called “efferocytosis”, reduces IL-23 production by myeloid cells, completing the feedback that regulates granulocytopoiesis (Stark, Huo et al. 2005). Several *in vivo* studies described that a circadian rhythm was involved in the patrol of circulating neutrophil. This mechanism was related to a feedback circuit that involves selectins, chemokine receptors and β2-integrins. As circulating neutrophils get older, the expression of CXCR4 and CD11b increases and L-selectin (CD62L) is downregulated. Neutrophils with a CD62^{Low}CXCR4^{high} phenotype re-enter the BM and are eliminated by the local macrophages (Casanova-Acebes, Pitaval et al. 2013). Phagocytosis of aged neutrophils by BM-associated macrophages reduces the production of CXCL12 by macrophages (Casanova-Acebes, Pitaval et al. 2013). This modulation of the neutrophil trafficking is caused by nervous system signals generated from the light cycle.

3.2 Neutrophil activation

After leaving the BM, mature neutrophils reach the sites of tissue inflammation through the circulation. The migration of neutrophils at sites of injury is a process referred to as “neutrophil recruitment” and involves sequential steps: tethering, rolling, adhesion, crawling and, transmigration (Hallett and Lloyds 1995). Some molecules, such as the bacterial-derived lipopolysaccharide (LPS) and Formylmethionyl-leucyl-phenylalanine (fMLP), as well as chemoattractants and cytokines, such as tumor necrosis factor (TNFα), interleukin (IL)-1β, and IL-17, increase the expression of adhesion molecules on endothelial cells (P-selectins, E- selectins and several members of the integrin superfamily, ICAMs) (Borregaard 2010). On the surface of neutrophils, two constitutively expressed proteins are critical for the recognition of the endothelial inflammatory signals: the P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin (Kolaczkowska and Kubes 2013). Upon random contact with the endothelium, these molecules engage P- selectins and E-selectins on endothelial cells, resulting in tethering of neutrophils to the vessel wall. Chemoattractants and cytokines induce changes in the conformation of the cell surface-expressed integrins. This process is important for the “firm adhesion” (Yago, Shao et al. 2010). Indeed, neutrophil adhesion is mediated by the β2-integrins LFA-1 and Mac-1 (Issekutz and Issekutz 1992). β2 integrin binding to ICAM1 is essential for firm adhesion. Some studies revealed that immediately upon adhesion, there is a significant crawling of neutrophils to distant sites (Phillipson, Heit et al. 2006). This integrin-dependent step of the recruitment cascade prepares neutrophils for transmigration. In fact, neutrophils crawl along the vessel wall until a preferred site of transmigration is reached. Crawling neutrophils move towards endothelial junctions localized between endothelial cells. Then, to go through the basement membrane, and finally migrate between pericytes. Then they leave the vasculature and reach the tissue site (Kolaczkowska and Kubes 2013).

3.3 Neutrophils and Inflammation

Inflammation is a physiological response against tissue damage, characterized by multiple signals that initiate and sustain this response (Majno 1998). Several cellular elements are attracted where tissue damage occurs, given the expression of chemokines and adhesion molecules (Coussens and Werb 2002). Neutrophils produce several mediators able to remodel the extracellular matrix, oxidants (ROS) and vasoactive mediators (de Visser, Eichten et al. 2006). Neutrophil effector functions are phagocytosis, degranulation, ROS production and the release of Neutrophil Extracellular Traps (NETs). In addition, neutrophils produce cytokines, which further fuel the inflammatory response (Tecchio, Scapini et al. 2013). When the inflammatory response is resolved, the neutrophil undergoes apoptosis.

Neutrophils are professional phagocytes (Lee, Harrison et al. 2003). The interaction between phagocytes and foreign body is accompanied by intracellular signals that trigger different cellular processes such as the rearrangement of the cytoskeleton, the alterations in the membrane traffic, the activation of killing mechanisms, the production of cytokines and chemokines (Aderem and Underhill 1999, Greenberg 1999). The particle internalization process involves a variety of distinct molecular and morphological processes (Aderem and Underhill 1999). Internalization of antibody-opsonized particles is characterized by extension of the phagocyte membrane around the particles, recruitment of Syk tyrosine kinase, and production of pro-inflammatory mediators. In contrast, phagocytosis of complement-opsonized particles occurs without appreciable membrane extension, does not require Syk and is often not accompanied by the production of inflammatory mediators (Aderem and Underhill 1999). Phagocytosis is a complex process in which a microorganism is internalized in a vacuole called "phagosome". Once the foreign body has been engulfed in the phagosome, a process of phagosome maturation begins: the NADPH oxidase enzyme complex is assembled on the phagosome membrane and ROS are produced. Then the phagosome environment becomes toxic to the phagocytosed elements (Lee, Harrison et al. 2003). ROS are highly reactive small molecules,

which constantly interact each other, resulting in the formation of a myriad of oxidant molecules that can be responsible for oxidative DNA damage that can promote mutagenesis in target cells (Knaapen, Gungor et al. 2006).

3.4 Neutrophil Extracellular Traps (NETs)

The formation of NETs is one of the neutrophil antimicrobial weapon, which was first discovered in 2004 (Brinkmann, Reichard et al. 2004). NETs are filamentous chromatin structures composed of histones, proteases, granular and cytosolic proteins (Jorch and Kubes 2017). The release of NETs favors the immobilization and capture of bacteria (Brinkmann, Reichard et al. 2004, Pilsczek, Salina et al. 2010, Amini, Stojkov et al. 2018, Robledo-Avila, Ruiz-Rosado et al. 2018), fungi (Muniz, Silva et al. 2018, Silva, Rodrigues et al. 2020) and viruses (Yazdani, Roy et al. 2019, Zuo, Yalavarthi et al. 2020). 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). The production of oxygen peroxide by NADPH oxidase and subsequent conversion by myeloperoxidase (MPO) is another important factor in NET formation. In fact, neutrophils lacking MPO or NAPDH (such as in patients with Chronic Granulomatous Disease, or CGD), show reduced NET synthesis, that further accounts for the increased susceptibility to infections of CGD (Bianchi, Hakkim et al. 2009, Metzler, Fuchs et al. 2011). The NETosis process is tightly regulated. Indeed, the activation of this process depends on the balance between two biological activities: nuclear translocation and phagolysosome homing for NE. 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, Lubojemska et al. 2014). Once in the nucleus, NE cleaves histones, decondenses

chromatin and drives NET formation (Papayannopoulos, Metzler 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 strategies to circumvent phagocytosis, NET formation can also be viewed as an immune response to overcome these resistance strategies (Branzk, Lubojemska et al. 2014).

Similarly to other neutrophil related molecules, NETs can also be dangerous for the host. Excessive and deregulated NET production causes a variety of pathologic diseases, including autoimmunity, sepsis, and thrombosis. A subset of systemic lupus erithemathosus (SLE) patients, for example, display an impaired DNAse-1 activity, due to the presence of DNAse-1 inhibitors or to antibodies that bind NETs and protect them from DNAse-1. The persistence of NETs correlated with anti-dsDNA titers and lupus nephritis. Anti-NETs antibodies and persistent NETs could also form "NET immunocomplexes", which are relevant in the exacerbation of SLE and could be pathogenic in the development of lupus nephritis (Hakkim, Furnrohr et al. 2010). 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, Brill et al. 2010). In a murine model of deep venous thrombosis, neutrophils were needed for activation of factor XII and propagation of the thrombosis cascade (von Bruhl, Stark 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, Shibamiya et al. 2007). Moreover, histones contained in NETs are cytotoxic for endothelium and can be responsible for thrombosis *in vivo* (Xu, Zhang et al. 2009).

The term "NETosis" was initially coined to indicate a mechanism of cell death in addition to necrosis and apoptosis, which follows the release of NETs (Nathan 2006). PMA is one of the main inducers of NETosis, dependent on ROS production (Takei, Araki et al. 1996). Beyond PMA, additional agonists responsible for NET release were discovered, such as IL-8/CXCL8, IFN-γ, IFN- γ/C5a, GM-CSF/C5a, and LPS (Brinkmann, Reichard et al. 2004, Yousefi, Mihalache et al. 2009, Caielli, Athale

et al. 2016, Amini, Stojkov et al. 2018). NETosis classically occurs within 2–4 h after neutrophil activation (Fuchs, Abed et al. 2007, Keshari, Verma et al. 2013, Douda, Yip et al. 2014) and is also called as "suicidal" NETosis, since the release of NETs is associated with cell death. Recently, a NADPH oxidase -independent NET formation connected to mitochondrial ROS generation was also identified (Brinkmann, Reichard et al. 2004, Remijsen, Vanden Berghe et al. 2011). During suicidal NETosis, PMA binds to protein kinase C (PKC), which induces the release of calcium from intracellular stores leading to Raf-MEK-ERK pathway activation (Takei, Araki et al. 1996, Brinkmann, Reichard et al. 2004, Hakkim, Furnrohr et al. 2010), as well as to the ROS-dependent activation of p38 MAPK (Keshari, Verma et al. 2013). MPO and NE are released from the azurophil granules and translocate into the nucleus (Papayannopoulos, Metzler et al. 2010, Metzler, Goosmann et al. 2014). The increase of intracellular calcium levels activates the PAD4. PAD4 catalyses the deamination of histones H2A, H3 and H4, changing the arginine residues into citrullines. As a result, histones lose their positive charge and chromatin loses its compaction (Neeli, Dwivedi et al. 2009). As a final result of this activation pathway, the neutrophil dies.

Quite recently, a different form of NETosis was described, named “vital NETosis”, in which the intracellular content is released in the extracellular space but the neutrophil remains alive (Yousefi, Mihalache et al. 2009, Yipp and Kubes 2013, Jorch and Kubes 2017, Amini, Stojkov et al. 2018, Cristinziano, Modestino et al. 2020). Vital NETosis was first described following the priming of granulocyte-macrophage colony stimulating factor (GM-CSF), and stimulation with C5a or LPS. The vital NET release occurred also within few minutes of bacterial stimulation with *Staphylococcus aureus* (Pilsczek, Salina et al. 2010). In vital NET formation, the multilobed nucleus was rapidly 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 release the chromatin. This entire process occurs via a unique, very rapid (5–60 min), oxidant-independent mechanism (Pilsczek, Salina et al. 2010).

Recently we demonstrated, in the context of cancer, that soluble mediators derived from anaplastic thyroid cancer (ATC) cell lines induced mitochondrial ROS activation and vital release of NETs containing mitochondrial DNA. We also found that ATC-induced NETs promoted *in vitro* the viability of cancer cells, suggesting an important tumor-promoting role of NETs in ATC (Cristinziano, Modestino et al. 2020).

3.5 Neutrophil and NETs in Cancer

Neutrophils were identified among the inflammatory cells infiltrating human tumors (Donskov 2013). Neutrophils can be recruited to sites of tumorigenesis by chemokines such as CXCL8/IL-8 and/or CXCL1/Gro-α (Eck, Schmausser et al. 2003). In the context of cancer-related inflammation, neutrophils are a source of inflammatory mediators (Mantovani 2009). TANs were proposed as key mediators of neoplastic transformation, tumor progression, angiogenesis and in the modulation of the antitumor immune response (Mantovani, Cassatella et al. 2011). In particular, recent experimental evidence indicates that neutrophils can be endowed with unsuspected plasticity. In fact, on the one hand they can inhibit tumor growth by killing tumor cells, on the other hand they can promote tumor progression (Piccard, Muschel et al. 2012, Galdiero, Bonavita et al. 2013, Galdiero, Varricchi et al. 2018). By mimicking the polarization of macrophages in M1 and M2 (Biswas and Mantovani 2010), it has been proposed that also neutrophils can undergo polarization into N1 and N2 phenotypes (Fridlender, Sun et al. 2009). N1 neutrophils, mainly induced by IFN-β-related signaling, are characterized by increased expression of TNF-α, poor production of VEGF-A and MMP-9 and poor cytotoxic functions. Upon the influence of TGF-β, neutrophils turn towards a defined N2 phenotype, with pro-tumorigenic, pro-angiogenic and immunosuppressive functions (Fridlender, Sun et al. 2009). In recent years, several experimental evidences demonstrate that tumor infiltration of neutrophils and their accumulation in the neoplastic tissue were associated with poor patient prognosis (Bellocq, Antoine et al. 1998, Wislez, Rabbe et al. 2003, Jensen, Donskov et al. 2009,

Kuang, Zhao et al. 2011, Jensen, Schmidt et al. 2012). In a mouse model of lung inflammation, sustained activation of neutrophils lead to the production of ROS which favored DNA damage, increased mutation rate and cancer initiation (Gungor, Knaapen et al. 2010). In mouse models of lung cancer, NE promoted tumor growth and progression. In fact, NE degraded the insulin receptor (IRS-1), favoring the interaction between phosphatidylinositol 3-kinase (PI3K) and the platelet-derived growth factor receptor (PDGFR): this binding promoted the proliferation of tumor cells (Houghton 2010, Houghton, Rzymkiewicz et al. 2010). GM-CSF stimulated neutrophils to produce Oncostatin M, which induced the release of VEGF from human breast tumor cells promoting their invasive potential (Queen, Ryan et al. 2005). Neutrophil infiltration in human tumors such as renal carcinomas (Jensen, Donskov et al. 2009), squamous cell carcinomas of the head and neck (Trellakis, Bruderek et al. 2011) and bronchoalveolar carcinomas (Wislez, Rabbe et al. 2003) was associated with poor prognosis. However, in some tumors such as gastric tumors, the density of TANs is associated with a favorable prognosis (Caruso, Bellocco et al. 2002) and therefore the role of neutrophils in cancer is still controversial.

Evidence on the role of NETs in cancer has increased in recent years. NETs were reported to drive endothelial-to-mesenchymal transition (EMT) which plays an important role in tumorigenesis (Visciano, Liotti et al. 2015, Pieterse, Rother et al. 2017). In mouse models of Lewis lung carcinoma and 4T1-induced breast cancer, tumor-bearing mice displayed higher plasma NET levels compared to control mice. In tumor-bearing mice, neutrophils displayed an increased susceptibility to produce NETs spontaneously, which correlated with thrombi formation and a pro-coagulation state (Demers, Krause et al. 2012, Park, Wysocki et al. 2016). In a mouse model of orthotopic pancreatic adenocarcinoma, genetic deletion of PAD4 decreased circulating NET levels and increased the survival of tumor-bearing mice (Miller-Ocuin, Liang 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, Yang et al. 2019).

CXCL8/IL-8, autocrinously produced by several human cancer cell lines, can induce NET formation from neutrophils and G-MDSCs (Alfaro, Teijeira et al. 2016, Bates, Gomez Hernandez et al. 2018, Cristinziano, Modestino et al. 2020). Beyond CXCL8/IL-8, several cancer-related stimuli (CXCR1/CXCR2-ligands, G-CSF, TGF-β) can induce the release of NETs from human and murine neutrophils, thus linking cancer and NET release (Alfaro, Teijeira et al. 2016, Azevedo, Paiva et al. 2018, Weiss, Hanzelmann et al. 2018, Teijeira, Garasa et al. 2020). Moreover, NETs predict improved survival in patients with head and neck squamous cell carcinoma and exert cytotoxic effects on melanoma cells (Millrud, Kagedal et al. 2017, Schedel, Mayer-Hain 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.

4. Melanoma

Melanoma of the skin is the second most prevalent cancer type in males and the fourth in women in United States (Miller, Nogueira et al. 2022). Metastatic melanoma is a fatal condition. New lifestyles and global atmospheric changes have gradually increased UV exposure of skin, which has mirrored an increase in the prevalence of melanoma (Karimkhani, Boyers et al. 2015). Malignant melanoma can arise from benign nevi or de novo. Malignant melanoma can arise on mucosal epithelium, uvea, and non-UV-exposed skin, and these melanoma forms typically lack the distinctive BRAF mutation (Timar and Ladanyi 2022). Skin and uveal melanoma both have a familial form, although they have different genetic basis. Besides the loss of CDKN2A (cyclin-dependent kinase inhibitor 2A), germline mutations of CDK4 (cyclin-dependent kinase 4), MITF (microphtalmia-associated transcription factor) and BAP1 (BRCA1-associated protein 1) are the most significant contributors for hereditary melanoma (Law, Macgregor et al. 2012). According to several assessments, the activating mutation of the BRAF oncogene in exon 15/codon 600, which is present in over half of these tumors, is the most common gene defect of cutaneous melanoma (Timar, Vizkeleti et al. 2016,

Timar and Ladanyi 2022). With a lower frequency (~20%), the NRAS (neuroblastoma RAS viral (v-ras) oncogene homolog) oncogene is mutated in melanoma in exon 3/codon 61. Interestingly, with similar frequency (<15%) the KIT (KIT proto-oncogene, receptor tyrosine kinase) gene is also mutated in melanoma (Doma, Barbai et al. 2020). The BRAF-mutant, RAS-mutant, NF1-mutant, and so-called triple wild-type forms are the four basic groups that can be currently classified using molecular biology (Cancer Genome Atlas 2015).

5. Role of Immune System in Melanoma

The prognostic value of immune cell infiltration in primary melanoma was analyzed in several studies. A prominent lymphocyte infiltration was an independent prognostic factor for better survival in several studies. However, these data were not confirmed in additional reports (Timar and Ladanyi 2022). Controversial results were reported on the prognostic value of infiltration by T lymphocytes (including CD4+ and CD8+ subsets), or macrophages by immunohistochemistry. A positive prognostic significance of mature dendritic cells and B cells, was described (Ladanyi 2015). With regards of melanoma, UVB modulate the recruitment of inflammatory cells into the skin, including macrophages and neutrophils (Wolnicka-Glubisz, Damsker et al. 2007, Zaidi, Davis et al. 2011, Bald, Quast et al. 2014, Senft, Sorolla et al. 2015). UV irradiation induced neutrophil chemotaxis in the skin, due to the release of HMGB1 from UV-damaged keratinocytes. This neutrophilic inflammation promoted angiotropism and distant metastasis of melanoma cells (Bald, Quast et al. 2014). We recently demonstrated that peripheral blood PD-L1+ PMN frequency efficiently predicted patient prognosis and response to the anti-PD-1 agent nivolumab in stage IV BRAF wild type melanoma patients (Cristinziano, Modestino et al. 2022).

AIM

The aim of this study was to investigate the role of neutrophils and their mediators in human melanoma.

MATERIALS AND METHODS

Cell cultures and preparation of tumor-conditioned media (CM)

Human melanoma cell lines SKMEL28, A375 were from ATCC, cultured and maintained in RPMI 1640 supplemented with 10% of heat-inactivated fetal calf serum (FCS; endotoxin level <0.1 EU/ml), 50 U/ml penicillin/streptomycin and 2 mM L-glutamine (Euroclone, Milan, Italy) at 37°C in a humidified atmosphere containing 5% of CO2 and 95% of air. Human Epidermal Melanocytes, adult, lightly pigmented donor, (HEMa-LP) were cultured and maintened, according to the manufacturer instructions (ThermoFisher, Waltham, MA, USA). Conditioned media (CM) were prepared and used as follows (Galdiero, Varricchi et al. 2018). 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 µm pore size filter), and stored at -20°C. All cell lines were routinely checked for *Mycoplasma* contamination.

Neutrophil purification and culture

The study protocol involving the use of human blood cells was approved by the Ethical Committee of the University of Naples Federico II (prot. n. 301/18), 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 samples (hepatitis B surface Ag-, hepatitis C virus -, and HIV-) obtained from a leukapheresis unit. Leukocytes were separated from erythrocytes by dextran sedimentation. PMNs were purified by Ficoll-Paque Histopaque-1077 (Sigma-Aldrich, Milan, Italy) density gradient centrifugation (400 x g for 30 min at 22°C), followed by Percoll (Sigma-Aldrich) (65%) density gradient centrifugation (660 x g for 20 min at 22°C), as previously described (Muzio, Re et al. 1994). Finally PMNs 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, Tamassia et al. 2017). These cells were >99% PMNs as evaluated by flow cytometric analysis with the following antibodies: anti-CD3, anti-CD14, anti-CD15, anti-CD11b, anti-CD193 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD62L (L-selectin) (BD Biosciences, San Jose, CA, USA) and anti-CD66b (BioLegend, CA, USA). Samples were analyzed on the MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and the FlowJo software, v.10. Dead cells, doublets, debris and eosinophils were excluded from the analysis. Data were expressed as percentage of positive cells or median fluorescence intensity (MFI) (Borriello, Iannone et al. 2016).

Cell migration assay

Migration of PMNs toward melanoma-CM was evaluated by means of a 3 µm cell culture insert in a 96-well companion plates (Corning Costar, New York, USA). The companion plates were loaded with 235 µL of a CM or control medium. PMNs (2.5 x 106 PMNs /ml per 75 µL) were placed in the insert and allowed to migrate at 37°C and 5% CO2 for 1 hour. At the end of incubation, the cells were centrifugated and resuspended in 100 µL of PBS and counted by flow cytometry (MACS Quant Analyzer 10, Miltenyi Biotec, Bergisch Gladbach, Germany). In some experiments, PMNs were pre-incubated with mouse monoclonal anti-CXCR1 and/or anti-CXCR2 blocking antibodies at 10 µg/mL (clone 42705 and clone 48311 respectively, R&D System, Minneapolis, MN, USA) or the corresponding control isotype (R&D System, Minneapolis, MN, USA) at 37°C and 5% CO2 for 60 minutes and then subjected to the migration assay as already described above.

Apoptosis assay

PMNs (2.5 x 106 cells/mL) were cultured in A375 CM with or without the mouse monoclonal anti-GM-CSF blocking antibody at 10 µg/mL (clone 3209, R&D System, Minneapolis, MN, USA) or the corresponding control isotype (R&D System, Minneapolis, MN, USA). PMNs were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) according to the protocol provided by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Quantification was performed on a MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Live cells were assumed to be double-negative annexin V- PI-. Analysis was performed by means of FlowJo v.10.

Flow cytometry

PMNs were kept in RPMI 1640 with 10% of FCS for 30 min at 37°C, then were washed with PBS solution and stimulated with SKMEL28 CM, A375 CM, HEMa CM or control medium with 5% of FCS for 90 minutes at 37°C with 5% of CO2. 2.5 x 105 cells were seeded in 96-well plate (ThermoFisher, Waltham, MA, USA) and Zombie Violet dye (BioLegend, CA, USA) was added to evaluate cell viability (20 min, +4°C). Then the cells were stained (20 min, +4°C) in PBS containing 1% FBS. The following antibodies were used: allophycocyanin (APC)-conjugated anti-CD66b (clone REA306, diluition 1:50, from Miltenyi Biotech, Bergisch Gladbach, Germany), VioBlue-conjugated anti-CD193 (clone REA574, 1:10, from Miltenyi Biotec, Bergisch Gladbach, Germany), peridinin chlorophyll protein (PerCP)-conjugated anti-CD11b (clone REA713, diluition 1:50, from Miltenyi Biotech, Bergisch Gladbach, Germany), fluorescein isothiocyanate (FITC)-conjugated anti-CD62L (clone 145/15, diluition 1:50, from Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were acquired by MACS Quant Analyzer 10 (Miltenyi Biotech, Bergisch Gladbach, Germany) and analyzed by FlowJo software, v.10. Doublets and debris (identified based on forward and side scatter

properties), dead cells (identified with Zombie Violet Fixable Viability Kit; BioLegend) and eosinophils (identified based on the CCR3+ exclusion gate) were excluded from the analysis.

Fluorescence, time-lapse, and high-content microscopy

Microscopy experiments were conducted using the Operetta High-Content Imaging System (PerkinElmer, Waltham, MA, USA), as previously described (Gobert, Treilleux et al. 2009, Borriello, Iannone et al. 2016). To evaluate NETs release, PMNs were seeded in 96-well black CellCarrier plates (PerkinElmer, Waltham, MA, USA) in SKMEL28, A375, HEMa CM or control medium, in the presence or absence of 0.5 µM of the cell impermeant SYTOX Green Nucleic Acid Stain (ThermoFisher, Waltham, MA, USA) at a controlled temperature (37°C) and CO2 concentration (5%). Nuclei were stained with the bisbenzimide DNA dye Hoechst 33342 (ThermoFisher, Waltham, MA, USA). For time-lapse experiments, PMNs were cultured for up to 60 minutes. Within this time period, fluorescence microscopy images of three fields per well were captured every 20 min through a 10X objective. PhenoLOGIC (PerkinElmer, Waltham, MA, USA) 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 PMNs were incubated with SKMEL28 or A375 CM with 10 µg/mL of monoclonal anti-CXCL8/IL-8 and/or anti-GM-CSF blocking antibodies (clone 6217 and clone 3209, respectively; R&D System, Minneapolis, MN, USA) or the relative control isotype (R&D System, Minneapolis, MN, USA) at 37°C and 5% CO2 for 60 minutes. 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 cell (b) was calculated using the following formula: (a/b) x 100. To quantify cell morphological features, PMNs were seeded in 96-well black CellCarrier plates (PerkinElmer, Waltham, MA, USA) and cultured overnight. Within this time window, digital phase contrast images of 15 fields/well were captured every 15 minutes via a 20X objective. PhenoLOGIC (PerkinElmer,

Waltham, MA, USA) was employed for image segmentation and for calculating the single-cell morphological results by the dedicated STAR analysis sequence (Borriello, Iannone et al. 2016). STAR morphology is an enhanced series of algorithms that provide a statistically powerful set of properties for analyzing phenotypes by characterizing cell morphology and the distribution of intensity within regions. The STAR method offer the possibility to calculate symmetry properties, threshold compactness, axial properties, radial properties, and profile (Borriello, Iannone et al. 2016, Borriello, Iannone et al. 2017).

Quantification of soluble mediators in culture supernatants or total protein lysates

CXCL8/IL-8, CXCL1/Gro-α, CXCL2/Gro-β, GM-CSF, MMP-9 and MPO concentrations in cell free CM, total protein lysates (0.1% Triton X-100) or patients sera, were assessed in duplicate with commercially available ELISA kits (R&D System, Minneapolis, MN, USA). MMP-9 and MPO levels in total protein lysates were normalized to total protein concentrations as determined by a Bradford protein assay (Bio-Rad, Hercules, CA, USA) and expressed in micrograms of protein per milligram of total protein. A microplate reader (Tecan, Grodig, Austria, GmbH) was used to determine sample absorbance at 450 nm. The ELISA sensitivity is 31.2-2000 pg/mL (CXCL8/IL-8 and CXCL1/Gro-α), 15.6-500 pg/mL (CXCL2/Gro-β), 7.80-500 pg/mL (GM-CSF), 31.2-2000 pg/mL (MMP-9) and 62.5-4000 pg/mL (MPO).

Serum NET detection

Serum circulating levels of DNA fragments (mono- and oligonucleosomes) and Citrullinated Histone H3 (CitH3) were measured by using two different assay. Serum circulating concentrations of mono- and oligonucleosomes were measured using Cell Death Detection ELISA kit (Roche, Basel, Swiss). Concentration of CitH3 in serum samples of melanoma patients and/or healthy controls was measured using ELISA kit developed by Cayman Chemicals (Ann Arbor, MI, USA). This kit used a specific monoclonal antibody for histone H3 citrullinated at residue R2, R8 and R17 (clone 11D3). A microplate reader (Tecan, Grodig, Austria, GmbH) was used to determine the mono- and oligonucleosomes and CitH3 sample absorbance respectively at 405 nm and 450 nm. The ELISA sensitivity range was 0.15 – 10 ng/ml (CitH3).

Patients

27 patients were recruited with a diagnosis of stage IV melanoma, according to VII edition of American Joint Committee on Cancer (Balch, Gershenwald et al. 2009) at the Istituto Nazionale Tumori—IRCCS—Fondazione "G. Pascale" of Naples, Italy. All patients had provided written informed consent for the use of samples according to the institutional regulations (prot. no 33/17). Peripheral blood samples were collected from all patients and freshly processed at diagnosis. Moreover, blood samples of 22 healthy donors, sex and age-matched, were collected at the University of Naples Federico II, Naples, Italy. The study was approved by the local Ethics Committee of the Istituto Nazionale Tumori - IRCCS - Fondazione "G. Pascale" of Naples (prot. no 33/17) and of University of Naples Federico II (n. 301/18) and was conducted in accordance with the international standards of good clinical practice and with the provisions of the Declaration of Helsinki. Serum samples were obtained (+4°C, 400 g, 20 min) and stored (-80°C) until used.

Statistical analysis

Statistical analysis was performed by using Prism 8 (GraphPad software). Data are expressed as mean ± SEM of the indicated number of experiments. D'Agostino & Pearson normality test was used to test for normality of distribution, and statistical methods were chosen to fit non-normal distribution when appropriate. Values from groups were compared by Student's t test or Mann-Whitney U test based on 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. Correlations between two variables were assessed by Spearman rank correlation analysis and reported as coefficient of correlation (r). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Melanoma conditioned media induced PMN chemotaxis

In a first set of in vitro experiments, we studied the capability of melanoma cell lines to induce the migration of PMNs, referred to as chemotaxis. Highly purified PMNs (≥99%) from peripheral blood of healthy donors were allowed to migrate toward a melanoma-CM from SKMEL28 or A375 cell lines conditioned media (SKMEL28 CM, A375 CM), primary melanocytes conditioned media (HEMa CM) or toward control medium. After 1 hour of incubation, migrating cells were counted by flow cytometry. SKMEL28 CM and A375 CM induced PMN migration as compared to the control medium as well as compared to the HEMa CM (**Figure 1A**). By contrast, HEMa CM was not able to induce PMN migration. These results suggested that SKMEL28 CM and A375 CM selectively released soluble factors able to induce PMN chemotaxis. Melanoma cell lines produce several CXC chemokines (Rossi, Cordella et al. 2018, Shang and Li 2019), which can be responsible for PMN chemotaxis (Trellakis, Bruderek et al. 2011). CXCL8/IL8, CXCL1/Gro-α, and CXCL2/Gro-β are well-known to have a chemotactic activity, through CXCR1/2, and have an important role in the tumor microenvironment (TME) (Cyster 1999, Dhawan and Richmond 2002, Waugh and Wilson 2008). Large quantities of CXCL8/IL8 and CXCL1/Gro-α were found in melanoma-CM (~ 8 ng/ml and ~ 2 ng/mL respectively) to be constitutively released (**Figure 1B-C**). A375 CM also contained some CXCL2/Gro-β compared to HEMa CM and SKMEL28 CM (**Figure 1D**). To better understand the mechanism of PMN chemotaxis, neutralizing antibodies against CXCR1 and/or CXCR2 were used. PMNs were allowed to migrate toward a SKMEL28 CM (**Figure 1E**) and A375 CM (**Figure 1F**) in the presence of CXCR1-blocking and/or CXCR2-blocking antibody or the relative isotype control. The results showed that blocking CXCR1 and CXCR2 significantly reduced PMN chemotaxis toward melanoma-CM.

A375 conditioned media promoted neutrophil survival

To investigate whether melanoma-derived soluble factors could modulate PMN lifespan, we tested the effect of melanoma-CM on PMN viability. PMNs from healthy donors were cultured in vitro in the presence of melanoma-CM (SKMEL28 CM, A375 CM), HEMa CM or control medium. After 24 hours, PMNs were stained with FITC-conjugated annexin V and PI and subjected to cytofluorimetric analysis. A375 CM markedly increased PMN survival compared to the control medium (**Figure 2A**). Interestingly, SKMEL28 CM or HEMa CM did not increase PMN survival, suggesting that only A375 cell line produced soluble factors able to increase PMN survival. To dissect the molecular mechanism behind this pro-survival effect, we evaluated the presence of soluble factors known to increase PMN lifespan in melanoma-CM. GM-CSF is a key mediator of proliferation and differentiation of granulocytes and macrophages (Guttman-Yassky, Nograles et al. 2011). Several type of cells, such as macrophages, T cells, endothelial cells fibroblasts and also tumor cells can produce GM-CSF (Shi, Liu et al. 2006). We evaluated the presence of GM-CSF in melanoma-CM by ELISA. A375 cell line constitutively produced high levels of GM-CSF compared to control medium, conversely SKMEL28 cell line or HEMa did not (**Figure 2B**). To confirm whether A375-derived GM-CSF was involved in PMN survival we performed similar experiments in the presence of GM-CSF neutralizing antibody. PMNs were purified and stimulated with A375 CM or with control medium in the presence of an anti-GM-CSF blocking antibody or with the relative isotype control. After 24 hours of incubation, PMNs were stained with FITC-conjugated annexin V and PI and subjected to cytofluorimetric analysis. The pro-survival effect of A375 CM was significantly inhibited by anti-GM-CSF blocking antibody (**Figure 2C, 2D, 2E, 2F and 2G**). **Figures 2D – 2G** illustrate representative flow cytometric panels of one out of five independent experiments.

A375 conditioned media induced PMN activation

To determine whether melanoma-derived soluble factors induce human PMN activation, we investigated CD66b, CD11b and CD62L (L-selectin) expression on PMNs by flow cytometry (Condliffe, Chilvers et al. 1996, Stocks, Ruchaud-Sparagano et al. 1996). PMNs were stimulated with a melanoma CM, primary melanocyte CM or with the control medium. Then PMNs were stained with antibodies against CD66b, CD11b and CD62L and evaluated by flow cytometry (Galdiero, Varricchi et al. 2018). Under basal conditions, PMNs showed minimal expression of CD66b and CD11b, which rapidly increased after the incubation with both SKMEL28 and A375 CM compared to control medium as well as compared to the HEMa CM (**Figure 3A, 3B, 3G and 3H**). In particular, A375 CM was the most efficient in inducing CD66b and CD11b up-regulation. Conversely, under resting conditions, PMNs highly expressed CD62L, which rapidly decreased (i.e., shedding) upon A375 CM stimulation (**Figure 3C and 3I**). Finally this data indicated that melanoma CM, in particular A375 CM, efficiently activated (CD66b and CD11b up-regulation, CD62L shedding) human primary normal PMNs. By contrast, primary melanocyte CM was not able to induce PMN activation. **Figures 3D – 3I** show representative flow cytometry panels for CD66b (**Figure 3A**), CD11b (**Figure 3B**) and CD62L (**Figure 3C**), with the specific gating strategy and related histograms (**Figure 3G, 3H and 3I**, respectively)

A375 conditioned media modified neutrophil morphology and kinetic properties

Using the high-content imaging system, we measured and tracked changes of morphological characteristics at single-cell level and quantitatively determined these morphological feature distributions in response to the culture conditions (Borriello, Iannone et al. 2016, Borriello, Iannone et al. 2017). PMNs were incubated with SKMEL28 CM, A375 CM, HEMa CM or with control medium for 16 hours at 37°C. PMNs treated with A375 CM showed lower axial length (**Figure 4A**) and roundness (**Figure 4B**), increased asymmetry (**Figure 4C**) cell area (**Figure 4D**). All these

morphological changes typically occur following PMN stimulation by inflammatory cytokines and growth factors (Kutsuna, Suzuki et al. 2004). Furthermore we evaluated kinetic properties by time-lapse microscopy. PMNs were cultured in SKMEL28 CM, A375 CM and HEMa CM and control medium for 16 hours at 37°C and 5% of CO_2. Within this time window, digital phase contrast images were captured every 15 minutes. PMNs stimulated with A375 CM showed an increased straightness (**Figure 4E**) and speed (**Figure 4F**) compared with control medium, as well as compared with HEMa CM and also compared with SKMEL28 CM. These results suggest that PMNs modified their kinetic properties under the influence of A375 CM.

Melanoma conditioned media induced MMP-9 and MPO release

We then tested the capability of melanoma CM to induce the release of MPO and MMP-9 from human primary neutrophils. PMNs were cultured in SKMEL28 CM, A375 CM and HEMa CM and control medium for 16 hours at 37°C and 5% of CO2. Then, the extracellular levels of MMP-9 and MPO in PMNs supernatants of SKMEL28 CM, A375 CM or HEMa CM or control medium were measured by ELISA. MMP-9 (**Figure 5A**) and MPO (**Figure 5B**) levels were not detectable in melanoma-CM, in HEMa CM and in control medium. MMP-9 and MPO concentrations were increased in PMN supernatants upon SKMEL28 CM or A375 CM stimulation compared with PMNs cultured within HEMa CM or control medium (**Figure 5A-B**). Accordingly, MMP-9 (**Figure 5C**) and MPO (**Figure 5D**)intracellular content were reduced following stimulation with SKMEL28 CM or A375 CM compared to PMNs cultured in control medium or in HEMa CM. These results suggests that SKMEL28 CM and A375 CM mediated the MMP-9 and MPO release from tertiary and primary granules respectively in human primary PMNs.

Melanoma conditioned media induced NET release from human neutrophils

To evaluate the ability of melanoma cell lines to induce NET release from PMNs, we used the Operetta High-Content Imaging Screening System. PMNs from peripheral blood of healthy donors were cultured in A375 CM, SKMEL28 CM, HEMa CM or control medium. NETing cells were analyzed using the PhenoLOGIC software with a dedicate analysis sequence. A375 CM and SKMEL28 CM were able to induce NET release from PMN, already after ~20 min of stimulation **(Figure 6A)**. HEMa CM and control medium did not induce NET release from PMNs (**Figure 6A**). Quant-iT™ PicoGreen™ dsDNA Assay Kit was performed on PMN supernatant to confirm dsDNA presence (**Figure 6D**). These results suggest that SKMEL28 CM and A375 CM rapidly and selectively induced NET release from PMNs.

Melanoma cell lines autocrinously produce significant quantities of CXC chemokines and GM-CSF (Sabatini, Chavez et al. 1990, Bardi, Al-Rayan et al. 2019). GM-CSF and CXCL8/IL-8 were able to induce NET release from PMNs (Yousefi, Mihalache et al. 2009, Alfaro, Teijeira et al. 2016). Neutralizing Abs against CXCL8/IL-8 and/or GM-CSF were used to investigate the mechanism behind melanoma CM-induced NET release. Anti-CXCL8/IL-8 blocking antibody inhibited the SKMEL28 CM-induced NET release **(Figure 6B and E)**. On the other hand, anti-GM-CSF blocking antibody inhibited the A375 CM –induced NET release **(Figure 6C and F)**. Anti-CXCL8/IL-8 blocking antibody did not exert any effect on NET release induced by A375 CM, nor in combination with anti-GM-CSF blocking antibody **(Figure 6C)**.

Serum levels of NET biomarkers and neutrophil-related mediators in melanoma patients

NETs can be released by PMNs upon a variety of immunologic and non-immunologic stimuli (Pilsczek, Salina et al. 2010, Yousefi, Stojkov et al. 2019). Several studies show elevated NETs concentrations in cancer patients compared with healthy controls, correlating with poor patient survival (Yang, Sun et al. 2015, Richardson, Hendrickse et al. 2017, Thalin, Lundstrom et al. 2018). We evaluated the serum levels of nucleosomes and CitH3 as NET biomarkers in serum of MMPs (Grilz, Mauracher et al. 2019). Circulating levels of nucleosomes and CitH3 were increased in MMPs compared with HCs (**Figure 7A-B**). Interestingly, circulating levels of nucleosomes and CitH3 were positively correlated (**Figure 7C**).

We also found that circulating levels of MPO, MMP-9, CXCL8/IL-8 and GM-CSF were significantly increased in MMPs compared with HCs (**Figure 8**). These results suggest that a neutrophil-related signature exists in melanoma patients.

DISCUSSION

In this study we investigated the effects of melanoma-derived soluble factors on human primary PMNs. We found that soluble factors derived from human melanoma cells can profoundly influence several characteristics of PMNs. Melanoma cells recruited PMNs and significantly promoted their survival and activation, modified PMN morphology and kinetic properties. Moreover, melanoma cells up-regulated neutrophils' proinflammatory activities as well as the expression of factors that promote tumor progression, such as MPO, MMP-9 and NETs. Finally, in a cohort of stage IV melanoma patients we found increased circulating levels of neutrophil-derived mediators, i.e. MMP-9, MPO, GM-CSF and CXCL-8, as well as increased levels of NET biomarkers, compared with HCs, suggesting that a neutrophil-related signature exists in stage IV melanoma patients.

Melanoma of the skin represents one of the most frequent cancers worldwide and, in the metastatic form, is one of the most fatal (Miller, Nogueira et al. 2022). Increasing evidence highlights the multiple roles of immune cells in human melanoma initiation and progression (Navarini-Meury and Conrad 2009, Umansky and Sevko 2012). The pivotal role of immunity in melanoma was established by the introduction of immune checkpoint inhibitors as new therapeutic options in advanced melanoma patients in the last 10 years (Larkin, Chiarion-Sileni et al. 2015, Eggermont, Blank et al. 2018, Eggermont, Robert et al. 2018, Larkin, Chiarion-Sileni et al. 2019). These immune-related therapeutic drugs significantly ameliorated patient prognosis and survival, even in the metastatic type (Miller, Nogueira et al. 2022).

Among innate immune cells involved in melanoma initiation and progression, few studies evaluated the roles of PMNs. In an *in vivo* model of melanoma, UV irradiation induced a TLR4/MYD88-driven neutrophilic skin inflammatory response that was initiated by HMGB1 release from UV-damaged keratinocytes. This TLR4-driven neutrophilic inflammation was in turn responsible for an increased angiotropism of melanoma cells and promoted distant metastasis (Bald, Quast et al. 2014). In humans, the circulating neutrophil-to-lymphocyte ratio (NLR) has been used as prognostic biomarker in

melanoma (Ferrucci, Gandini et al. 2015, Zaragoza, Caille et al. 2016, Cassidy, Wolchok et al. 2017, Mei, Shi et al. 2017, Capone, Giannarelli et al. 2018), even though with some limitations, due to PMN heterogeneity (Scapini, Marini et al. 2016). We recently demonstrated that peripheral blood PD-L1^{+} PMN frequency efficiently predicted patient prognosis and response to the anti-PD-1 agent nivolumab in stage IV BRAF wild type melanoma patients (Cristinziano, Modestino et al. 2022). To go deeper inside the mechanisms linking melanoma cells and PMNs, in this paper we investigated the bidirectional cross-talk between melanoma cell lines and primary human PMNs purified from peripheral blood of healthy donors. A375 and SKMEL-28 cell lines, two of the most studied human melanoma cells, were chosen as model of different aggressiveness and malignancy, as demonstrated *in vitro* and *in vivo* (Pal, Baxter et al. 2015, Rossi, Cordella et al. 2018).

In a first series of *in vitro* experiments we found that melanoma derived CM were able to induce PMN chemotaxis *in vitro*, whereas CM from human primary melanocytes did not. We then evaluated the mediators responsible for this chemotactic effect. The expression and the production of chemokines is a well-known characteristic of melanoma cells (Navarini-Meury and Conrad 2009) (Zhou, Peng et al. 2021). Melanoma-derived chemokines act as both autocrine and paracrine mediators. Indeed, autocrine loops sustain tumor cell activation, reprogramming and metastasis. Paracrine loops orchestrate tumor behavior through the engagement of receptors expressed by stromal and immune cells. In an interesting study investigating gene expression of a series of immunomodulatory genes on human melanoma biopsies, CXCL1/Gro-α and CXCL2/Gro-β were significantly over-expressed in the metastatic patient samples compared with not metastatic ones. CXCL1/Gro-α showed a particularly high predictive value and independently predicted the occurrence of distant metastases in the patient cohort (Erdrich, Lourdault et al. 2022). CXCL8/IL-8 also acts as a major key-player in melanoma progression, since autocrinously activates oncogenic signaling, pro-metastatic behavior, drug-resistance and angiogenesis (Filimon, Preda et al. 2021). In our experimental setting, we confirmed that melanoma cells constitutively release CXCL8/IL-8, CXCL1/Gro-α and CXCL2/Groβ

and, accordingly, CM-induced PMN chemotaxis was dependent on CXCR1/2. It is important to note that CM derived from primary melanocytes failed to induce PMN chemotaxis and, indeed, primary melanocyte CM did not contain relevant levels of CXCL8 nor CXCL1/2. These results suggest that the production of chemokines and the chemotactic effect exerted on PMNs is specific of cancer cells and is related to the acquisition of specific features by melanoma cells during the initiation and progression of the disease. Noteworthy, the inhibition of both CXCR1 and CXCR2 was required to block PMN chemotaxis, highlighting the pleiotropic and redundant potential of melanoma-derived chemokines.

Once recruited, melanoma cells were able to significantly increase PMN survival trough the release of GM-CSF. Interestingly, only the more aggressive melanoma cell line A375 produced high levels of GM-CSF and was able to increase PMN survival, whereas the less aggressive SKMEL28 was not. Similarly, the primary melanocytes did not produce GM-CSF, nor modified PMN survival. In addition, we also found that the A375 CM significantly activated PMNs, and profoundly modified PMN morphology and kinetic properties. In contrast with the chemotactic activity, the effects on PMN survival, activation and kinetic properties were differentially regulated by the two different melanoma cell lines, according with their aggressiveness. Indeed, only CM derived from the more aggressive A375 cell line was able to increase PMN survival, induce their activation, modify their morphology and kinetic properties, whereas the CM from the less aggressive SKMEL28 cell line did not. Coherently, CM derived from the primary melanocytes did not modify any PMN property at all. These results suggest that, beyond PMN recruitment, which could be a “bystander effect” of the production of chemokines by melanoma cells, the modulation of PMN behavior is finely tuned according with melanoma progression, suggesting a key role for these cells in the latest stages of melanoma.

PMNs contain a lot of preformed granular enzymes that can be released quickly (Lehman and Segal 2020). We demonstrated that melanoma-CM, but not primary melanocytes CM, were able to induce PMN degranulation and release of MPO and MMP-9, a well-known angiogenic and pro-tumorigenic factor.(Nozawa, Chiu et al. 2006, Hanahan and Weinberg 2011, Moloney and Cotter 2018). MPO is able to activate proMMP-8 and -9, and regulates matrix MMP-7 activity *in vitro* (Saari, Sorsa et al. 1990, Fu, Kassim et al. 2001). MPO has been implicated in tumor initiation through the support of a hypermutagenic environment due to the action of MPO-derived oxidants that are able to oxidize and modify DNA (Valadez-Cosmes, Raftopoulou et al. 2022). An association between MPO and disease progression has been reported in ovarian and cervical cancers, as well as in CRCs (Droeser, Mechera et al. 2016). MPO stored in primary/azurophilic granules plays important roles in NET formation and ROS production (Yousefi, Mihalache et al. 2009, Hendrix and Kheradmand 2017). MMP-9 exerts several functions in the different phases of metastatic melanoma and could retain clinical value during melanoma progression (Nikkola, Vihinen et al. 2005). MMP-9 is one of a major contributor of tumour progression and a play a key role in extracellular matrix degradation (Ardi, Kupriyanova et al. 2007). Additionally, the production of VEGF and MMP-9 in TANs makes them the primary regulators of angiogenesis and tumour progression in a mouse model of transplantable melanoma and fibrosarcoma (Jablonska, Leschner et al. 2010).

NETs were first described as a protective mechanism through which PMNs performed antibacterial functions (Brinkmann, Reichard et al. 2004, Brinkmann and Zychlinsky 2012). Growing data suggests that NETs contribute to a number of inflammatory diseases, including cancer (Brinkmann, Reichard et al. 2004, Hakkim, Furnrohr et al. 2010, Cristinziano, Modestino et al. 2020, van den Hoogen, van der Linden et al. 2020, Varricchi, Modestino et al. 2022). In an experimental melanoma model, NETs within the tumor microenvironment (TME) promoted tumor progression (Demers, Wong et al. 2016). By contrast, in an *in vitro* model, NETs decreased melanoma cell viability and migration (Schedel, Mayer-Hain et al. 2020). In our experimental setup, we found that the melanoma-

derived CM induced NET release from human primary PMNs. Compared with primary melanocytes CM and control medium, both the SKMEL28- and A375-derived CM induced NETs release. GM-CSF is a pleiotropic cytokine that influences the differentiation, activation, and survival of a variety of immune cells, including PMNs (Hamilton 2020). GM-CSF is also one of the main mediators responsible for vital NETosis (Yousefi, Mihalache et al. 2009). Serum levels of GM-CSF are a key predictors of metastasis in early melanomas (Mancuso, Lage et al. 2020). CXCL8/IL8 also induced NETs release and play important roles in cancer (Alfaro, Teijeira et al. 2016, Liu, Li et al. 2016). The inhibition of GM-CSF selectively inhibited NETs release induced by A375 CM-primed human PMNs, and the blockage of CXCL8/IL8, selectively inhibited NETs release induced by SKMEL28 CM-primed human PMNs. To evaluate NET release, we used a High-Content Image System, a high-throughput system that allows uniform standardization of cell image acquisition and analysis using specialized software (Gupta, Chan et al. 2018). Although the majority of published approaches use automated NET quantification based on microscopy, they are often not fully automated and rely on an increase in staining area as the only criteria determining the extent of NET formation without knowledge at the single-cell level (Brinkmann, Goosmann et al. 2012, Kraaij, Tengstrom et al. 2016, Mohanty, Sorensen et al. 2017). Automated image quantification has many benefits since it can process numerous images quickly using parameters that define NETs (Gupta, Chan et al. 2018). In order to further verify these results, we also investigated the release of dsDNA, which is usually used as NETs biomarker (Xiao, Jiang et al. 2018).

In this study we found that serum concentrations of PMN-related factors (i.e. MPO, MMP-9, CXCL8/IL8 and GM-CSF) and NETs (i.e. nucleosomes and CitH3) are increased in stage IV melanoma patients compared to the healthy controls. Some of these mediators such as MMP-9 and GM-CSF they have been shown to play a role as a key predictor of metastasis and melanoma progression (Nikkola, Vihinen et al. 2005, Mancuso, Lage et al. 2020). In a recent study it has been proposed an involvement of CXCL8/IL8 and NETs, through measurement of MPO-DNA complexes,

in different types of solid tumours including melanoma (de Andrea, Ochoa et al. 2021). The amount of NET formation *in vivo*, however, is not always reflected by the quantitative presence of circulating DNA. DNA complexes may actually develop as a result of any type of cell death brought on by neutrophilic inflammation (Yousefi, Simon et al. 2020). For this reason we decided to use two different methods (i.e. nucleosomes and CitH3) to detect the serum levels of NETs. In particular CitH3 serum levels, which are thought to be a more reliable indicator of NETs formation, were specifically measured to corroborate our findings (Grilz, Mauracher et al. 2019, Cristinziano, Modestino et al. 2022). Collectively, these results show that four different neutrophil-derived mediators are increased in stage IV melanoma patients and suggest that the PMNs are activated *in vivo* and that NET formation could be associated with the aggressiveness of melanoma.

Although our results indicate the possible involvement of PMNs and NETs in human melanoma, this paper suffers from some limitations. Additional experiments are necessary to understand the role of these "melanoma-educated" PMNs in modifying melanoma behaviour as well as to elucidate the mechanism by which PMNs can promote melanoma progression. *Ex vivo* evidences should be useful to corroborate these data. More in details, immunohistochemical investigations could be performed to evaluate whether TANs locally produce NETs in melanomas. Finally, a larger cohort of patients should be recruited to corroborate these data and, to evaluate any differences in the circulating levels of neutrophil-related mediators as well as in NETs among patients bearing melanomas at different stages of progression.

FIGURES

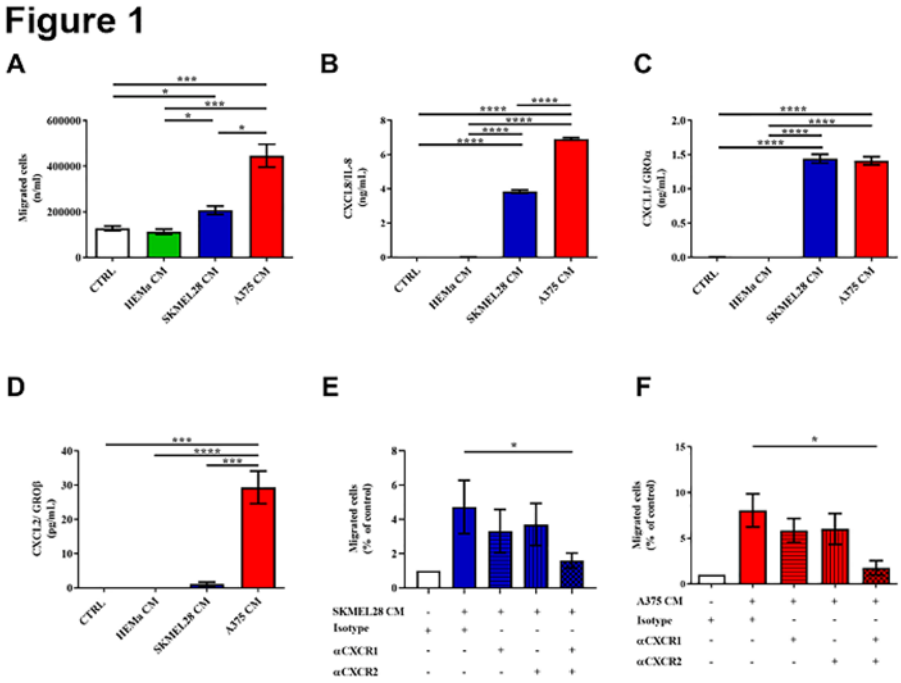

Fig 1. Melanoma-derived soluble mediators induced neutrophil chemotaxis. A. Neutrophil chemotaxis toward melanoma-CM or the control medium was evaluated using 3 µm cell colture inserts in 96-well companion plates. Neutrophils (2.5 x 10^6 cells/mL per 75 µL) were allowed to migrate (37°C, 60 minutes) toward a melanoma-CM (SKMEL28 CM, A375 CM), HEMa CM or control medium (235 µL per well). At the end of the incubation, the cells were centrifugated and resuspended in PBS (100 µL) and counted by flow cytometry. Data are expressed as migratory cells relative to the control medium (mean ± SEM of five independent experiments). One-Way Anova and Dunn's multiple comparison test; *** $p < 0.005$; * $p < 0.05$. **B-D.** The CXCL8/IL-8 (**B**), CXCL1/GROα (**C**) and CXCL2/GROβ (**D**) release by HEMa, SKMEL28 and A375 cells was evaluated by ELISA in a conditioned media or in the control medium. Results are expressed as mean ± SEM of five independent experiments; One-Way Anova and Dunn's multiple comparison test; **** $p < 0.001$. **E and F**. Chemotactic activity of neutrophils *via* a SKMEL28-derived (**E**) or A375-derived (**F**) conditioned medium was analyzed in the presence of blocking antibodies directed against CXCR1 and/or CXCR2 (10 µg/mL) or the related isotype control. Migratory neutrophils were counted by flow cytometry. The results are expressed as percentage of isotype control (mean ± SEM of ten independent experiments); One-Way Anova and Dunn's multiple comparison test; * $p < 0.05$.

Figure 2

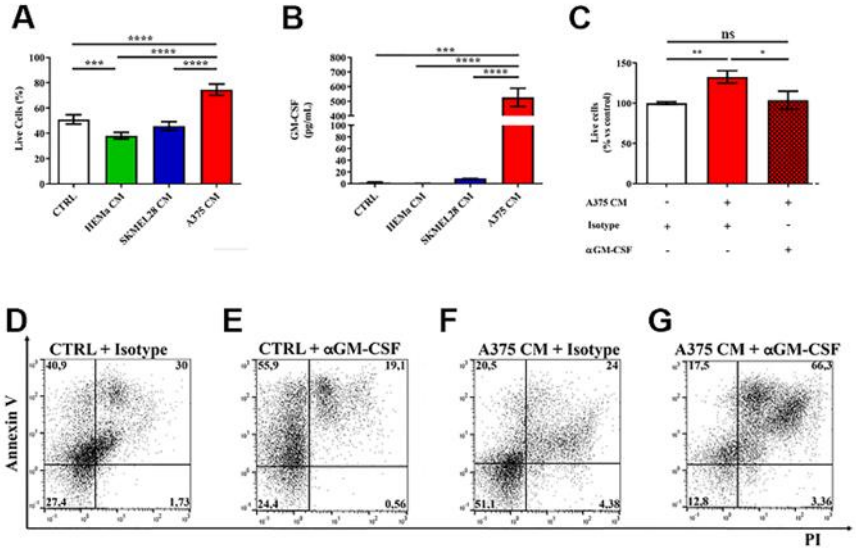

Fig 2. A375-derived soluble factors promoted neutrophil survival. A. PMNs were cultured in melanoma-CMs (SKMEL28 CM, A375 CM), HEMa CM or control medium for 24 hours. PMNs were then stained with FITC-conjugated annexin V and PI and were evaluated by flow cytometry. Results were expressed as percentages of live cells (mean ± SEM of five independent experiments); One-Way Anova and Dunn's multiple comparison test; **** $p < 0.001$; *** $p < 0.005$. **B.** The GM-CSF release by HEMa, SKMEL28 and A375 cells was evaluated by ELISA in conditioned media or in the control medium. Results were expressed as mean ± SEM of five independent experiments; One-Way Anova and Dunn's multiple comparison test; **** $p < 0.001$; *** $p < 0.005$. **C.** Neutrophil survival in A375-derived conditioned medium was evaluated in the presence of an anti-GM-CSF blocking antibody or the relative isotype control (10 µg/mL). At 24 hours, live cells were stained with FITC-conjugated annexin V and PI and analyzed by flow cytometry. The results were expressed as mean ± SEM of five independent experiments; ** $p < 0.01$; * $p < 0.05$. **D-G**. Illustrate representative flow cytometric panels of one of out of five indipendent experiments.

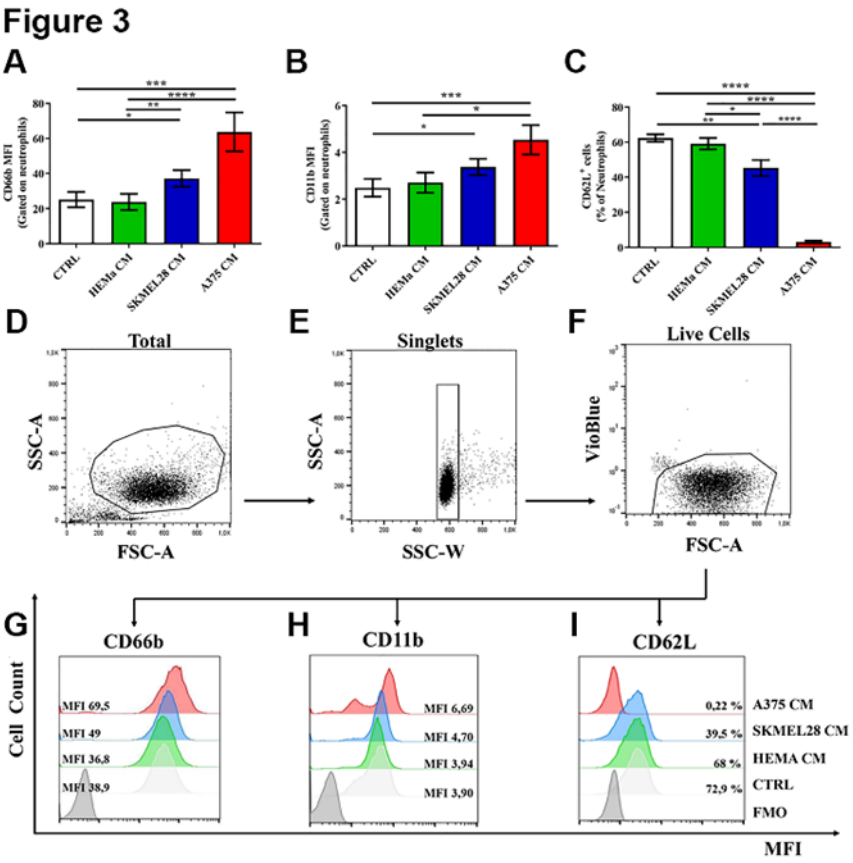

Fig 3. A375 conditioned media induced PMN activation. A-C. Neutrophils were stimulated with melanoma-CM (SKMEL28 CM, A375 CM), HEMa CM or control medium for 90 minutes, stained for neutrophil activation markers CD66b (**A**), CD11b (**B**) and CD62L (**C**) and subjected to cytofluorimetric analysis. The results were expressed as mean fluoscence intesity or percentage of positive cells gated on neutrophils (mean ± SEM of seven independent experiments); One-Way Anova and Dunn's multiple comparison test; **** $p < 0.001$; *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$. **D-F.** Representative flow cytometric panels were gated on live single cells and show forward (FSC) and side scatter (SSC) of EasySep-purified untouched neutrophils (**D, E**), since Vioblue-positive cells included both dead cells and CCR3+ cells (eosinophils), both cells were excluded based on a negative gate (**F**). **G-I.** Representative histograms illustrating mean fluorescence intensity (MFI) and cell count for CD66b (**G**), CD11b (**H**) and CD62L (**I**) for one out of seven experiments. MFI = mean fluorescence intesity; FMO = fluorescence minus one.

Figure 4

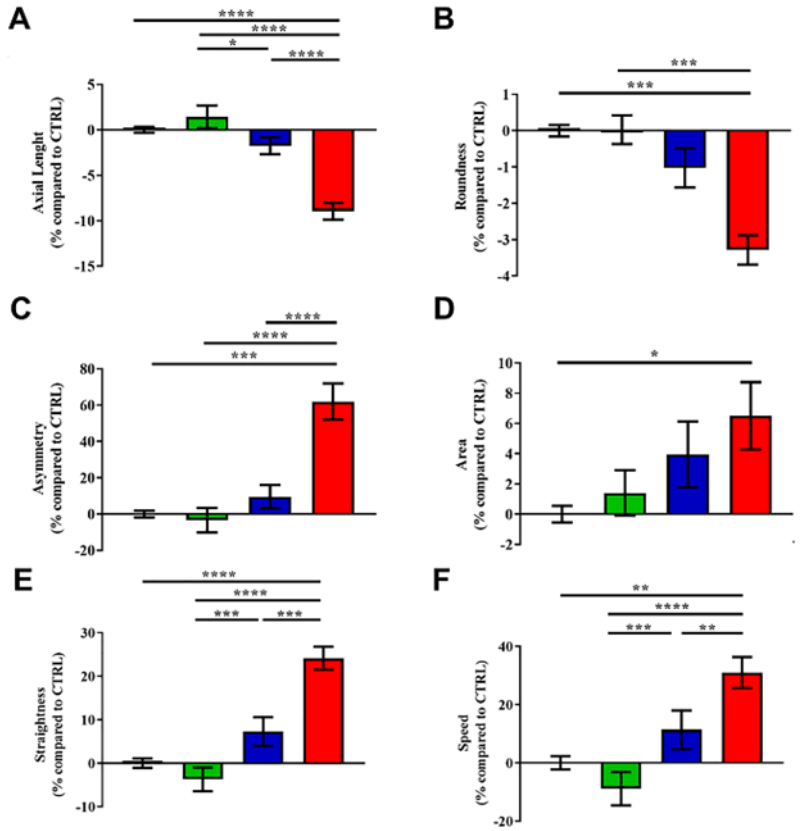

Fig 4. A375 conditioned media induced morphological changes and kinetic properties in neutrophils. A-D. Neutrophils were stimulated with melanoma-CM (SKMEL28 CM, A375 CM), HEMa CM or control medium for 16 hours and then were imaged by means of an Operetta high-content imaging system at 20x magnification. The images were analyzed in the Harmony software with PhenoLOGIC (PerkinElmer) and a dedicated analysis sequence (morphological properties, method STAR) to evaluate axial lenght (**A**), roundeness (**B**), asymmetry (**C**) and cell area (**D**). The results were expressed as an increase or decrease compared to the control (mean ± SEM of five independent experiments); **** $p < 0.001$; *** $p < 0.005$; * $p < 0.05$. **E-F.** In the same time window, digital phase contrast images of 15 fields/well captured every 15 minutes via a 20x objective in the Operetta high-content imaging system. PhenoLOGIC (PerkinElmer) was employed for image segmentation and to calculate the single-cell kinetic properties straightness (**E**) and speed (**F**). The results were expressed as mean ± SEM of five independent experiments; One-Way Anova and Dunn's multiple comparison test **** $p < 0.001$; *** $p < 0.005$; ** $p < 0.01$.

Figure 5

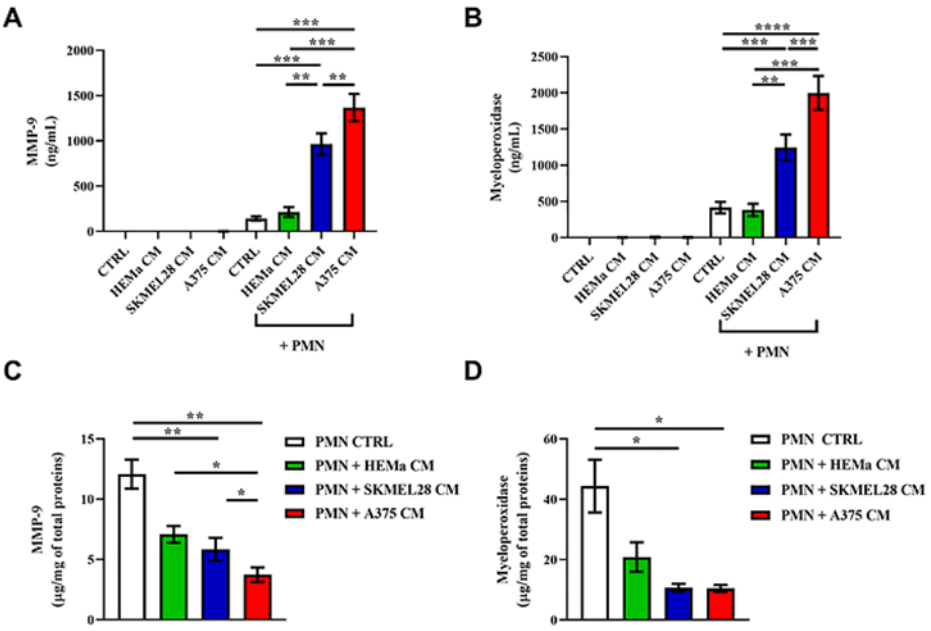

Fig 5. Melanoma-CMs induced MMP-9 and Myeloperoxidase release. A-D. Neutrophils were cultured in melanoma-CM (SKMEL28 CM, A375 CM), HEMa CM or control medium for 18 hours. At the end of the incubation, neutrophils were harvested and centrifugated (600 x g, 4°C, 5 minutes), and the supernatants were collected. The extracellular content of MMP-9 and Myeloperoxidase from melanoma cell lines and neutrophils (**A**) and (**B**) as well as intracellular concentration of MMP-9 and Myeloperoxidase in neutrophils (**C**) and (**D**) after cell lysis (Triton X-100, 0,1%) were evaluated by an ELISA. The results were expressed as mean ± SEM of five independent experiments; One-Way Anova and Dunn's multiple comparison test; **** $p < 0.001$; *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$.

Figure 6

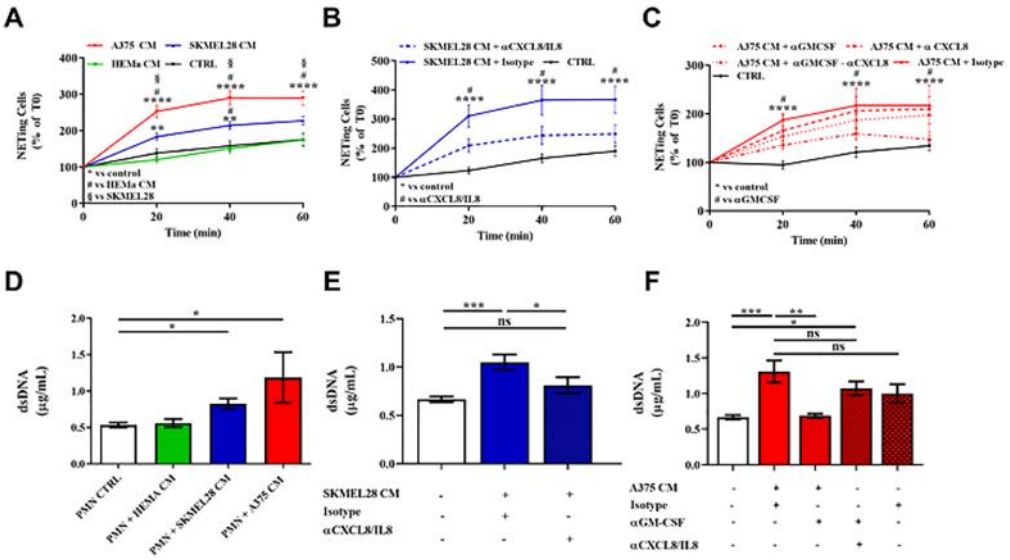

Fig 6. Melanoma-CMs induced NETs release from highly purified human neutrophils. A, B, C. Neutrophils (1 x 10^6 cells/mL) were seeded in a 96-well CellCarrier plate and cultured in A375, SKMEL28 or HEMa CM or in a control medium (37°C, up to 60 min) in the presence or absence of anti-CXCL8/IL-8 and/or anti-GM-CSF blocking antibodies or the relative isotype control (10 µg/mL) 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 NETing cells versus time 0 (mean ± SEM of five independent experiments using five different donor samples). Two-Way Anova and Bonferroni multiple comparison test (**A**) or Dunnett's multiple comparison test (**B, C**); **** $p < 0.001$; *** $p < 0.005$; ** $p < 0.01$; * $p < 0.05$. **D, E, F.** dsDNA levels in supernatants of neutrophils cultured with A375, SKMEL28 or HEMa CM or in control medium, in the presence or absence of anti-CXCL8/IL-8 and/or anti-GM-CSF blocking antibodies or the relative isotype control (10 µg/mL), were measured by Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher). Results were expressed as mean ± SEM of five independent experiments; * $p < 0.05$. One-Way Anova and Dunn's multiple comparison test; *** $p < 0.005$; * $p < 0.05$.

Figure 7

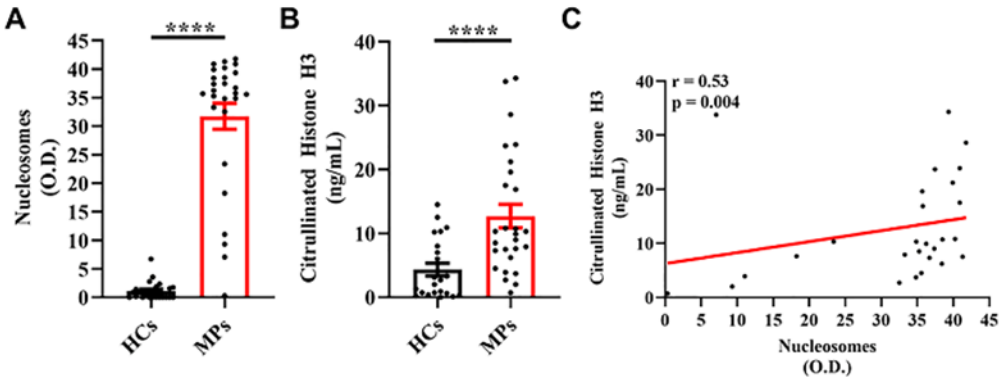

Fig 7. Serum levels of NETs in melanoma patients. Serum concentrations of nucleosomes (**A**) and citrullinated histone H3 (CitH3) (**B**) in melanoma patients (red borders) and healthy controls (black borders) were measured by Cell Death Detection ELISA (Roche) and Citrullinated Histone H3 (clone 11D3) ELISA kit (Cayman), respectively. Results were expressed as mean ± SEM; Student's t test or Mann-Whitney U test according to the parametric or nonparametric distribution of the variables. **** $p < 0.001$. **C.** Correlation between serum concentrations of nucleosomes and citrullinated histone H3 (CitH3) in melanoma patients. Sperman correlation test; r=0.53; p=0.0043.

Figure 8

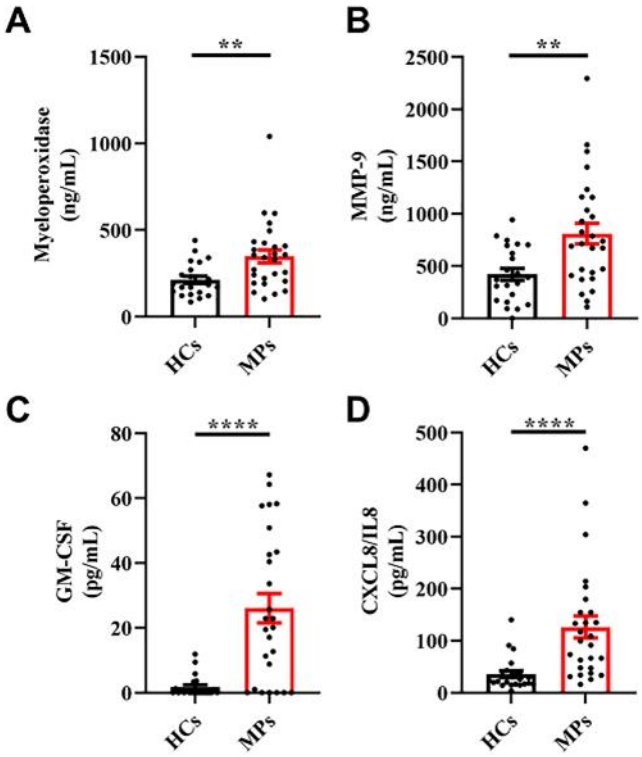

Fig 8. Serum levels of neutrophil-related mediators in melanoma patients. Serum concentrations of Myeloperoxidase (**A**), MMP-9 (**B**), GM-CSF (**C**) and CXCL8 (**D**) in melanoma patients (red borders) and healthy controls (black borders) were measured by ELISA. The results were expressed as mean ± SEM. Student's t test or Mann-Whitney U test according to the parametric or nonparametric distribution of the variables. **** $p < 0.001$; ** $p < 0.01$.

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