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Coordinatore: Prof. Gianni Marone

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*Clinical significance and functional roles
of tumor-associated neutrophils in colorectal cancer*

TUTOR

Chiar.mo
Prof. Gianni Marone

CANDIDATA

Dott. ssa
Maria Rosaria Galdiero

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ABSTRACT

Immune cells actively take part to the tumor microenvironment, where they play several roles in the multistep process of carcinogenesis. Among the tumor-infiltrating immune cells, myeloid cells represent a “double-edged sword” for cancer. Indeed, they can both promote or inhibit cancer initiation and progression. Neutrophils have long been viewed as short-lived cells, pivotal for the elimination of extracellular pathogens, with a limited role in the orchestration of the immune response. However, recent lines of evidence have challenged this limited point of view, revealing a number of unexpected functions for neutrophils in several pathological contexts. Indeed, neutrophils were also found among inflammatory cells infiltrating cancer tissues, such as colorectal cancer (CRC). Today, the functional roles of neutrophils in cancer and the association between neutrophil infiltration, clinicopathological features and outcome of CRC patients remain to be clarified. Here, we found that neutrophils are recruited within CRC tissues through a spatiotemporal dynamism. Infiltration of neutrophils increased from stage I to stage III CRC and decreased at stage IV. In a large cohort of stage III CRC, neutrophil infiltration was preferentially found in the intratumoral compartment compared with the invasive margin. Higher density of neutrophils within the tumor was found to be an independent prognostic factor for better patients’ outcome (n=128, Stage I-Stage IV CRC) and a predictive factor for 5-FluoroUracyle chemotherapy response in Stage III CRC patients (n=178). Moreover, CRC cell lines released soluble factors, which promoted neutrophil chemotaxis and survival. In turn, neutrophils displayed a cytostatic activity towards CRC cell lines. Our data suggest that neutrophils are critical components of antitumor immunity and that neutrophil infiltration within tumors could be a useful predictive marker to identify patients with cancer who would likely benefit from chemotherapy. Therefore, neutrophils have to be considered as central participants in the regulation of the innate and adaptive immune responses, with important roles in the resolution or exacerbation of various pathologies, including cancer.

INTRODUCTION

Immune cells are important participants of the tumor microenvironment, where they play several roles in all the steps of cancer development. Among the immune cells infiltrating tumors, myeloid cells can play a dual role in sculpting cancer behavior. Indeed, they can promote or inhibit cancer initiation and progression. Neutrophils have recently emerged as key component in the regulation of the innate and adaptive immunity in several inflammatory conditions, including cancer.

I. 1. The hallmarks of cancer

The gradual transformation of a normal cell to a neoplastic state is complex and requires the acquisition of a series of skills necessary to overcome the multiple natural mechanisms that control cell homeostasis, growth and proliferation. For instance, accumulation of genetic mutations can overwhelm mechanisms naturally designed to control DNA integrity and, therefore, lead to malignant cell transformation. In 2000, Hanahan and Weinberg proposed that all cancers share six common traits, called “hallmarks”, responsible for the transformation of a normal cell (Hanahan and Weinberg, 2000) (**Figure 1**). These hallmarks are: self-sufficiency in growth signals; evading growth suppressors; evading apoptosis; enabling replicative immortality; sustained angiogenesis; activating invasion and metastasis.

Following this first description, four new hallmarks have been added. They consist in the deregulation of the metabolic pathways, the capacity of cancer cells to evade the immune system, the role of chronic inflammation and the presence of unstable DNA (**Figure 2**) (Hanahan and Weinberg, 2011). The role of the immune system in preventing malignant transformation consists of three functions: a) eradication of oncogenic viruses; b) prevention and control of tumor-promoting inflammation; c) direct recognition and

destruction of neoplastic cells, a process initially referred as “cancer immunosurveillance” (Hanahan and Weinberg, 2011).

I. 2. Cancer Immunoediting: the immunosurveillance hypothesis revisited

In 1909 a protective role for the immune system in defense against cancer was proposed by Paul Ehrlich (Ehrlich, 1909). However, knowledge about mechanisms and functions of the immune system were immature to sustain this hypothesis. Fifty years later, Burnet and Thomas have elaborated the “immunosurveillance hypothesis”, suggesting that the adaptive immune system was able to prevent tumor development in immunocompetent hosts (Burnet, 1957; Thomas, 1959). However, further findings did not support this hypothesis and the immunosurveillance theory was abandoned for years (Stutman, 1974). Only in late Nineties and with the advent of genetically modified animal models this theory came to light again. In fact, evidence that mice genetically deficient in IFN- γ signaling (*Ifng^{-/-}*) or in adaptive immune components (e.g. *Rag2^{-/-}*) displayed higher spontaneous or carcinogen-induced tumor susceptibility renewed the interest in the role of the immune system in defense against tumor development (Engel et al., 1996; Engel et al., 1997; Kaplan et al., 1998). Few years later, another line of evidence led to a further revision of the immunosurveillance hypothesis. In fact, chemically induced sarcomas from immunodeficient mice (*Ifng^{-/-}* or *Rag2^{-/-}*) were rejected in immunocompetent mice, whereas sarcomas from immunocompetent mice were not rejected (Shankaran et al., 2001). This result suggested that tumors occurring in absence of a functional immune system were more immunogenic compared to analogous tumors arising in immunocompetent mice and that the immune system was able not only to detect tumor cells and potentially eliminate them, but also to shape tumor features, a process called “tumor immunogenicity” (Shankaran et al., 2001). These observations put the basis of the cancer immunoediting theory, as the property of the immune system to modify cancer behavior and

immunogenicity, articulated in three phases: elimination, equilibrium and escape (**Figure 3**) (Schreiber et al., 2011).

The elimination phase is a reviewing of the immunosurveillance. This concept defines the capacity of both innate and adaptive immunity to identify transformed cells and eliminate them. In the best epilogue, if tumor cells are eliminated, the elimination phase can be sufficient. In the equilibrium phase, the immune system maintains the tumor in a functional state of dormancy and the tumor can rest latent in the host for long time (Dunn et al., 2002). Under the pressure of the immune system, tumor clones can lack immunodominant rejection antigens via a process called “immunoselection” of the most immunoevasive clones. This is the escape phase, in which tumor cells have acquired a number of mechanisms to evade immune system. As an alternative way to escape immunosurveillance, tumor cells use the immune cells to their advantage. In a so-called “immunosubversion” mechanism, tumor cells recruit immune cells and properly educate them to shut down the anti-tumor immune response, supporting tumor growth and dissemination. The immunosubversion process is the most evolved form of immunoescape (Zitvogel et al., 2006).

Tumor-infiltrating immune cells have long been recognized by pathologists, mirroring inflammatory conditions arising in non-neoplastic tissues (Dvorak, 1986). Such immune responses were considered an attempt by the immune system to eliminate tumors. However, the tumor-associated inflammatory response had also the paradoxical effect of promote tumorigenesis and progression, favoring emerging neoplasias to acquire hallmark capabilities. In the next decade, several lines of research analyzed the connections between inflammation and cancer concluding that inflammatory immune cells display tumor-promoting properties on neoplastic progression (Colotta et al., 2009; DeNardo et al., 2010; Grivennikov et al., 2010; Qian and Pollard, 2010). Indeed, in the most recent revision of cancer hallmarks, Hanahan and Weinberg described cancer associated inflammation as an

“enabling characteristic”, necessary to acquire the cancer hallmarks and to make possible the neoplastic transformation (Hanahan and Weinberg, 2011).

II. CANCER RELATED INFLAMMATION

Inflammation is a physiological process naturally occurring in tissues, following a stress, infection or damage (Medzhitov, 2008). In a controlled inflammatory response, specialized cells are recruited at the inflammatory sites to limit the insult and avoid the extent of the damage. Usually, inflammatory response is self-limited and homeostatic conditions are reestablished during the process of resolution of inflammation (Zamarron and Chen, 2011). However, inflammation can evolve to a chronic state, which was associated with the development of several human cancers (Grivennikov et al., 2010; Zamarron and Chen, 2011).

In 1860 Rudolf Virchow first observed leukocyte infiltration in neoplastic tissues and suggested a link between inflammation and cancer (Balkwill and Mantovani, 2001). A number of evidence coming from epidemiological studies and animal models have suggested that cancer and inflammation were linked (Mantovani et al., 2008). For instance, several cancer types are due to inflammatory reactions following infectious causes, such as Hepatitis C virus for Hepato-Cellular Carcinoma (HCC) or *Helicobacter pylori* for gastric cancer (Mantovani et al., 2008). However, also tumors without an inflammatory background present signs of “smoldering inflammation”. In fact, inflammatory cells and mediators are found in most tumors microenvironment (Mantovani et al., 2008).

Chronic inflammation plays a role in all stages of tumorigenesis, including initiation (through genotoxic damage), promotion (favoring cell proliferation) and progression (promoting invasiveness and metastasis) (Grivennikov et al., 2010). Given these assumptions, it has been proposed that inflammation and anti-tumor immune response exert opposite effects on cancer development (Karin et al., 2002; Mantovani et al., 2008). However, cancer-related inflammation and anti-tumor immunity can co-exist in the same

tumor in a delicate balance, which can be altered in favor of one during the process of immunoediting (Bui and Schreiber, 2007). Moreover, inflammation exerts pivotal functions during tumor escape, when inflammatory cells, recruited at the tumor site and activated by cancer-derived molecules, suppress the anti-tumor immune response (Grivennikov et al., 2010). Tumor cells can generate an immunosuppressive state through the inhibition of effector functions of immune cells or the active recruitment of regulatory immune cells (Vesely et al., 2011). For instance, many malignant cells constitutively produce small amounts of TNF- α , which is known to enhance growth and spread of syngeneic, xenogeneic and carcinogen-induced tumors of skin, ovary, pancreas, pleural cavity and bowel (Balkwill, 2009; Schioppa et al., 2011). Malignant-derived TNF- α can also exert pro-tumorigenic functions targeting macrophages or T CD4⁺ cells (Charles et al., 2009; Hagemann et al., 2005). Tumor cells exert also systemic effects through the production of the immunosuppressive molecules TGF- β and IL-10, which inhibit dendritic cell (DC) activation as well as T cell and natural killer (NK) cell functions (Aruga et al., 1997; Wrzesinski et al., 2007). A number of non-neoplastic cells, which constitute the tumor-associated stroma, such as fibroblasts and immune cells, take part in tumorigenesis as active contributors rather than passive bystander cells and actively contribute to the acquisition of cancer hallmarks (Dudas, 2015).

III. TUMOR-INFILTRATING IMMUNE CELLS

III. 1. Adaptive immune cells

III. 1.1. T cells

T cells are found in the tumor microenvironment of most solid tumors (Galon et al., 2006; Senovilla et al., 2012). Both CD4⁺ T helper cells and CD8⁺ T cytotoxic cells can be present.

CD8⁺ cytotoxic T cells (CTL) play a central role in tumor immune surveillance (Fridman et al., 2012). They express T cell receptors (TCRs), which interact with specific peptides

presented in the context of MHC class I (MHCI/pep) by the antigen presenting cells (APCs) (Cross et al., 2008). Together with the co-stimulatory signal derived from the interaction between CD28 on the T cell and CD80/CD86 on the APC, the MHCI/pep-TCR interaction activates T cells. In this favorable context and with the help of cytokines (e.g. IL-12, IFN- γ), naive CD8⁺ T cells undergo clonal expansion and acquire effector functions, such as Granzyme and Perforin production, which finally destroy target cells (Hwang and Nguyen, 2015). In the context of tumor immunosurveillance, APC, such as DC, can capture tumor antigens released from tumor cells or immunogenic dying tumor cells, a process induced by some forms of chemotherapy (Palucka and Banchereau, 2012; Zitvogel et al., 2013). DC process the antigens and present them in the context of MHC I, MHC II or non-classical CD1 molecules. Extracellular peptides can be presented to CD8⁺ T cells in the context of MHC I in a mechanism called cross-priming (Chen et al., 2004). Activated DC express cellular (such as co-stimulatory ligands) and soluble mediators (such as IL-12) for activation of CD8⁺ T cells (Scapini et al., 2002). CD8⁺ T cells undergo clonal expansion and differentiate in effector cells in secondary lymphoid organs, exit the organs and are recruited to the tumor microenvironment, where they exploit their effector functions and kill target cells (Banchereau and Steinman, 1998; von Andrian and Mempel, 2003).

CD4⁺ T helper (Th) lymphocytes are an heterogeneous population of cells (Eyles et al., 2006). The T helper cell subsets are defined by the production of cytokines and/or the expression of characteristic lineage defining transcription factors (**Table 1**). Five principal subsets of Th cells have been identified: Th1, Th2 and Th17 cells, which respond to distinct classes of pathogens (Korn et al., 2007; Romagnani, 1997), regulatory T cells (Treg), which are involved in the process of tolerance (Sakaguchi, 2005) and follicular helper T cells (T_{FH}) that provide help to B cells for antibody production (Crotty, 2011).

Following the antigen-specific signal, mediated by the interaction between TCR and MHC II, and the co-stimulatory signal, mainly mediated by the interaction between CD28

on the T cell and CD80 and CD86 on the APCs, specific T helper subset polarization depends on the signal derived from the APCs, consisting in soluble or membrane-bound factors (Kapsenberg, 2003). Th1 cells are classically induced in presence of IL-12, mainly produced by cells infected by intracellular pathogens and following TLR activation (Netea et al., 2005; Nizzoli et al., 2013). They express the transcription factor T-bet and produce IFN- γ , which activates macrophages and NK cells to eliminate invading viruses and bacteria (Nizzoli et al., 2013; Romagnani, 1997). In contrast, in presence of IL-4, Th cells undergo Th2 differentiation. Th2 cells express the transcription factor GATA3, produce IL-4, IL-5, IL-10 and IL-13, and play pivotal roles in defense against helminthic infection and in allergic responses (Robinson et al., 1992). Early studies in humans demonstrated that IFN- γ and IL-4 production were not exclusive, since some T cell clones co-produced both molecules (Maggi et al., 1992). Moreover, some T cells co-express the Th1 and Th2 markers CXCR3 and CCR4 (Rivino et al., 2004), as well as the transcription factors T-bet and GATA3 (Peine et al., 2013). T cell plasticity was also demonstrated by the production of IL-10 in Th1 cells (Gerosa et al., 1996). This property was related to the capacity of Th1 cells to prevent an uncontrolled immune response (O'Garra and Vieira, 2007). Thus, Th1 cells can switch to an immunomodulatory IL-10-producing phenotype, named Type 1 regulatory (Tr1)-like T cell, to maintain the integrity of the host (Roncarolo et al., 2001). In addition, in a mouse model of helminthic infection, Th2 cells were shown to give rise to BCL6⁺, IL-21⁺ and GATA3⁺ T_{FH} (Glatman Zaretsky et al., 2009). T_{FH} are professional B helper cells, which express the transcription factor BCL6 and the chemokine receptor CXCR5 and produce IL-21 in B cell follicles (Crotty, 2011). Murine Th2 cells, in the presence of TGF- β , can switch to Th9 cells, which express the transcription factor PU.1, produce IL-9 and participate in the allergic response in asthma and helminthic infections (Anuradha et al., 2013; Chang et al., 2010; Veldhoen et al., 2008; Xie et al., 2012).

CD4⁺ T cells contribute to anti-tumor or pro-tumor immunity in different ways (Blankenstein et al., 2012). They can sustain the activity of CD8⁺ T cells through the production of IL-2 and IL-12. Indeed, these cytokines promote CD8⁺ T cells expansion, activation and survival and improve the affinity of MHCI/peps to ameliorate CD8⁺ T cell response (Mescher et al., 2007). Moreover, the interaction between CD4⁺ T cell and DC presenting cognate MHCII/peps, can give rise to DC reconditioning, through interactions between CD40 on DC and CD40L on T cells. These interactions result in the up-regulation of a number of co-stimulatory signals and polarizing stimuli, such as IL-12 production, which in turn favor the activation of CD8 cells (Melief, 2008; O'Sullivan and Thomas, 2003). CD4⁺ T cells play also a role in immunosurveillance independent of CD8⁺ cells since cytokines such as IL-2, IL-12 and IFN- γ also activate monocytes, macrophages and DC, to display an anti-tumor response (Jacobson et al., 1995; Mucida and Cheroutre, 2010). In contrast, IFN- γ can also trigger the expression of programmed cell death ligand (PDL-1) on cancer cells, which engages PD-1 on cytotoxic T cells, providing off signals and favoring tumor development (Sharpe et al., 2007) (Matsushita et al., 2012). Th2 cells retain a well-known tumor-promoting potential. In human and murine breast cancer, tumor-promoting Th2 cells can be induced by IL-13 and TNF- α produced by OX40L-expressing DC, which in turn are activated in response to tumor-derived Thymic-Stromal lymphopietin (TSLP) (Pedroza-Gonzalez et al., 2011). In murine models of breast cancer, CD4⁺ Th2 cells accelerated tumor growth through the production of IL-13 (Aspard et al., 2007) and favored macrophage polarization toward a pro-tumorigenic phenotype, induced by the local production of IL-4 (Gocheva et al., 2010). In human breast and pancreatic cancers, the Th2 signature was increased in lymph nodes and associated with reduced survival (De Monte et al., 2011; Kristensen et al., 2012).

Local production of cytokines and the expression of selected chemokine receptors by tumor infiltrating lymphocytes (TILs) define their homing, their activation and their

polarization states. For instance, CD8⁺ T cells and Th1 cells express CXCR3 and CX3CR1 that make them responsive to CXCL9 and CXCL10 and to CX3CL1, respectively. Moreover, the chemokines CXCL9 and CXCL10 are produced by cancer or stromal cells and promote anti-tumor responses by recruiting CD8⁺ and Th1 cells, as documented in colo-rectal cancer (CRC) (Mlecnik et al., 2010; Musha et al., 2005) (Zumwalt et al., 2014), renal cancer (Pan et al., 2006) and gastric cancer (Ohtani et al., 2009). Thus, within the tumor microenvironment, tumor and stromal cells modulate the microenvironment, which shape the immune infiltrate, towards a pro-tumor or anti-tumor response.

CD4⁺ CD25⁺ regulatory T cells (Treg) express the transcription factor Foxp3 and include natural (acquiring regulatory functions in the thymus) or adaptive (induced in the periphery from mature CD4⁺ T cells under the influence of TGF- β) Treg (Stephens et al., 2001; Tran et al., 2007). These cells are required to maintain self-tolerance and their functional specialization is shaped by the tissue microenvironment (Burzyn et al., 2013). Treg play important roles in tumor progression and accumulate in peripheral blood and tumor of cancer patients (Halvorsen et al., 2014) (Whiteside, 2012). Tumor-infiltrating immune cells or tumor cells themselves produce soluble factors able to recruit Treg, such as CCL17 or CCL22 (Mizukami et al., 2008; Sun et al., 2015; Wertel et al., 2015). Increased recruitment of Treg contributes to a tumor-promoting microenvironment (Whiteside, 2013; Zou, 2006). Indeed, through the production of immunomodulatory cytokines, such as IL-10 and TGF- β , Treg efficiently dampen the functions of CD8⁺ T cells, including IFN- γ production (Roncarolo et al., 2006). Treg express high levels of the IL-2 receptor IL-2R α , which acts as a decoy for IL-2 and reduce the local levels of this cytokine, impairing T cell activation and proliferation (Shevach, 2009). Treg affect also DC development. Indeed, they prevent DC maturation and induce the expression of IL-10, as well as immunosuppressive molecules of the B7-H family, in DC (Mahnke et al., 2007). Priming of T cells by these tolerogenic DC results in T cells tolerance in the form of anergy or clonal deletion

(Maldonado and von Andrian, 2010; Raimondi et al., 2007). Interestingly, the immunoregulatory functions of Treg can be also beneficial for the host. Indeed Treg limit the inflammatory component of tumors and it has been hypothesized that this mechanism is responsible for the positive prognostic effect exerted by the T regulatory infiltrate in CRC (Fridman et al., 2012).

Th17 cells are induced by the combination of IL-1 β , IL-6, TGF- β and IL-23, express the transcription factors ROR-c in humans and ROR γ t in mice respectively, are characterized by the production of IL-17 and play important roles in combating extracellular pathogens and fungi (Annunziato et al., 2007; Unutmaz, 2009) (Manel et al., 2008). In cancer, Th17 cells can exert pro- or anti-tumor functions depending on the tissue microenvironment. The major pro-tumor functions of Th17 cells are due to angiogenesis promotion and recruitment of tumor-promoting myeloid cells (Wei et al., 2012). For instance, in a murine model of subcutaneous fibrosarcoma cell graft, the increased expression of IL-17 was associated with tumors displaying higher vessel density, due to the increased expression of VEGF, keratinocyte-derived pro-angiogenic chemokines (CXCL1 and CXCL2) and prostaglandins by fibroblasts and stromal cells (Numasaki et al., 2003). In some tumor murine models, upon treatment with anti-VEGF antibodies higher tumor infiltration by mature Th17 was found, associated with higher CD11b⁺Gr1⁺ cells and amplified expression of both G-CSF and IL-17. This “IL-17-G-CSF” axis was associated with angiogenesis independent of anti-VEGF therapy and promotion of tumor growth via the recruitment of immune-suppressive and pro-angiogenic CD11b⁺Gr1⁺ myeloid cells (Chung et al., 2013). In addition, in a mouse model of intestinal spontaneous carcinogenesis, microbial products derived from the adenoma-induced epithelial barrier alterations, induced IL-23 production in myeloid cells, which in turn activated an IL-17-mediated pro-tumoral response (Grivennikov et al., 2012). In humans, IL-17 was responsible for the recruitment of neutrophils in peritumoral stroma of Hepatocellular Carcinoma tissues through the induction of CXC chemokines in epithelial

cells. The recruited peritumoral neutrophils were an important source of matrix metalloproteinase-9 (MMP-9), which *in vitro* stimulated proangiogenic activity in hepatoma cells. Accordingly, *in vivo* neutrophil depletion inhibited tumor angiogenesis and growth (Kuang et al., 2011). On the other hand, Th17-derived IL-17 can synergize with IFN- γ to induce CXCL9 and CXCL10 production in tumor cells, thus favoring the recruitment of cytotoxic T cells (Wei et al., 2012).

III. 1.2. B cells

Beyond their classical antibody-production function, B cells work together with other cell types, mainly T cells, in a number of antibody-independent effects (Bao and Cao, 2014). Indeed B and T cells co-localize in secondary lymphoid organs, where T cells modulate B cell functions, through the release of cytokines (e.g. IL-21) that promote B cell clonal expansion, Immunoglobulin (Ig) production and class switching (Bao and Cao, 2014). In turn, B cells modulate T cell functions, through their antigen-presenting capability and providing co-stimulatory molecules necessary for T cell activation and proliferation (Bindea et al., 2013; Nelson, 2010). Depending on the type of response in which they are involved, B cells produce a wide spectrum of cytokines, which orientate the immune response (Bao and Cao, 2014). For instance, in presence of Th1-related stimuli, B cells produce IL-12 or IFN- γ , whereas in a Th2 setting they produce IL-4 and IL-13 (Harris et al., 2000; Lund and Randall, 2010). In the skin, squamous carcinogenesis is limited in the absence of B cells (Andreu et al., 2010; Schioppa et al., 2011). Two mechanisms are responsible for the B cell-dependent skin carcinogenesis. Firstly, an increased deposition of IgG and Immunocomplexes (IC) within the tumor activates the Fc γ receptors on myeloid cells (e.g. mast cells and macrophages), favoring angiogenesis and immunosuppression (Andreu et al., 2010; de Visser et al., 2005). Moreover, B cells produce IL-10 in a TNF- α -dependent manner, which in turn impaired anti-tumor IFN- γ CD8⁺ T cell activity (Schioppa et al., 2011). In addition, in a transgenic murine model of cutaneous squamous cell

carcinoma, B cell depletion therapy improved platinum or paclitaxel based chemotherapy response. This effect was due to a macrophage “reprogramming” characterized by the increased production of CCR5-ligands responsible for the enhancement of CD8⁺ T cells recruitment (Affara et al., 2014). Moreover, in a murine model of prostate cancer, B cell derived lymphotoxin β activated the I κ B kinase IKK α and STAT3 in cancer cells to enhance androgen refractory tumor growth and metastatic progression in a cell autonomous, NF- κ B independent mechanism (Ammirante et al., 2010). However, leukocyte pro-tumorigenic activities depend on tissue-specific signals, as highlighted by the fact that, in contrast to skin and prostate cancers, B cells lack of functional significance during mammary carcinogenesis (DeNardo et al., 2009).

III.2. Innate Immune cells

III. 2.1. NK cells

NK cells are a family of innate immune cells, which play a central role in tumor immunosurveillance along with T cells (Pardoll, 2003). NK cells have the capacity to recognize infected or transformed cells and kill them. They are an important source of IFN- γ , a potent immunostimulatory cytokine, which enhance antigen presentation and Th1 responses (Koch et al., 2009). Unlike T cells, NK cells do not express restricted receptors (such as TCR), but two alternative classes of receptors. The first type is an inhibitory receptor, called “killer cell immunoglobulin like receptor” (KIRs), which binds MHC-I. When NK cells encounter normal MHC-I expressing cells, KIRs mediate the inactivation of the NK, keeping them in resting condition and preventing reactions against self (Vivier et al., 2012). When a cell down-regulates its MHC-I expression, the NK cell is activated (Moretta et al., 1997). As a mechanism of immunoescape, tumor cells can down-regulate MHC-I expression to evade CD8⁺ T cell recognition (Ferrone and Marincola, 1995). Therefore, NK cells can recognize the transformed cells and eliminate them (Ljunggren and Karre, 1990). The second class of receptors activates NK cells and is named NKG2D

(Natural Killer group 2, member D). These receptors bind a class of stress-induced ligands, such as MHC I polypeptide A (MICA) and B (MICB), and activate NK cells in presence of IL-15 or IL-2 (Nausch and Cerwenka, 2008). Under stress condition, tumor cells can express MICA or MICB and can easily activate NK cells (Carbone et al., 2005). Because of their crucial role in combating tumor cells, NK cells have been considered a promising tool for anticancer therapy. For instance, autologous NK-cell transfer has been experimented for the treatment of renal cell carcinoma, malignant glioma and metastatic breast cancer. However, the inhibition of NK cells by self MHC-I expressed by tumor cells significantly limited the efficacy of this approach. Subsequently, allogeneic NK transplantation efficiently prevented relapse, graft reaction and Graft versus Host disease (GvHD) in myeloid leukemia patients (Moretta et al., 2011; Ruggeri et al., 2002). Later, adoptive cellular transfer of allogeneic NK cells was also attempted for solid tumors such as neuroblastoma and ovarian cancer (Castriconi et al., 2004; Miller et al., 2005). Quite recently in patients with HCC treated by surgical resection, radiofrequency ablation, or percutaneous ethanol injections of activated cytokine-induced killer cells ($CD3^+/CD56^+$ and $CD3^+/CD56^-$ T cells and $CD3^-/CD56^+$ natural killer cells) significantly prolonged recurrence-free survival (Lee et al., 2015).

III. 2. 2. Dendritic Cells (DC)

Dendritic cells are a subset of myeloid cells, which play essential roles in activating adaptive immunity (Gabrilovich et al., 2012). Considered at the crossroad between innate and adaptive immune responses, DC act as professional APC that recognize foreign antigens in peripheral tissues, process and present them to T cells (Ley, 2014). To activate T cells, DC have to display a mature phenotype, consisting in the expression of co-stimulatory molecules (CD80/CD86) and appropriate cytokines. Indeed, whether immature DC interact with T cells, the last undergo anergy, since immature DC express low levels of co-stimulatory molecules, low levels of MHC class II and express immunoregulatory

molecules such as surface-bound TGF- β (Guerder et al., 2013). Moreover even mature DC can display a tolerogenic phenotype, characterized by the production of immunoregulatory molecules such as Arginase 1 (ARG1), indoleamine 2,3 dioxigenase (IDO), TGF- β and IL-10 (Hurwitz and Watkins, 2012; Novitskiy et al., 2008). In tumor-bearing hosts, the maturation of DC can be impaired, thus favoring T cell suppression (Lee et al., 2003; Mitsuka et al., 2013). In the tumor microenvironment, the functions of mature DC can also be compromised. For instance, tumor-related hypoxia negatively affected the functions of DC, by inducing the production of VEGF, IDO and ARG1 (Mancino et al., 2008). Moreover, tumor-conditioned DC tend to preferentially promote the function of CD4⁺CD25⁺ Foxp3⁺ Treg (Gai et al., 2013).

III. 2. 3. Macrophages

Macrophages are the most represented leukocytes in the microenvironment of solid tumors. Classically viewed as terminally differentiated cells, they were thought to derive from circulating monocytes and to differentiate at sites of inflammation under the influence of distinct molecules, such as M-CSF or GM-CSF (Allavena et al., 2008). However, a recent line of evidence suggests the existence, at least in mice, of a self-renewing population of macrophages, independent on bone marrow-derived monocytes, able to locally proliferate in addition to differentiate (Davies et al., 2011; Jenkins et al., 2011; Robbins et al., 2013). To date, only few studies in atherosclerosis and cancer indicate that macrophage proliferation also exists in humans (Lutgens et al., 1999) (Campbell et al., 2011).

A number of evidence in the last 20 years highlighted the multifunctional properties of macrophages, which are actually considered highly plastic cells, able to modify their phenotype in response to microenvironmental signals (Galdiero et al., 2013b). Under the influence of mediators such as IFN- γ , alone or together with microbial stimuli such as LPS, macrophages undergo classical M1 polarization, characterized by an immunostimulatory

phenotype able to induce cytotoxic and Th1 responses. M1 macrophages produce significant amount of IL-12 and IL-23 as well as Th1-attracting chemokines (i.e. CXCL9 and CXCL10) and high levels of reactive oxygen and nitrogen species. In contrast, they are scarce producers of the immunomodulatory cytokine IL-10. On the other hand, IL-4 or IL-13 are able to induce alternative M2 macrophages, characterized by a high production of chemokines such as CCL17, CCL22 or CCL24, involved in the recruitment of Th2 cells, Tregs, eosinophils and basophils, as well as a high production of IL-10. In contrast, M2 macrophages produce low levels of IL-12 or IL-23 and are mainly involved in immunoregulatory networks, regulating tissue remodeling and angiogenesis (Mantovani et al., 2013) (**Figure 4**).

M1 macrophages are responsible for resistance against intracellular pathogens such as *Listeria monocytogenes*, *Salmonella typhi*, *Salmonella typhimurium* and *Mycobacterium species* (Cavaillon and Adib-Conquy, 2006; Chacon-Salinas et al., 2005; Jouanguy et al., 1999; Shaughnessy and Swanson, 2007). Moreover, M1 inflammation can lead to pathologic consequences. Indeed, during acute *Escherichia coli* or *Streptococcus sp.* infections, uncontrolled M1 inflammation is associated with gastroenteritis, urinary tract infections, neonatal meningitis and sepsis (Cavaillon and Adib-Conquy, 2006). M1 to M2 switch is important in containing the inflammatory reaction, limiting tissue damage, such as during the respiratory syncytial virus-induced (RSV-induced) bronchiolitis (Shirey et al., 2010). Failure to switch from an M1 to an M2 phenotype can lead to unresolved chronic inflammation, such as in chronic venous ulcers in humans (Sindrilaru et al., 2011). Macrophage activation has also been found in autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Haringman et al., 2005; Zhang et al., 2010). In contrast, M2 polarization is associated with experimental and human parasitic infections (Noel et al., 2004).

In cancer, due to their Th1-immunostimulatory profile, M1 macrophages have been described to play important anti-tumor roles. For instance, Th1-driven macrophage activation mediates elimination of senescent hepatocytes, responsible for carcinogenesis (Kang et al., 2011). In contrast, tumor progression has been associated to a switch from M1 to M2 phenotype. Indeed, tumor-associated macrophages (TAM) have been described to express a number of M2-like properties (**Figure 5**). In particular they promote growth, invasive behavior and metastatic potential of several tumors, such as renal cancer (Daurkin et al., 2011), breast cancer (Movahedi et al., 2010), pancreatic cancer (Gocheva et al., 2010), lung (Koukourakis et al., 1998) and cervical cancer (Schoppmann et al., 2002), and as described by *in vivo* and *in vitro* models (DeNardo et al., 2009; Joyce and Pollard, 2009; Vasiljeva et al., 2006). TAM express a number of pro-angiogenic factors (such as VEGF and TGF- β) and release immunosuppressive molecules (such as TGF- β and IDO), thus shifting the balance towards a pro-tumor microenvironment (Galdiero et al., 2013b).

IV. THE NEUTROPHIL

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., 2011b; Tecchio et al., 2013).

IV. 1. Granulopoiesis

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 (Kato 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 granulocytogenesis because G-CSF^{-/-} mice, even though neutropenic, still display residual granulocytogenesis 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 “neurostat 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).

Recent 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).

IV. 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 (**Figure 6**). 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).

IV. 3. Neutrophil recruitment

The sequential process by which neutrophils leave circulation and migrate to tissues is defined as “neutrophil recruitment cascade”, and comprises five steps: tethering, rolling, adhesion, crawling and transmigration (Ley et al., 2007; Muller, 2013).

In the first phases of the inflammatory response, inflammatory stimuli activate endothelial cells, which up-regulate P- and E- selectins on their surface. Neutrophils constitutively express selectin ligands, such as P-selectin glycoprotein ligand 1 (PSGL-1), which engage P- and E-selectins on endothelial cells and give rise to the tethering of neutrophils on the surface of the vessel wall. Tethering is followed by the rolling of neutrophils along the vessel in the direction of the blood flow. The engagement of PSGL-1 on neutrophils also activates intracellular signals leading to neutrophil activation and firm adhesion (Wang and Arase, 2014; Yago et al., 2010). Indeed, during the rolling phase, chemokines lead to the β_2 -integrin clustering on neutrophils, enhancing their binding to the ligand Intracellular Adhesion Molecule- 1 (ICAM-1) expressed on activated endothelium and leading to firm adhesion of neutrophils to the endothelium. During early stages of inflammation, neutrophils extend a PSGL-1-rich microdomain into the vessel lumen that scans for activated platelets in the bloodstream. The intact distribution and signaling through PSGL-1 regulate neutrophil crawling, at least in part, orchestrating the appropriate distribution of the adhesive and chemotactic receptors CD11b/CD18 (Mac-1) and CXCR2. Only when productive interactions occur with platelets, neutrophils organize these additional receptors needed for intravascular migration or generate NETs, resulting in inflammation (Sreeramkumar et al., 2014). After adhesion, neutrophils cross the endothelium to gain the underlying tissue. Usually this process occurs in the endothelial cell junctions, through a mechanism involving cell adhesion molecules (Woodfin et al., 2009). As an alternative way and in conditions of high ICAM-1 expression, neutrophils can migrate through pores in the cytoplasm of endothelial cells (transcellular migration) (Yang

et al., 2005). Beyond this generally accepted view, neutrophil recruitment mechanisms also display some variations in distinct organs. For instance, in the liver, neutrophils directly adhere to the activated endothelium, skipping the rolling step. Moreover, in sterile conditions neutrophil adhesion is ICAM-1-dependent, but during infections it occurs through hyaluronan – CD44 interactions between endothelial cells and neutrophils (McDonald and Kubes, 2012; McDonald et al., 2008).

IV. 4. Neutrophil activation at sites of inflammation: beyond ROS and proteases

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 have the capacity to trap different types of microbes (e.g. *Escherichia coli*, *Shigella flexneri*, *Staphylococcus aureus*), fungi (e.g. *Candida albicans*, *Aspergillus fumigatus*) and human immunodeficiency virus-1 (HIV-1), supporting their interaction with effector molecules and their clearance (Brinkmann and Zychlinsky, 2012; Saitoh et al., 2012; Yousefi et al., 2009). 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).

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 NADPH (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 neutrophil extracellular traps 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 necessary 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).

IV. 5. 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-1 β following LPS stimulation, in a NLRP3 inflammasome 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)

(Table 2). 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., 2011b; Tamassia et al., 2013). Furthermore, Tamassia and colleagues recently 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).

IV. 6. Neutrophils as a source of Pattern Recognition Molecules (PRMs)

In addition to be a reservoir of proteolytic and antimicrobial molecules and to express a set of cytokines and chemokines, neutrophils have also been found to be a source of pattern recognition molecules (PRMs). These membrane-bound or soluble molecules retain the capability to identify pathogen associated molecular patterns (PAMPs), to discriminate self versus non-self and to initiate a coordinated immune response (Jaillon et al., 2013).

PRMs expressed by neutrophils include all the known TLRs excepted TLR3 (Hayashi et al., 2003), the C-type lectin receptors Dectin-1, Mincle, CLEC2 and CLECSF8 (Graham et al., 2012; Kennedy et al., 2007; Kerrigan et al., 2009; Lee et al., 2012) and a number of

cytosolic DNA sensors, such as MDA5, STING and RIG1 (Tamassia et al., 2012; Tamassia et al., 2008). Dectin-1, the main β -glucan receptor, plays a major role in defense against fungi such as *Candida albicans* and *Aspergillus fumigatus* (Kennedy et al., 2007). Cytosolic DNA sensors recognize microbial nucleic acids and activate the expression of IFN- β , thus playing important roles in defense against intracellular pathogens such as *Listeria monocytogenes*, *Legionella pneumophila* and adenovirus (Tamassia et al., 2012). Neutrophils also express the Formyl Peptides Receptors 1 (high-affinity) and 2 (low-affinity), which recognize bacterial and mitochondrial formyl peptides. Once activated, these receptors mediate neutrophil activation and migration through p38 or Erk activation respectively (Liu et al., 2012; McDonald et al., 2010).

Neutrophils also display a number of soluble PRMs, stored in granules and released upon activation. These molecules are part of the humoral arm of innate immunity, share similar functions with the antibodies and include PTX3, proteoglycan recognition receptor (PGRP-S) and M-ficolin.

PTX3, a member of the long pentraxin family, is stored in secondary granules of neutrophils in a ready to use form and is rapidly mobilized upon neutrophil activation. PTX3 has been reported to be associated with NETs (Jaillon et al., 2007). PTX3 acts as an opsonin against *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. Following opsonisation, PTX3 activates phagocytes through Fc γ RIIA engagement and amplifies C3b-opsonized pathogen phagocytosis (Moalli et al., 2010; Moalli et al., 2011). Interestingly, PTX3 acts also as a regulator of neutrophil recruitment and activation. Indeed, PTX3 attenuated neutrophil recruitment at sites of inflammation, through the interaction with P-selectin (Deban et al., 2010) and translocated from granules to membrane of apoptotic neutrophils to favor their recognition and clearance by macrophages (Jaillon et al., 2009). In addition, PTX3 has recently emerged as key regulator of complement cascade in modulating cancer susceptibility in mice and humans. Indeed, in murine models of

carcinogen-induced tumors, lack of PTX3 was associated with increased cancer susceptibility, due to a deregulated complement activation and increased macrophage M2-polarization (Bonavita et al., 2015).

PGRP-S and M-ficolin are stored in secondary and tertiary granules (Cho et al., 2005; Rorvig et al., 2009). As the main proteoglycan receptor, PGRP-S plays major roles in defense against microbes such as *Staphylococcus aureus* and *Bacillus subtilis*, exerting bacteriostatic and bactericidal functions (Cho et al., 2005; Kashyap et al., 2011). M-ficolin is a member of the lectin family, activates complement cascade, acts as an opsonin and plays important roles in defense against Gram-positive and Gram-negative bacteria. Neutrophil-derived M-ficolin is also able to activate neutrophils themselves through the interaction with the surface molecule CD43 (Moreno-Amaral et al., 2012; Rorvig et al., 2009).

IV. 7. Neutrophils in immune cell cross-talk

Given their complex and multi-faced functions, neutrophils can be part of a dynamic interplay with innate immune cells, such as DC, monocytes, macrophages and NK cells, and adaptive immune cells, such as T and B cells. By establishing these interactions, neutrophils receive and integrate signals modulating their activation and survival and, on the other hand, modulate effector immune responses.

IV. 7. 1. Neutrophils and innate immune cells

Activated neutrophils were shown to promote maturation of human monocyte-derived DC (mo-DC), through interaction between CD18 and CEACAM-1 (carcinoembryonic antigen-related cell adhesion molecule-1) expressed by neutrophils and DC-SIGN (DC-specific ICAM3-grabbing non-integrin) on mo-DC (Schuster et al., 2013). In mice, neutrophils are also able to induce maturation of bone marrow-derived DC, through the production of TNF- α (van Gisbergen et al., 2005a; van Gisbergen et al., 2005b). Mo-DC matured in the presence of neutrophils acquire the potential to induce T cell proliferation and polarization towards Th1 phenotype (Megiovanni et al., 2006). Supernatants from

parasite-infected neutrophils induced IL-12 and TNF- α production and CD40 and CD86 up-regulation in DC, in a TNF- α dependent manner. Splenic DC from neutrophil-depleted mice showed a significantly lower IL-12 and TNF- α production, thus confirming the pivotal role exerted by neutrophils in microbial-triggered DC maturation (Bennouna et al., 2003).

In contrast, neutrophils can also negatively affect DC maturation and functions. For instance, neutrophil elastase significantly impaired the DC allostimulatory ability switching them into TGF- β 1-secreting cells (Maffia et al., 2007). Accordingly, neutrophil-derived extracellular vesicles (or ectosomes) modified mo-DC morphology, reduced their phagocytic activity, and increased the release of TGF- β 1. Even under LPS stimulation, the DC maturation process was partially inhibited as suggested by reduced expression of cell surface markers (i.e. CD40, CD80, CD83, CD86), inhibition of cytokine-release (IL-8, IL-10, IL-12, and TNF- α), and reduced capacity to induce T cell proliferation (Eken et al., 2008).

Neutrophils can modulate cytokine production and microbicidal activity of macrophages. For instance, apoptotic neutrophils further enhanced macrophage proinflammatory properties following *Mycobacterium tuberculosis* infection, through the activation of the NLRP3 inflammasome and IL-1 β signaling (Andersson et al., 2014). During *Mycobacterium tuberculosis* infection, close interactions between macrophages and neutrophils were observed. Indeed, macrophages phagocytosed NETs and produced IL-6, TNF- α , IL-1 β and IL-10 when co-cultured in presence of NETs from Mtb-activated neutrophils (Braian et al., 2013). In contrast, apoptotic neutrophil phagocytosis can also induce an IL-10^{high} IL-12^{low} immunomodulatory phenotype in macrophages, favoring tissue repair and return to homeostasis (Filardy et al., 2010).

Neutrophils also participate in an important bidirectional cross-talk with NK cells. On the one hand, NK-derived soluble factors, such as IFN- γ and GM-CSF significantly improve

neutrophil life span and ROS production (Bhatnagar et al., 2010; Costantini and Cassatella, 2011). On the other hand, neutrophils are required for NK maturation and activation. Indeed, in a murine model of genetically induced neutropenia, poor survival, hyporeactivity and impaired development of NK were observed (Jaeger et al., 2012; Ordonez-Rueda et al., 2012). Similar defects in maturation and activation of NK were also described in patients suffering from cyclic neutropenia, suggesting that neutrophil-NK interaction is important also in humans (Jaeger et al., 2012).

Interestingly, a peculiar “*ménage a trois*” has been described between neutrophils, NK cells and a specific myeloid DC subset, namely 6-sulfo-LacNAc myeloid DC (slanDC). Indeed, neutrophils induced the release of IL-12p70 by slanDC via the interaction between CD18 and ICAM-1. SlanDC-derived IL-12p70 stimulated NK cells to produce IFN- γ , which in turn further enhanced neutrophil-induced IL-12p70 production in SlanDC, thus fueling a peculiar positive feedback loop (Costantini et al., 2011). Neutrophils and NK cells can also negatively influence each other. For instance, neutrophil-derived microparticles (ectosomes) inhibited the production of IFN- γ and TNF- α but enhanced the release of TGF- β 1 by IL-2/IL-12-activated NK cells, switching NK from an immunostimulatory to an immunomodulatory phenotype (Pliyev et al., 2014). In contrast, NK cells can induce neutrophil apoptosis in a contact-dependent manner through the interaction between NKp46 and Fas (Thoren et al., 2012).

Even though quite neglected, the interaction between neutrophils and invariant NKT cells (iNKT) also plays a role in modulating inflammatory reactions. For instance, a cell-cell contact between these cells has been described *in vitro*, leading to iNKT function impairment. Accordingly, during neutrophil-mediated inflammatory reactions, cytokine production and activation of iNKT cells were significantly reduced (Wingender et al., 2012).

IV. 7. 2. Neutrophils and adaptive immune cells

Neutrophils also communicate with adaptive immune cells, such as T lymphocytes. Indeed, neutrophils induce Th1 and Th17 cells migration through the release of CXCL10 or CCL20, respectively. Association between neutrophils and Th17 cells was observed in colonic mucosa of Crohn's patients and in the synovial fluid from rheumatoid arthritis patients (Pelletier et al., 2010a). In addition, Th17 and Tregs are able to release CXCL8, and activated T cells are able to modulate neutrophil survival and activation through the production of GM-CSF, IFN- γ or TNF- α (Davey et al., 2011a; Himmel et al., 2011; Pelletier et al., 2010b). Moreover, neutrophils migrate to the lymph nodes in a CCR7-dependent manner and can exert APC functions. Indeed, neutrophils were able to recognize and process the antigen in MHC-I context, present it to CD8⁺ T cells through a process of cross-presentation and prime CD8 T cells to become cytotoxic effector cells (Abi Abdallah et al., 2011; Beauvillain et al., 2011; Beauvillain et al., 2007).

Neutrophils interact with B cells, influencing several aspects of B cell functions. Indeed, a specific neutrophil population colonizing the peri-marginal zone of the spleen was identified, named "B-helper neutrophils" (N_{BH}). Compared to circulating neutrophils, these cells displayed a high expression of B-cell supporting molecules such as BAFF, APRIL, IL-21 and CD40L, as well as B-cell attracting chemokines such as CXCL12 and CXCL13. B helper neutrophils formed B cell-interacting NETs-like structures and favored Immunoglobulin (Ig) class switch and somatic hypermutations. Patients with genetically induced neutropenia also displayed a reduction in T-independent antigen specific Igs, whereas T-dependent antigen Ig production was intact. These data supported the role of neutrophils in creating an innate antibody-mediated antimicrobial humoral defense through their direct interaction with B cells also in humans (Puga et al., 2012).

IV. 8. Neutrophils in the resolution of inflammation

Neutrophils content is deleterious for microbes but also, potentially, for the host. Therefore, the clearance of recruited neutrophils at sites of inflammation is an important

phase of the resolution of inflammation (Buckley et al., 2013). Failure of neutrophils to undergo apoptosis or failure of macrophage to eliminate apoptotic neutrophils can lead to prolonged and deregulated inflammation. Apoptotic neutrophils undergo several modifications, including alterations of cell surface markers (e.g. increased expression of phosphatidylserine) and membrane relocalization of intracellular components (e.g. PTX3, endoplasmic reticulum proteins and nucleosomes) that promote their clearance by phagocytes, such as macrophages (Fox et al., 2010; Jaillon et al., 2009) (Soehnlein and Lindbom, 2010). Within the inflammatory microenvironment neutrophil clearance is modulated by a number of factors, such as IL-1 β , G-CSF and GM-CSF as well as hypoxia that delay neutrophil apoptosis (Thompson et al., 2014; Witko-Sarsat et al., 2010) or TNF- α , which can both accelerate apoptosis or enhance neutrophil survival in a concentration-dependent manner (Cross et al., 2008). As a mechanism aimed at limiting the inflammatory response, during apoptosis neutrophils release soluble factors, such as lactoferrin or annexin A1, which reduce the recruitment of inflammatory cells (Perretti and D'Acquisto, 2009) (Bournazou et al., 2009). Moreover, apoptotic neutrophils and macrophages following phagocytosis of apoptotic neutrophils increase the production of lipid pro-resolving mediators, such as lipoxins, resolvins and protectins (Dalli and Serhan, 2012; Serhan et al., 2008). Indeed, in the latest stages of the inflammatory cascade, neutrophils switch the eicosanoid biosynthesis from Leukotriene B4 to Lipoxin A4, thus limiting further neutrophil infiltration. Lipoxin A4 acts on its specific G-protein coupled receptor (GPCR) expressed on neutrophils, modifies the phosphorylation state of cytoskeleton proteins and causes cell arrest (Serhan et al., 2008). In addition, Lipoxin A4 prevents neutrophil L-selectin shedding and integrin up-regulation (Filep et al., 1999). Also neutrophil derived resolvins limit neutrophil infiltration, as demonstrated in a number of *in vivo* models (Chiang et al., 2012; Krishnamoorthy et al., 2010; Serhan et al., 2008; Spite et al., 2009). The two members of this family RvD1 and RvE2 directly interact with specific

GPCRs, and RvE2 interact with LTB₄ receptor, limiting neutrophil chemotaxis (Arita et al., 2007; Serhan et al., 2008). In addition, they also impair neutrophil adhesion to the endothelium by modulating neutrophil integrin expression and CD62L shedding (Krishnamoorthy et al., 2010; Spite et al., 2009). In addition, resolvins up-regulate CCR5 expression on apoptotic neutrophils, enhancing the clearance of chemokines such as CCL3 and CCL5 (Ariel et al., 2006). Also live neutrophils retain the ability to capture and entrap chemokines and cytokines, thus limiting their effects. For instance, neutrophils express IL-1Ra, which engages IL-1R without any signal transduction and the decoy IL-1R type II, which captures IL-1 β preventing its interaction with the functional IL-1R1 (Bourke et al., 2003; Cassatella et al., 1994).

V. NEUTROPHILS IN DISEASES

From the classical point of view, neutrophils have long been studied as immune cells with a pivotal role in defense against pathogens. However, recent lines of evidence shed new light on neutrophils as key contributors of many diseases such as chronic inflammatory diseases, autoimmune diseases and cancer (Galdiero et al., 2013b; Jaillon et al., 2013).

V. 1. Infections

Traditionally, neutrophils have been considered crucial soldiers in the battle against extracellular pathogens. However, recent lines of evidence also suggest a role for neutrophils in combating intracellular microbes. For instance, a genome-wide transcriptional analysis of peripheral blood neutrophils purified from patients with active tuberculosis indicated a type I IFN-related signature, suggesting a role for these cells in the pathogenesis of *M. Tuberculosis* infection (Berry MP Nature 2010). In the same line of evidence, Tamassia and colleagues demonstrated the ability of neutrophils to produce IFN- β upon infection by intracellular pathogens (e.g. *L. pneumophila*, *L. monocytogenes*) through the activation of cytosolic DNA sensors (Tamassia et al., 2012). Moreover, neutrophils express a functional and active TLR9 on their membrane surface. This receptor

was activated by oligonucleotides containing unmethylated CpG motifs, providing an additional tool to recognize microbial nucleic components when endosomes were not accessible to TLR9 ligands (Lindau et al., 2013). TLRs also act as modulators of neutrophil recruitment. Indeed, engagement of TLRs on neutrophils led to activation of G-coupled receptor kinase 2 and to desensitization and internalization of CXCR2, limiting neutrophil migration at sites of inflammation (Alves-Filho et al., 2009; Trevelin et al., 2012). Accordingly, in a murine model of sepsis, TLR9 deficiency was associated to enhanced neutrophil infiltration, lower inflammatory response and improved outcome (Trevelin et al., 2012). In addition, the IL-1 family member IL-33 inhibited the down-regulation of CXCR2 induced by TLR4 activation, thus increasing neutrophil recruitment and improving outcome in sepsis (Alves-Filho et al., 2010). Indeed, septic patients who did not survive displayed higher levels of soluble ST2, a circulating decoy receptor for IL-33, thus supporting the important role played by IL-33 in modulating neutrophil response and outcome of septic patients (Alves-Filho et al., 2010). In addition, in a murine model of *C. Albicans* infection, IL-33 promoted neutrophil recruitment at sites of infection through the enhanced CXCL1 and CXCL2 production from macrophages and the inhibition of CXCR2 down-modulation on neutrophils. Moreover, IL-33 pretreatment enhanced neutrophil CR3 expression, through a synergism between TLR and dectin-1 pathways, resulting in improved phagocytosis and killing of the opsonized *C. albicans* (Alves-Filho et al., 2010; Le et al., 2012).

V. 2. Chronic inflammation and autoimmune diseases

An uncontrolled neutrophil activation or impaired neutrophil clearance can be responsible for chronic inflammatory or autoimmune diseases, with dangerous consequences for the host.

Chronic Obstructive Pulmonary Disease (COPD) is a chronic disease and a major cause of morbidity and mortality in western countries. From the pathological point of view it is

characterized by a significant neutrophil infiltration, due to chemotactic stimuli such as CXCL8 and leukotriene B4 (LTB4). It has been shown that COPD patients display high levels of immunoglobulin-free light chains in serum and lungs, which activate neutrophils and induce the release of CXCL8, favoring a positive feedback loop which further promotes neutrophil infiltration in damaged lungs (Braber et al., 2012). In addition, LTB4 is synthesized by Leukotriene A4 hydrolase (LTAH4), an enzyme that also retains an aminopeptidase activity, which is responsible for the catabolism of the tripeptide proline-glycine-proline PGP, a potent neutrophil chemoattractant. Interestingly, cigarette smoke inhibits the aminopeptidase activity but not the hydrolase activity of the enzyme, increasing neutrophil recruitment and inflammation (Snelgrove et al., 2010). In contrast, chloride ions are able to activate the aminopeptidase activity of LTA4 hydrolase. However, in cystic fibrosis patients, due to the impaired functions of the cystic fibrosis transmembrane conductance regulator (CFTR), chloride ions extracellular levels are reduced, with consequent high levels of PGP and enhanced neutrophil pulmonary infiltration, which partially contributes to the emphysematous aspect of the lungs in these patients (Snelgrove et al., 2010). In addition, once recruited, neutrophils release high levels of gamma-glutamyl-peptidase, an enzyme involved in the catabolism of glutathione, thus reducing the local levels of anti-oxidant and impairing the respiratory functions of cystic fibrosis patients (Corti et al., 2012). In addition, in a murine model of acute intestinal inflammation, transmigrating neutrophils modified the transcriptional profile of the epithelial cells. More in details, during the acute phase of inflammation, recruited neutrophils gave rise to the respiratory burst with consequent local oxygen depletion. Local hypoxia stabilized hypoxia-inducible factor (HIF1- α) in epithelial cells, which preserves mucosal barrier homeostasis, by increasing goblet cell number and mucus production (Campbell et al., 2014).

Neutrophils play also important roles in pathogenesis of autoimmune diseases. Indeed, NETs are a source of self-antigens, such as nucleic acids and ribonucleoproteins in SLE, deaminated histones in Felty syndrome, proteinase 3 or MPO in Anti-neutrophil Cytoplasmic Autoantibody (ANCA)-associated vasculitis, citrullinated histones in RA (Khandpur et al., 2013; Radic and Marion, 2013).

ANCA-related vasculitis is a subset of autoimmune diseases characterized by chronic inflammation of blood vessels. ANCAs are a class of autoantibodies mainly directed against the cytoplasmic neutrophil proteins MPO or proteinase 3. The mechanism by which intracellular neutrophil proteins become antigenic remains still a matter of debate, but a role for NETosis, responsible for the exposure of self-antigens to the immune response has been proposed. For instance, serum of SLE patients contains immune complexes composed of antibodies anti-ribonucleoproteins and antimicrobial peptides. Following exposure to SLE-derived anti-ribonucleoprotein antibodies, neutrophils release NETs, which activate dendritic cells, in a TLR9-dependent manner. DC activation by NET-related immunocomplexes induces the release of IFNs by DC, which in turn activate neutrophils, and give rise to a positive feedback loop responsible for the chronic pathogenesis of the disease (Garcia-Romo et al., 2011; Lande et al., 2011). Moreover, NETs have been found in glomeruli and interstitium of kidney biopsies in vasculitis patients, supporting the role for NETs in tissue damage (Kessenbrock et al., 2009; Mulder et al., 1995). ANCAs directly activate neutrophils, leading to the release of ROS, proteases and cytokines, responsible of tissue damage. In addition, ANCAs activate the complement cascade, leading to the formation of C5a, a potent neutrophil chemoattractant, thus sustaining an autonomous loop that continuously fuels vascular inflammation (Schreiber et al., 2009). *In vivo* studies further support the central role of neutrophils in vasculitis pathogenesis, because neutrophil-depleted mice were protected from anti-MPO IgG-induced ANCA vasculitis (Mulder et al., 1995).

Rheumatoid arthritis (RA) is a chronic autoimmune polyarthritis and is characterized by neutrophil infiltration in the affected joints. Neutrophils play a pivotal role in tissue damage, through the release of ROS and proteases and recruit other immune cell populations through the release of chemokines, thus orchestrating the various phases of the inflammatory reaction (Wright et al., 2014). For instance, RA synovial fluid neutrophils produce high levels of the Receptor Activator of the Nuclear Factor κ -B Ligand (RANKL) and BAFF, which activate osteoclasts and B cells, respectively (Assi et al., 2007; Chakravarti et al., 2009). Moreover, circulating immunocomplexes, which are typical of RA, activate synovial neutrophils via Fc γ Rs. Cross-linking of IgGs bound on neutrophils lead to degranulation, ROS release and enhancement of neutrophil adhesion to endothelial cells, thus amplifying tissue damage (Rollet-Labelle et al., 2013). Finally, NETs play also a central role in neutrophil-mediated RA pathogenesis. Indeed, peripheral blood and synovial fluid neutrophils from RA patients displayed an increased susceptibility to produce NETs in basal conditions. NETosis was mediated by circulating autoantibodies (Rheumatoid Factor and/or Anti-Citrullinated Peptides antibodies – ACPA) together with inflammatory cytokines such as TNF- α and IL-17A. During NETosis, neutrophils extrude citrullinated autoantigens, such as citrullinated vimentin, which in turn further enhance NET release. NETs also directly activate fibroblast-like synoviocytes to produce proinflammatory molecules such as IL-6 and IL-8 (Khandpur et al., 2013).

Neutrophils have also been described to play important roles also in a murine model of multiple sclerosis and type I diabetes, mainly due to CXCR2-related mechanisms (Battaglia, 2014; Liu et al., 2010).

V. 3. Cancer

Among myeloid cells infiltrating the tumor stroma, tumor-associated macrophages (TAM) have been widely described as central cells orchestrating tumor growth, stroma remodeling, angiogenesis and T cell-dependent immunity (Balkwill et al., 2005; Biswas

and Mantovani, 2010; Pollard, 2004). Because of their short half-life and terminally differentiated phenotype, the role of neutrophils in tumor development has been considered negligible. However, new *in vivo* and *in vitro* techniques have completely changed this point of view and recent evidences suggest important roles of neutrophils in modulating tumor behavior. More in details Tumor-Associated Neutrophils (TAN) have been shown to exert anti-tumoral as well as pro-tumoral functions and findings derived from murine models suggest that neutrophils are endowed with unsuspected plasticity (Fridlender et al., 2009; Mantovani, 2009). In the context of cancer related inflammation, neutrophils emerge as candidates that modulate all steps of cancer initiation and growth as well as antitumor immunity.

V. 3. 1. Neutrophil occurrence in tumors and their prognostic significance

Within the tumor microenvironment, tumor and stromal cells produce a number of CXC chemokines (CXCL8, CXCL1, CXCL2, CXCL3, CXCL5) which are associated to cancer growth and progression and with the promotion of tumor angiogenesis and metastasis (Keeley et al., 2010; Lazennec and Richmond, 2010; Mantovani et al., 2011). For instance, murine models suggest a central role for CXCR2 in promoting lung and pancreatic cancers (Ijichi et al., 2011; Keane et al., 2004). More recently, in elegant murine models of inflammation-associated skin cancer, colitis-associated or spontaneous intestinal cancer, Jamieson and colleagues demonstrated that both inflammation-induced and spontaneous carcinogenesis were significantly suppressed following CXCR2 abrogation or neutrophil depletion (Jamieson et al., 2012). Moreover, the last identified CXC chemokine, CXCL17 promoted cancer growth together with the increased infiltration of a myeloid subset of CD11b⁺Gr1⁺F4/80⁻ in a murine model of graft tumor (Matsui et al., 2012).

In a conditional genetic murine model of lung cancer driven by K-ras activation and p53 inactivation, the spleen was found as a reservoir for TAM and TAN precursors. Indeed these cells were relocated from the spleen to the tumor and splenectomy significantly

reduced myeloid infiltration in tumors (Cortez-Retamozo et al., 2012). In the same model, the authors identified Angiotensin II as a factor playing a central role in amplifying hematopoietic self-renewal and myeloid cells progenitors (Cortez-Retamozo et al., 2013).

In humans, Head and Neck Squamous Cell Carcinoma (HNSCC) cell lines recruited neutrophils through the release of CXCL8 and Macrophage Inhibiting Factor (MIF), both identified as mediators of CXCR2-dependent neutrophil chemotaxis (Dumitru et al., 2011; Trellakis et al., 2011b). Hepatocellular carcinoma (HCC) cells were also able to recruit neutrophils through the production of CXCL8 (Kuang et al., 2011). Moreover, an extensive immunohistochemical study of 919 HCC patient biopsies showed association between increased CXCL5 expression, neutrophil infiltration and poor patients' survival (Zhou et al., 2012).

Epidemiological studies indicated an association between TAN and poor clinical outcome in metastatic and localized renal cell carcinoma, bronchioloalveolar carcinoma, hepatocellular carcinoma, colorectal cancer and head and neck cancer (Donskov, 2013; Jensen et al., 2009; Kuang et al., 2011; Rao et al., 2012; Trellakis et al., 2011a; Wislez et al., 2003). Moreover, neutrophil infiltration has been associated with higher glioma grade (Fossati et al., 1999) and higher aggressive behavior of pancreatic cancer (Reid et al., 2011). However, in some tumor types, such as gastric and colorectal cancer, the association between neutrophil infiltration and patient clinical outcome is not so clear and remains controversial (Caruso et al., 2002; Hirt et al., 2013) (**Table 3**). It is important to take in mind that variability in techniques (e.g. immunohistochemistry versus hematoxylin-eosin staining), patient datasets, definition, outcomes may account for the variability in the results and may explain these controversies (Donskov, 2013).

V. 3. 2. Neutrophils in tumor initiation and progression

Genetic instability is a hallmark of cancer (Hanahan and Weinberg, 2011) and neutrophil-derived reactive oxygen species (ROS) and nitroxic derivatives have been linked

to carcinogenesis already 30 years ago (Weitzman et al., 1985). Indeed, neutrophil-derived ROS and derivatives, such as MPO-mediated HOCl have been described to be associated with DNA point mutations (Gungor et al., 2010). Moreover, HOCl activates a number of neutrophil proteolytic enzymes such as MMP-2, MMP-7, MMP-8 and MMP-9 and inactivates the tissue inhibitor of proteases (TIMP-1), thus favoring matrix remodeling, invasiveness and metastatic behavior of cancer cells (De Larco et al., 2004).

Granule proteins were involved in tumor progression. For instance, Neutrophil Elastase (NE) could directly promote cancer. In fact, NE was taken up by lung cancer cells and was responsible for the degradation of Insulin Receptor Substrate-1 (IRS-1), a natural inhibitor of the PI3K, which usually modulated Platelet-Derived Growth Factor Receptor (PDGFR) intracellular signaling. Reduced levels of IRS-1 promoted increased PI3K activation and signaling of PDGFR, thus favoring tumor cell proliferation (Houghton et al., 2010). NE has also been shown to play a role in neutrophil-related epithelial-to-mesenchymal transition (Grosse-Steffen et al., 2012). On the other hand, NE has also an anti-tumoral role. Indeed, breast cancer cells took up NE, which cleaved cyclin E, which was then presented as a truncated form in HLA-I context and efficiently activated a Cytotoxic T Lymphocytes (CTL)-mediated anti-tumor response (Mittendorf et al., 2012).

Neutrophils have also emerged as an important source of cytokines, which also play important roles in cancer initiation and progression. In an *in vitro* model of breast cancer, tumor cells produced GM-CSF, which induced the production of Oncostatin M by neutrophils. This IL-6-like cytokine was able to up-regulate VEGF production in breast cancer cells, thus promoting tumor cell detachment and invasiveness (Queen et al., 2005). In bronchoalveolar carcinoma (BAC), high levels of Hepatocyte Growth Factor (HGF) in bronchoalveolar fluid (BAL) correlated with neutrophil infiltration and poor patients' prognosis and this cytokine was able to promote tumor cell migration and invasiveness (Wislez et al., 2003) (Imai et al., 2005). In HNSCC patients, neutrophil infiltration was

correlated with the expression of CORTACTIN, a protein involved in cellular migration. In addition, neutrophils and CORTACTIN significantly correlated with poor patients' outcome (Dumitru et al., 2013). Neutrophils also release TRAIL, which retains important anti-tumoral activities (Cassatella, 2006; Hewish et al., 2010). *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) induced the release of TRAIL from neutrophils, thus potentially mediating the anti-cancer effects of BCG in bladder cancer (Kemp et al., 2005). Moreover, upon IFN- α stimulation, neutrophils from Chronic Myeloid Leukemia (CML) patients released TRAIL, which in turn promoted apoptosis of leukemic cells (Tanaka et al., 2007; Tecchio et al., 2004).

Recently, in surgically resected lung cancer patients, TAN were phenotypically and functionally characterized as T-stimulatory cells (Eruslanov et al., 2014). Indeed, compared with circulating neutrophils, TAN displayed the activated phenotype CD62L^{low}CD54⁺CXCR1^{low}CXCR2^{low}, expressed CCR7, CXCR3, CXCR4 and CCR5 and produced significant amount of the proinflammatory molecules CCL2, CCL3, CXCL8 and IL-6. From the functional point of view, TAN were able to stimulate T cell proliferation and IFN- γ release, mainly in a contact-dependent manner (Eruslanov et al., 2014). These interactions with T cells up-regulated neutrophil expression of co-stimulatory molecules, such as CD86 and OX40L, giving rise to a positive feedback loop and suggesting an anti-tumoral role for TAN in early stages human lung cancers (Eruslanov et al., 2014).

V. 3. 3. Neutrophils in angiogenesis and metastatic behavior modulation

Neutrophils can also play a dual role in modulating metastatic behavior of cancer cells. Melanoma cells have been described to produce CXCL8, which up-regulated β_2 -integrin expression on neutrophils and favored the interaction with melanoma cell-expressed ICAM-1. In turn, this dangerous alliance allowed melanoma cells to transit across the endothelium, giving rise to distant metastasis (Huh et al., 2010). Neutrophil-derived NETs during sepsis were able to capture circulating tumor cells and favor their engraftment to

distant organ sites (Cools-Lartigue et al., 2013). In contrast, in a murine model of transplanted breast cancer, neutrophils were accumulated in the pre-metastatic lung and inhibited metastasis in a H₂O₂-dependent manner and upon stimulation with G-CSF and tumor-derived CCL2. Accordingly, in this model, neutrophil depletion significantly increased the metastatic load (Granot et al., 2011).

Neutrophils express several angiogenic factors. Firstly, neutrophils are a major source of VEGF-A, which is also responsible for the angiogenic activity exerted by CXCL1 *in vivo* (Scapini et al., 2004). In an *in vivo* model of subcutaneous melanoma and fibrosarcoma and in the absence of IFN- β , TAN displayed pro-angiogenic features such as increased expression of CXCR4, VEGF-A and MMP-9, associated with a better developed tumor vascular architecture (Jablonska et al., 2010). MMP-9 has been described as a potent modulator of angiogenesis, able to release the active form of VEGF-A from the extracellular matrix (ECM) (Nozawa et al., 2006). In parallel, neutrophils have been identified as the major MMP-9 producers in HNSCC and HCC patients (Dumitru et al., 2012; Kuang et al., 2011; Nozawa et al., 2006) and, unlike other cell types, they deliver MMP-9 in a peculiar TIMP-free manner, which enhanced the pro-angiogenic activity of MMP-9 (Ardi et al., 2007). On the other hand, intratumoral injection of adenovirus carrying MMP-9 gene decreased tumor growth and angiogenesis, suggesting that MMP-9 also retains anti-angiogenic functions (Leifler et al., 2013).

Bv8 (also known as prokineticin-2) is a well-known molecule that promotes neutrophil mobilization and angiogenesis. In a tumor xenograft murine model, expression of Bv8 by neutrophils was increased by G-CSF (Shojaei et al., 2007) and Bv8 neutralization significantly impaired angiogenesis, neutrophil recruitment and tumor development (Shojaei et al., 2007). Interestingly, tumors displaying resistance to anti-VEGF therapy showed high infiltration of neutrophils and drug resistance was likely related to G-CSF-induced Bv8 expression. Indeed, anti-G-CSF or anti-Bv8 therapy reduced tumor growth

and angiogenesis (Shojaei et al., 2008; Shojaei et al., 2009). In contrast, neutrophils also express a number of anti-angiogenic molecules. NE itself can exert anti-angiogenic activity, through the cleavage of VEGF and FGF-2 and the generation of angiostatin-like fragments from plasminogen, which suppress VEGF and FGF-2 induced angiogenesis (Ai et al., 2007) (Scapini et al., 2004).

V. 3. 4. Neutrophil plasticity and heterogeneity in cancer

Challenging the classical point of view, neutrophils have emerged as cells with unsuspected plasticity. In murine models of mesothelioma and lung tumor, it has been elegantly demonstrated that neutrophils can be driven by TGF- β to acquire a pro-tumoral phenotype (Fridlender et al., 2009). TGF- β block gave rise to a massive neutrophil infiltration displaying increased cytotoxicity against tumor cells and characterized by high expression of TNF- α , CCL3 and ICAM-1 and low level of the T-cell detrimental molecule arginase-1. TGF- β block also lead to a T-cell mediated anti-tumor response, in which neutrophils played a pivotal role as effector cells (Fridlender et al., 2009). The potential dual role played by neutrophils in tumor microenvironment has been resumed in a new proposed paradigm, in which neutrophils can be polarized towards an anti-tumor N1 or towards a pro-tumor N2 phenotype in response to signals derived from the microenvironment (**Figure 7**).

Cancer context is not the only one in which neutrophil plasticity has been proposed. Indeed, 15 years ago human neutrophils were shown to acquire a dendritic cell (DC)-like phenotype, under the influence of TNF- α and IL-4 *in vitro* (Oehler et al., 1998) and more recently to be reprogrammed into macrophages (Araki et al., 2004). These data have been revised by Matsushima and colleagues, who demonstrated that, following *in vitro* stimulation with GM-CSF, both immature and mature murine neutrophils differentiated into hybrid populations, with functional properties of both neutrophils and DC (Matsushima et al., 2013). The authors also obtained human data, showing neutrophil differentiation into

a hybrid population expressing both neutrophil and DC markers upon *in vitro* treatment with GM-CSF, TNF- α and IL-4 for 7 days (Matsushima et al., 2013). Following methicillin-resistant *Staphylococcus aureus* (MRSA) infection, in addition to normal neutrophils, two distinct neutrophil subsets were identified, distinguished on the basis of cytokine and chemokine production and TLR expression, and displayed different susceptibilities to MRSA infection (Tsuda et al., 2004). In addition, a subset of neutrophils was identified capable of survive *in vitro* after 7 days of culture on absence of growth factors and presenting phenotypic and functional aspects of giant phagocytic cells (Dyugovskaya et al., 2014).

Taken together, this increasing body of evidence emphasizes the high versatility of neutrophils dependent on different settings and could offer new therapeutic approaches based on the biology of neutrophils.

V. 3. 5. Neutrophils, TAN and MDSC

Cancer development is accompanied by the appearance of a heterogeneous population of myeloid cells, namely Myeloid Derived Suppressor Cells (MDSC), which possess immunosuppressive and cancer-promoting properties. MDSC are classically distinguished between Monocytic (Mo-MDSC) and Granulocytic (G-MDSC) subsets based on morphological and phenotypical aspects (Youn and Gabrilovich, 2010). However, this distinction is not so defined. Indeed, in tumor-bearing mice, M-MDSC were found to acquire phenotypic, morphological and functional features of G-MDSC, due to epigenetic silencing of the retinoblastoma gene (Youn et al., 2013).

In addition, in mice, G-MDSC share many phenotypic and functional features with TAN, such as the morphology or the capability to promote immunosuppression via arginase-1 production (Gabrilovich et al., 2012). Moreover, neutrophils and G-MDSC are usually identified by the same membrane markers (CD11b, Gr1 and Ly6G), thus providing an

additional confounding element. Therefore, the distinction between neutrophils, TAN and MDSC is still debated.

Some reports describe that renal cell cancer patients display a subset of circulating activated neutrophils responsible for immunosuppression of T cell-mediated immune response via arginase-1 production, thus accounting for a pro-tumor activity (Rodriguez et al., 2009; Schmielau and Finn, 2001). Therefore, in this view, activated neutrophils acquired immunosuppressive properties and are considered as G-MDSC. In a genetic conditional lung adenocarcinoma model, TAN precursors physically relocated from spleen to tumors (Cortez-Retamozo et al., 2012) and, since MDSC accumulated in the spleen of tumor-bearing animals, MDSC activities have been at least partially attributed to immature neutrophils (Solito et al., 2011; Trellakis et al., 2011b). Accordingly, following GM-CSF stimulation, G-MDSC displayed phenotypical and functional characteristics proper of neutrophils, suggesting that G-MDSC are immature neutrophils (Youn et al., 2012). These immature neutrophilic MDSC have also been reported in cancer patient peripheral blood, and their levels correlated with poor patients' prognosis (Trellakis et al., 2011b). Recently, in a multistage mouse model of breast cancer, atypical $CD11b^+Ly6G^+Rb^{low}$ neutrophils accumulated during tumor progression, in peripheral tissues but not in the primary tumor of animals. This neutrophil subset showed T cell-suppressive properties via ROS production. Tumor-derived G-CSF was identified as the main mediator for the expansion and differentiation of hematopoietic stem cells toward the myeloid lineage and the activation of a peculiar myeloid differentiation program in bone marrow (Casbon et al., 2015). These evidences further highlight the poorly defined distinction between immunosuppressive TAN and G-MDSC. In contrast with these evidences, Fridlender and colleagues performed a transcriptomic analysis on peripheral neutrophils, TAN and G-MDSC in tumor-bearing mice, and found that TAN and G-MDSC are distinct populations of cells and that naïve

neutrophils and G-MDSC are more closely related to each other than to TAN (Fridlender et al., 2012).

VI. COLORECTAL CANCER

Colorectal cancer (CRC) is the third most frequent tumor in men and the second in women worldwide (Ferlay et al., 2010). More than half of the cases occurs in developed countries. There is a wide geographical heterogeneity in incidence with the highest estimated rates in Oceania, together with part of Europe and North America, and the lowest in Western Africa (<http://globocan.iarc.fr>). Nevertheless, increase in previously low-risk populations have been recently described, attributed to changes in dietary habits and behaviors associated with Western lifestyle (Center et al., 2009). However, in USA and several other countries death rates display a slightly decline, likely to be the result of improvements in early detection and treatment (<http://globocan.iarc.fr>).

VI. 1. Risk factors and histopathological classification

A number of risk factors have actually been identified, such as age, male sex, smoking, excessive alcohol intake, inflammatory bowel disease, family history of CRC, high consumption of red meat, diabetes and obesity (Chan et al., 2011; Fedirko et al., 2011; Jess et al., 2012; Jiang et al., 2011; Liang et al., 2009; Ma et al., 2013b; Taylor et al., 2010). In contrast, physical activity, aspirin intake and endoscopy with removal of precancerous lesions have been described as protective factors (Bosetti et al., 2012; Boyle et al., 2012; Brenner et al., 2011). Moreover, low levels of circulating 25-Hydroxy-vitamin D have been associated with an increased risk to develop CRC, but the relevance of these data needs to be confirmed (Ma et al., 2011). Family history is a well-known risk factor for CRC. However, beyond the hereditary forms, which account for less than 10% of cases, genetic polymorphisms identified by Genome-Wide association studies and involved in CRC development are a matter of debate (Hutter et al., 2012).

The current histopathological classification is based on local invasion (T stage), lymph node involvement (N stage) and distant metastasis occurrence (M stage). The combination of these elements gives rise to the Tumor Node Metastasis (TNM) system, which represents the starting point for clinicians to make therapeutic decisions (**Table 4 and Table 5**). However, the usefulness of the TNM system for patients' outcome prediction has been questioned (Galon et al., 2006). For instance, a percentage of stage III and high-risk stage II patients, who are recommended for chemotherapy, do not respond to the treatment (Brenner et al., 2014). Therefore, additional prognostic and predictive factors are needed to optimize patient therapeutic approaches and follow-up.

VI. 2. Diagnosis and staging

Diagnosis of CRC is based on histological analysis of biopsy samples obtained during endoscopy. Synchronous cancers, present in 2-4 % of patients, need to be excluded by complete colonoscopy or Computed Tomography (CT) colonography (Arnaud et al., 1989; Park et al., 2012). If not possible before surgery, a complete colon analysis should be performed within 6 months after curative intervention. For rectal cancers accurate local staging at diagnosis is essential for making therapeutic decisions. To precisely define the local extent of the tumor and to distinguish between invasive or non-invasive tumors, Ultrasound (US)-endoscopy is the method of choice, together with Magnetic Resonance Imaging (MRI) (Puli et al., 2010; Puli et al., 2009). If these methods are employed after radiotherapy the results may not be accurate because of the local changes due to radiations.

Following diagnosis, metastatic localizations, present in about 20% of patients at diagnosis, need to be investigated. The most frequent localization is liver and detection by imaging (e.g. US, CT and MRI) is mandatory. In a recent meta-analysis including 3391 patients, MRI was shown to be more sensitive than CT, with the highest sensitivity for smaller lesions (Niekel et al., 2010). The sensitivity of abdominal US was considered lower compared to the other imaging techniques, even though improvable by using contrast

(Floriani et al., 2010; Rafaelsen and Jakobsen, 2011; Sietses et al., 2010). The second most frequent localization of metastatic lesions is the lung. The rate of lung metastasis is higher in rectal cancers compared to colon cancers (9-18% versus 2% respectively). Therefore, in colon-derived tumors chest radiograph can be enough, but in rectal cancer chest CT can be justified (Brenner et al., 2014). Even though other organs can be target of metastatic foci (e.g. brain, bones), no routine investigation is recommended. Finally, no evidences support a role for PET-CT in patients without suspected metastatic lesions (Brenner et al., 2014).

VI. 3. Etiopathogenesis and Molecular classification

Classically, CRC classification was based on clinic-pathological aspects (e.g. histological type, tumor grade and clinical stage). However, new evidences proposed that molecular pathways are involved in CRC initiation and can be associated with patients' outcome. Therefore and despite its heterogeneity and complexity, a classification of CRC based on molecular pathways has been proposed (Heinimann, 2013). Based on etiopathogenesis, CRC can be classified into inherited or sporadic syndromes.

VI. 3. 1. Inherited syndromes

Inherited syndromes account for about 3-5% of CRC and, in these cases, a clear genetic alteration can be identified (Jasperson et al., 2010).

Familial Adenomatous Polyposis (FAP) is an hereditary syndrome in which hundreds of adenomatous colorectal polyps occur from the second decade of life, with a risk of developing CRC by 100% (Schmoll et al., 2012; Vasen et al., 2015). The key target in the management of patients with FAP is cancer prevention. Therefore, annual colonoscopy is recommended, with the removal of the suspicious adenomas. In some cases, given the wide number of adenomas, prophylactic colectomy can be necessary (Schmoll et al., 2012).

FAP is an autosomal dominant disease, caused by a germline mutation in the adenomatous polyposis coli (APC) gene. In 25% of cases, FAP derives from a "de novo" mutation in the APC gene (Half et al., 2009). In a subset of patients with clinical FAP, the

phenotype is due to a biallelic mutation (and loss of function) in the base excision repair (BER) gene MUTY Homolog (MUTYH), which normally codes for a protein that excises from the DNA 8-oxoguanine, an abnormal guanine derived from oxidative stress (Markowitz and Bertagnolli, 2009). Given the loss of function of this gene, a highly significant excess of somatic G:C-T:A mutations in the APC gene accumulates, giving rise to the neoplastic transformation (Cheadle and Sampson, 2003).

APC gene is a tumor suppressor gene playing important roles in the regulation of the Wnt/ β -catenin pathway. The oncoprotein β -catenin is part of a transcription factor involved in the activation of a number of genes regulating epithelial cell proliferation. APC plays a major role in the degradation of β -catenin, thus contributing to the homeostasis of the epithelium in the intestinal crypt (Goss and Groden, 2000). Therefore APC gene fulfills the criteria of a “gatekeeper gene” which are genes responsible for controlling and inhibiting cell growth and enhancing cell death. FAP-related mutations give rise to a truncated APC protein, with a subsequent loss of its function, leading to an uncontrolled epithelial proliferation and early onset of polyps (Goss and Groden, 2000). Given this accelerated proliferation rate, the risk of further genetic mutations involved in cancer development is significantly increased.

Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome is the most common inherited colon cancer syndrome (Lynch et al., 2015). HNPCC accounts for 2-5% of all CRC and is caused by a germline mutation in one of the mismatch repair (MMR) genes, which include mutL homolog 1 (MLH1), mutS homolog 2 (MSH2), mutS homolog 6 (MSH6) and postmeiotic segregation increased 2 (PMS2). MMR genes maintain genomic stability by repairing replication errors in DNA. Therefore, impaired MMR can lead to the accumulation of mutations in others genes (oncogenes or tumor suppressor genes), involved in neoplastic transformation. Given the “ubiquitous” functions of MMR genes, HNPCC can

also be characterized by the occurrence of extra-intestinal tumors, such as endometrial cancer (Lynch et al., 2015).

MMR genes represent an example of “caretaker genes”, which are responsible for the maintenance of the genome integrity and, unlike gatekeepers, are not directly linked to tumor promotion. However, mutation in a caretaker gene and loss of protein expression or function increased genetic instability and mutation in gatekeeper genes. If a patient inherits a mutated caretaker gene copy, he needs three additional mutations to develop a neoplasia: a second one in the second allele of the caretaker gene and two additional mutations in both copies of a gatekeeper gene. In contrast, if a patient inherits a mutated copy of a gatekeeper gene, he only needs an additional mutation, in the second allelic copy, to develop a neoplasia. Consequently, mutations in caretaker genes are not expected to cause sporadic cancers, since four mutations are needed to give rise to neoplastic transformation (Kinzler and Vogelstein, 1997).

The loss of MMR function is associated with the epiphenomenon of microsatellite instability (MSI). Microsatellites consist in short DNA repeats (1-6 base pairs), normally scattered in the genome (Hewish et al., 2010). Due to the repetitive nature of these short sequences, the susceptibility to replication errors is increased, caused by the slippage of DNA polymerases over tandem repeats (Hewish et al., 2010). In normal conditions, these mutations are readily recognized and removed by the MMR system. When this machinery is not functional, insertions and/or deletions in microsatellites become fixed and the length of microsatellites is altered, defining the so-called “Microsatellite Instability”. MSI tumors display a peculiar phenotype: poor or mixed differentiation (often mucinous tumors), large size, proximal colon location, high level of tumor-infiltrating lymphocytes, rarely associated to metastasis (Brenner et al., 2014).

VI. 3. 2. Sporadic CRC

Sporadic cancers account for about 90% of CRC cases and the molecular pathogenesis can be schematically represented in three groups.

In a first group, the chromosomal instability (CIN) molecular pathway represents the most common (70%) molecular pathway responsible for genetic instability and is characterized by the accumulation of chromosomal abnormalities (numerical or structural), which result in karyotypic variability among cells. These chromosomal rearrangements are responsible for the accumulation of mutations in tumor suppressor genes (such as APC, P53 and SMAD4) causing loss of expression or function, and possible initiation of pathways involved in CRC tumorigenesis (Markowitz and Bertagnolli, 2009).

In a second group of cancers, genomic instability can derive from the inactivation of MMR genes. Indeed, MMR mutations can be inherited, such as in HNPCC, or acquired. In HNPCC patients, the germline inactivation of one of MMR genes (usually MLH1 or MSH2) increases the risk of cancer of 80%. In 15% of CRC, MMR defects are not inherited but acquired by biallelic silencing of the promoter region of MLH1 by methylation (Markowitz and Bertagnolli, 2009).

The last group is named CpG Island Methylation Phenotype (CIMP) and is found in cancers that present aberrant methylation in selected promoter-associated CpG islands. These epigenetic events can lead to loss of expression of the gene or loss of function of the protein (Grady and Pritchard, 2014).

The classification of these three pathways is not strictly defined. Indeed, tumors can exhibit aspects of more than one pathway. For instance, CIMP pathway can be responsible for sporadic MSI tumors, due to somatic epigenetic silencing of MLH1. In addition, activating mutations of the oncogene BRAF take place almost completely in MSI tumors when CIMP features are also present. Indeed, BRAF mutations are often associated with MLH1 hypermethylation. Therefore, the presence of BRAF mutations can be useful to distinguish MSI tumors due to MLH1 methylation from MSI tumors due to germline MMR

mutations (Parsons et al., 2012) (Grady and Pritchard, 2014). Moreover, CIMP positive tumors can be distinguished in two types: CIMP high, associated with MLH1 methylation and BRAF mutations and CIMP low, associated with KRAS mutations (Thiel and Ristimaki, 2013).

Genes and molecular pathways involved in CRC pathogenesis are schematically represented in **Figure 8**.

VI. 4. Chronic inflammation and CRC

Patients with Inflammatory Bowel Disease (IBD) display higher risk to develop CRC, that increases with duration of colitis, with a risk of 2% at 10 years, 8% at 20 years and 18% after 30 years of disease (Eaden et al., 2000). Another important risk factor is represented by the extent of inflammation, with a risk increasing proportionally to the colonic surface involved in colitis. Clinically evaluated disease activity is not associated with incidence of CRC (Eaden et al., 2000). Indeed, the degree of active inflammation should not be evaluated on the basis of patient symptoms, but it should be estimated on the basis of endoscopic and/or histologic criteria. This aspect is supported by the fact that CRC can arise in areas of microscopic colitis regardless of clinical activity, supporting a major role of the histologically and endoscopically defined inflammation in determining the risk of cancer (Mathy et al., 2003). Moreover, patients with severe inflammation non responsive to medical therapy (e.g. aminosalicylates, corticosteroids and immunomodulators) often undergo colectomy at early stages of disease. Because these patients are no longer at risk for colon cancer, this may lead to the false conclusion that severe colitis is not related to CRC risk. Patients with a stable active inflammation do not need early-stage colectomy and remain at risk for developing CRC. Thus, most patients with IBD who develop CRC have quiescent chronic inflammation (Ullman and Itzkowitz, 2011).

Sporadic and colitis-associated colon cancers (CAC) share genetic and signaling pathways alterations, such as Wnt, β -catenin, KRAS, P53, TGF- β and MMR proteins

(Ullman and Itzkowitz, 2011). Accordingly, similar frequencies of chromosome instability, microsatellite instability and DNA methylation were found between CAC and sporadic cancers (Willenbacher et al., 1999) (Ullman and Itzkowitz, 2011). However, CAC presents distinct pathogenic sequences compared to sporadic cancer. In particular, sporadic cancer arises from a dysplastic lesion, usually the adenomatous polyp, whereas CAC evolves from chronic inflammation to dysplasia and carcinoma, without the formation of a well-defined adenoma (Ullman and Itzkowitz, 2011). Thus, the classical adenoma-carcinoma sequence occurring during sporadic colorectal tumor development is an inflammation-dysplasia-carcinoma sequence in CAC (Ullman and Itzkowitz, 2011) (**Figure 9**). Genetic and signaling pathways also are altered with a different timing. For instance, the loss of APC function occurs early in sporadic CRC but lately and less frequently in CAC development (Aust et al., 2002; Lakatos and Lakatos, 2008).

Inflammation takes part into CRC carcinogenesis through the promotion of chromosomal and microsatellite instability, CpG island methylation, by inducing oxidative stress. Indeed, in chronically inflamed tissue, tumor initiation can be due to radical oxygen and nitrogen species (RONS), released by cells of the innate immune system (Terzic et al., 2010).

VI. 4. 1. Immune cell infiltration in CRC

Similarly to other solid malignancies, various types of immune cells infiltrate CRC. Innate immune cells, such as neutrophils, mast cells, NK cells, DC and TAM can be distinguished in CRC (Atreya and Neurath, 2008). Adaptive immune cells are also recruited into colitis-associated and sporadic CRC. The presence of immune cells within the tumors can be functionally interpreted as part of immunosurveillance or as tumor promoting inflammation. Since sporadic CRC do not occur in the context of pre-existing inflammation, it is improbable that inflammation plays a key role for sporadic CRC initiation. In contrast, in CAC, IBD always precedes tumor initiation and the risk to develop CAC is directly correlated with the severity and duration of active disease. Thus, in CAC,

the inflammatory response appears to essentially exert a pro-tumorigenic role. For instance, in a CAC mouse model chemically induced by azoxymethane/dextran sodium sulfate (AOM/DSS), M2 macrophages were described to exert a critical role in CRC initiation, promotion, and metastasis (Wang et al., 2015). In contrast, in sporadic CRC, a well-defined balance between immunosurveillance and tumor-promoting inflammation has been described. In both CRC and CAC, immunosurveillance can early identify and eliminate transformed cells and aberrant crypt foci, keep small tumors at quiescent state (equilibrium) and control metastatic foci (Terzic et al., 2010). However, in later stages of carcinogenesis, inflammation is involved in tumor-cell survival, proliferation, angiogenesis, and other hallmarks of cancer, including immunosuppressive effects (Terzic et al., 2010). Indeed, after cancer initiation, the local inflammatory microenvironment favors accumulation of further mutations and epigenetic changes. For instance, activated inflammatory cells produce reactive oxygen and nitrogen intermediates that can promote DNA damage and mutations (Westbrook et al., 2009).

VI. 4. 2. Role of cytokines in CRC

A number of tumor-promoting cytokines exert their effect on intestinal epithelial cells and trigger activation of oncogenic transcription factors and signaling pathways, such as Akt/mammalian target of rapamycin (mTOR), NF- κ B and STAT3, which play many roles in the development of CAC and CRC (Bollrath and Greten, 2009; Bromberg and Wang, 2009). The role of cytokines in regulation of CAC has been clarified in mouse models of CAC and similar mechanisms can be applied to sporadic CRC. For instance, wild type mice exposed to AOM/DSS displayed high circulating levels of TNF- α and developed multiple colon tumors compared to the controls. In contrast, mice deficient in the receptor TNFR1 exposed to AOM/DSS displayed reduced inflammation and cancer development. Accordingly, the phenotype of wild type mice was reverted following anti-TNF- α treatment (Popivanova et al., 2008). A number of pathways are activated downstream TNFR1, but the

final activation of NF- κ B plays a major role in CAC development. Indeed, mice lacking NF- κ B in colon epithelium developed fewer tumors following AOM/DSS treatment; mice lacking NF- κ B in the myeloid compartment developed few and smaller tumors and displayed reduced expression of pro-inflammatory mediators such as IL-1 β , IL-6 and TNF- α (Greten et al., 2004). In addition, in an *in vivo* model of intestinal spontaneous carcinogenesis, Karin and colleagues demonstrated that adenoma formation altered the epithelial barrier, favoring the invasion of microbial products. These molecules induced myeloid cells to produce IL-23, which in turn activated an IL-17-mediated pro-tumoral response (Grivennikov et al., 2012). Cytokines such as TNF- α , IL-17, IL-6, IL-1 play a major role in promoting colitis-associated tumor development, even though many others were found up-regulated in these tumor settings. By contrast, cytokines such as IL-10 and TGF- β have been shown to inhibit CRC tumorigenesis (Becker et al., 2004; Popivanova et al., 2008; Tang et al., 2005; Terzic et al., 2010).

VI. 5. Management and therapeutic approaches

Similarly to other cancer types, CRC should be managed by a multidisciplinary team, composed by a gastroenterologist, a medical oncologist, a colorectal surgeon, a pathologist, a radiologist and a radiotherapist. All patients should be screened for metastatic localizations before starting the treatment. Similarly, patients with rectal cancers should undergo further investigations to define the local extent of the disease to assess the need for neo-adjuvant therapy.

Surgery is the first therapeutic approach. For colon cancers, tumor and related lymph nodes need to be removed, with the extent of the removal previously determined by staging procedures (Stintzing, 2014). For rectal cancers the reference surgical intervention consists in the mesorectal excision, i.e. the removal of rectum together with mesorectum and mesorectal fascia. The removal of mesorectum is essential, since it contains lymph nodes and tumor foci. Moreover, for both colon and rectal tumors, several evidences suggest the

importance of clear circumferential margins, i.e. a distance of at least 1 mm between the tumor and the resection margin. Indeed, patients with involved resection margins display a higher risk of local recurrence and distant metastases (Caricato et al., 2006; Nagtegaal and Quirke, 2008). Following the introduction of mesorectal excision in surgical practice, the rate of rectal cancer local recurrence has significantly been reduced. Moreover, an important trial has shown a further increase in recurrence free survival following neo-adjuvant therapy (van Gijn et al., 2011). However, which patients need to undergo adjuvant chemotherapy and which regimen is the optimal one need to be clearly established. Given the optimal prognosis, stage I colon and rectal cancer patients should not undergo any adjuvant treatment following surgery. Stage III patients will benefit of the adjuvant treatment, whereas for stage II patient the adjuvant treatment-derived benefit is not so clear. Actually, for stage II rectal cancer patients benefit is generally accepted for locally invasive T4 or T3 tumors infiltrating the mesorectal fascia. In Stage II colon cancer patients, adjuvant chemotherapy is actually recommended only in selected “high-risk” conditions, such as T4 tumors, perforation or bowel obstruction and < 12 lymph nodes removed (Brenner et al., 2014).

Stage III colon cancer patients are recommended to undergo adjuvant chemotherapy, given the risk of local recurrence (about 20%). Standard regimens include 5-FluoroUracyle (5-FU) or platin compounds and significantly reduce the risk of recurrence (Gill et al., 2004). 5-FU is a pyrimidine analogue, which inhibits the enzyme thymidylate synthase, thus decreasing the production of deossiThymidineMonoPhosphate (dTMP). It can also mimic pyrimidine function, since 5-FU is incorporated into DNA (via dUMP or dTMP) or RNA (via UTP), leading to cytotoxic actions (e.g. DNA strand breakage and decreased protein synthesis). As an alternative, capecitabine, which is a pro-drug of 5-FU, has been also shown to be efficient (Twelves et al., 2012). To further improve disease free survival, oxaliplatin has been added to 5-FU (FOLFOX) or capecitabine (XELOX), showing a

significant increase in 5-years DFS (Andre et al., 2009; Haller et al., 2011). However, one study showed that therapeutic advantage of oxaliplatin was observed only in younger patients (Yothers et al., 2011) suggesting that further investigations are needed. Other approaches have been tested such as anti-VEGF or anti-Epidermal Growth Factor Receptor (EGFR) drugs. However and surprisingly, in stage III patients, addition of these drugs to oxaliplatin-based regimens as well as addition of the topoisomerase I inhibitor irinotecan to 5-FU did not show any significant effect on patient DFS (Alberts et al., 2012; de Gramont et al., 2012; Van Cutsem et al., 2009).

With the regard of stage IV patients, resectable metastases should be surgically removed; if unresectable, patients should undergo palliative chemotherapy. With the advent of new biological drugs such as anti-VEGF (bevacizumab, aflibercept) and anti-EGFR (cetuximab or panitumumab) given alone or in combination to standard chemotherapy regimens, the survival of stage IV patients is improved, up to 20 months in some cases (Arnold and Seufferlein, 2010; Heinemann et al., 2014; Schwartzberg et al., 2014; Stintzing, 2014). In some cases, metastasis unresectable at time of diagnosis become resectable following chemotherapy, thus conferring a significant advantage in DFS (Brenner et al., 2014). Expectedly, the characteristics of the chemotherapeutic programs need to be carefully defined depending on patient clinical conditions and features, such as age and comorbidities.

VI. 6. Molecular markers of prognosis and prediction of therapeutic response

The milestone of the current guidelines for CRC treatment is the TNM system described by the American Joint Committee on Cancer (AJCC) in collaboration with International Union for Cancer Control (UICC) (**Tables 4 and 5**). As already mentioned, this well-known standard classification scheme is based on the anatomical extent of the tumor at diagnosis and is widely established. However, the clinical reliability and usefulness of this system have been recently questioned (Galon et al., 2006). Indeed, clinical practice showed

that patients at the same TNM stage could differently respond to a similar treatment and have different disease progression. For stage I or low-risk stage II patients, guidelines do not recommend adjuvant therapy after radical surgery because these patients are considered completely cured and display long term survival. However, about 10% of stage I and 20% of stage II patients undergo recurrence or metastasis (Mei et al., 2014). To move to a “patient-tailored” medicine, in the last decade a huge amount of work has been made in order to find new prognostic and predictive markers useful to predict progression rates and response to chemotherapy (Di Caro et al., 2013).

V. 6. 1. Microsatellite instability

Besides the identification of hereditary cases of CRC, microsatellite instability investigation provides additional information to orientate prognosis and predict therapy response. Stage II and stage III patients with MSI cancers display better prognosis than patients with microsatellite stable (MSS) tumors (Barrasa Shaw et al., 2009; Benatti et al., 2005; Chang et al., 2006; Malesci et al., 2007). Indeed, a metanalysis including 7642 CRC patients distributed in 32 studies showed a survival advantage for patients with MSI compared to patients with MSS (hazard ratio (HR) of 0.65 (95% CI 0.59-0.7) (Popat et al., 2005). In contrast, MSI CRC patients do not respond to 5-FU-based adjuvant therapy but show a better response to irinotecan (Bertagnolli et al., 2009; Fallik et al., 2003; Sargent et al., 2010). These results support the need of molecular tumor analysis in all CRC patients that are candidate to undergo adjuvant chemotherapy.

V. 6. 2. KRAS and BRAF

The proto-oncogene KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) encodes for a hydrolase enzyme that can bind and hydrolyze guanosine triphosphate (GTP) (Schubbert et al., 2007). K-RAS is involved in the RAS-RAF-MAPK pathway, which is activated during carcinogenesis (Williams et al., 2006). Accordingly, KRAS mutations are found in about 40% of CRC and have been proposed to be an early event in CRC

carcinogenesis (Grady and Pritchard, 2014). Prognostic role for KRAS mutations in CRC remains controversial, likely due to the need of considering KRAS mutations together with the specific mutations on specific codons (Cerottini et al., 1998; Moerkerk et al., 1994). In addition, contrasting results found in different studies can be due to variability in sample sizes and patient datasets.

The BRAF (v-raf murine sarcoma viral oncogene homolog B) gene encodes for a serine/threonine kinase, activated by KRAS-GTP. More in details, BRAF protein is recruited to the membrane and activated by KRAS. In turn, active BRAF induces the activation of MAPK (Mitogen-activated protein kinases) leading to activation of many transcription factors, which regulate cell proliferation and survival. KRAS and BRAF mutations are actually considered mutually exclusive in CRC (Rajagopalan et al., 2002). BRAF mutations were associated with poor patient survival especially in MSS CRC. The prognostic role of BRAF mutations in MSI tumors is not clearly defined (Ogino et al., 2009; Thiel and Ristimaki, 2013). Even though the effects of KRAS and BRAF mutations on patient prognosis remain controversial, their predictive role in response to anti-EGFR therapy in metastatic CRC patient is accepted. Indeed, EGFR engagement activates a signaling cascade via KRAS/BRAF and in case of mutations in KRAS anti-EGFR therapy response rates were reduced from 20% to about 0% (Amado et al., 2008). Similarly, KRAS wild-type BRAF mutated tumors do not respond to anti-EGF-based chemotherapy (Di Nicolantonio et al., 2008).

V. 6. 3. Prognostic and predictive role of immune cell infiltration in CRC

In the view of an even more personalized medicine, infiltration of leukocytes within the tumor may have a prognostic role for patients with CRC (Bindea et al., 2013).

In 1986 infiltration of immune cells was first described as an independent prognostic factor in CRC patients (Jass, 1986). Following this first report, number of studies showed that type, density and location of immune cells have a prognostic role for patient outcome

(Galon et al., 2006; Mei et al., 2014). However, differences in methodological approaches (e.g. tissue microarray, patient datasets) and discrepancies in results suggest that further studies are needed to define the prognostic role of leukocyte subsets in CRC. Recently, a study reported that the immune infiltrate composition changes according to tumor stage (Bindea et al., 2013).

Among immune cells infiltrating CRC, lymphocytes (CD3⁺ cells, CD8⁺ cells and FOXP3⁺ cells) were significantly associated with favorable patient prognosis (Laghi et al., 2009; Mei et al., 2014; Pages et al., 2005; Salama et al., 2009). Microarray analysis revealed two clusters of patients with CRC. Cluster 1 was characterized by Th1, T γ δ , cytotoxic T cells and macrophage signature and was associated with good prognosis. Cluster 2 was characterized by a Th2, Th17, Treg and NK cell signature and was associated with poor prognosis (Bindea et al., 2013). Analysis of immune populations *in situ* revealed that most of T cell markers, including CD8⁺ and Th1 were significantly associated with a good prognosis, whereas Th17 cells negatively influenced the patients' outcome (Bindea et al., 2013). In addition, B cell infiltration was associated with patient increased survival (Bindea et al., 2013). Accordingly, a recent study identified the tertiary lymphoid tissue (TLT) as a peculiar histological entity, rich in CD20⁺ B cells and CD3⁺ lymphocytes, which dynamically interact. In this study, CD3⁺ tumor-infiltrating lymphocytes efficiently correlated with TLT and predicted better patients' outcome (Di Caro et al., 2014).

Recently, a study proposed the calculation of an "immunoscore" based on the density of total CD3⁺ and cytotoxic CD8⁺ T lymphocytes, both in the core and at the invasive margin. This immunoscore could be a useful prognostic marker in patients with rectal cancer treated with primary surgery and could be a predictive marker for response to preoperative chemio-radiotherapy. This new scoring system is named "Immunoscore" and represents a new promising instrument for prognostic stratification of colorectal cancer (Galon et al., 2014).

In contrast to the adaptive immunity, the prognostic role of innate immune cell infiltration has not been analyzed in a such complete extent and controversial data have been reported for macrophages (Forssell et al., 2007; Grizzi et al., 2013; Kaler et al., 2010) and neutrophils (Hirt et al., 2013; Rao et al., 2012). More in details, the role of macrophages in CRC is controversial, with some evidence suggesting that their pro- or anti-tumor functions may depend on their tumor location (e.g. invasive front *versus* intratumoral compartment) (Erreni et al., 2011). Studies are mostly in favor of an association between high macrophage infiltration and better patient outcome (Edin et al., 2013; Forssell et al., 2007).

The prognostic role of neutrophil infiltration in tumors has been described above (see pg 38). In CRC, first studies had indicated an association between neutrophil infiltration and good patients' prognosis (Klintrup et al., 2005; Nagtegaal et al., 2001; Nielsen et al., 1999). In contrast, more recent works proposed that tumor-infiltrating neutrophils were associated with poor patients' outcome (Akishima-Fukasawa et al., 2011; Rao et al., 2012). The most relevant published data on prognostic role of neutrophil infiltration in tumors are summarized in **Table 3**.

AIM

The aim of this study was to evaluate the role of neutrophils in human CRC, and in particular:

- to find a specific marker to identify neutrophils in CRC tissues by Immunohistochemistry, with particular regard to Myeloperoxidase (MPO) and CD66b;
- to evaluate neutrophil infiltration in Stage I-IV Colorectal Cancer (CRC) tissues from patients who underwent surgical resection for CRC at Humanitas Clinical and Research Center, Rozzano (MI), by immunohistochemistry;
- to investigate correlations between clinic-pathological aspects and tumor-infiltrating neutrophils in CRC and evaluate their prognostic value on patient survival;
- to evaluate the role of tumor-infiltrating neutrophils in predicting chemotherapy response in Stage III CRC patients;
- to define the phenotypical and functional aspects of tumor-infiltrating neutrophils in CRC, in particular whether a cross-talk exists between tumor cells and infiltrating neutrophils;
- to investigate whether tumor cells could modify neutrophil biology and whether tumor-infiltrating neutrophils could modulate tumor cell behavior.

RESULTS

The temporal dynamics of CD66b⁺ neutrophil infiltration during CRC

Today, there is no consensus on the method for the staining of neutrophils in cancer tissues. Therefore, we conducted a first set of experiments to characterize a specific neutrophil marker in CRC tissues, focusing our attention on myeloperoxidase (MPO) and CD66b, two molecules used as neutrophil markers in CRC tissues (Rao et al., 2012; Roncucci et al., 2008). Double staining in immunohistochemistry for CD66b plus MPO and CD68 plus MPO revealed that all CD66b⁺ cells were MPO⁺, corresponding to neutrophils. However, a population of CD66b⁻ MPO⁺ cells and CD68⁺ MPO⁺, likely to be not fully mature macrophages, was also observed (**Figure 10a**). Therefore, we evaluated the pattern and density of neutrophil infiltration in CRC tissues by using CD66b as neutrophil marker and a computer-aided imaging analyzing system. To bring a robust and uncontroversial conclusion, we analyzed the immunoreactive area (IRA) for CD66b both in the intratumoral compartment (IT) and in the invasive margin (IM) of whole tissue section (**Figure 10b**). Three distinct regions both in the IT and IM compartments were blindly analyzed.

We compared the infiltration of neutrophils found in whole CRC tissue sections at different cancer stages. Importantly, the infiltration of neutrophils appeared to evolve with tumor progression. Indeed, the density of tumor-infiltrating neutrophils increased from stage I to stage III, followed by a dramatic decrease from stage III to stage IV ($p=0.005$; **Figure 10c**).

The association between neutrophil infiltration, clinicopathologic features and survival of CRC patients

Patient demographics and clinicopathologic features are detailed in **table 6**. ROC curve analysis was employed to define the cut-off value for high CD66b⁺ IRA. Linear regression analysis revealed that no clinicopathological parameter was associated with CD66b⁺ IRA both in the IM and in the IT (**Table 6**). However, we observed a trend towards a positive

association between vascular invasion and neutrophil infiltration both in the IM and the IT (**Table 6**).

As expected, univariate analysis revealed that microsatellite status, TNM staging and vascular invasion significantly influenced disease-specific survival (DSS) and disease-free survival (DFS) (**Table 7**). Surprisingly, high CD66b⁺ IRA, both in the IM (i.e. 49.2% of tumor samples) and in the IT (i.e. 50.7% of tumor samples), was significantly associated with better DSS (p=0.003 and p=0.002, respectively) and better DFS (p= 0.03 for both IM and IT) (**Table 7**). These results were confirmed by Kaplan-Meier curves showing significantly better DSS and DFS in patients with tumors highly infiltrated by CD66b⁺ cells (**Figure 11**).

In order to assess whether the prognostic value of CD66b⁺ IRA can be influenced by other variables, we combined clinicopathologic features with CD66b⁺ IRA scores in multivariate analysis. We found that higher density of neutrophils found in the IT was an independent prognostic marker for better DSS (Hazard ratio (HR)=0.47; p=0.03) and DFS (HR=0.58; p=0.05) (**Table 7**). Regarding the other variables, clinical stage and vascular invasion were also independent prognostic predictors but as risk factors for poor DSS and DFS (**Table 7**).

Predictive significance of neutrophil infiltration for chemotherapy response

Several lines of evidence have reported that successful chemotherapy depends on both innate and adaptive immunity (Anitei et al., 2014b; Ma et al., 2013a; Medina-Echeverz et al., 2011). However, the clinical significance and role of neutrophils remain to be discovered. Thus, we recruited new DNA mismatch repair-proficient patients with Stage III CRC (n=178), for whom 5-fluorouracil (5-FU) chemotherapy was recommended (Schmoll et al., 2012). Firstly and to gain deeper insight about the localization of neutrophils in CRC tissue, we compared the density of neutrophils found in the IM and in the IT. Interestingly, higher neutrophil density was found in the IT compared with the IM (p=0.02, **Figure 12a**).

Since primary tumor is surgically removed before chemotherapy treatment, we evaluated whether a correlation existed between the CD66b⁺ IRA found in primary tumor (IT) and in metastatic lymph node. Analysis showed that metastatic lymph node CD66b⁺ IRA was significantly associated with primary tumor CD66b⁺ IRA (p=0.03, **Figure 12b**). Therefore, our data suggest that the density of neutrophils found in the metastatic lymph nodes mirrors their density in the primary tumor.

Thus, we next assessed whether CD66b⁺ IRA found in the IT had a predictive significance for 5-FU-based chemotherapy response. Analysis of the whole cohort showed that age, tumor grade, vascular invasion and nodal status were prognostic markers for poor survival in stage III CRC patients (**Table 8**). Importantly, we found that a trend towards better DFS was seen in patients treated with 5-FU (HR=0.61; p=0.06) and in patients with higher neutrophil infiltration in the IT (HR=0.61; p=0.12). Multivariate regression Cox analysis revealed that an interaction exists between 5-FU treatment and CD66b⁺ IRA (p=0.009). Accordingly, 5-FU-based chemotherapy and CD66b⁺ IRA were not independent predictive markers for DFS in these patients (**Table 8**).

The predictive significance of CD66b⁺ IRA could be influenced by the heterogeneity of the population, due to the combined presence of treated and untreated patients. Therefore, we assessed the predictive significance of CD66b⁺ IRA in treated versus untreated patients. As shown in **figure 12**, higher density of neutrophils in the IT was associated with better prognosis in 5-FU treated patients (**Figure 12c**, HR=0.42; p=0.01) but was irrelevant to predict the prognosis of untreated patients (**Figure 12d**, HR=1.79; p=0.26) or combined treated and untreated patients (**Figure 12e**, HR=0.64; p=0.12).

All together, these results indicate that density of neutrophils found in the primary tumor had predictive significance for chemotherapy response and could reflect a more systemic effect of neutrophils, such as an antitumor activity beyond the primary tumor.

Regulation of neutrophil chemotaxis and survival by CRC cell lines

In a next set of *in vitro* experiments, we assessed whether CRC cell lines (i.e. SW480, HT29 and SW620) have the ability to modulate the biology of neutrophils. We found that CRC cell lines-derived conditioned media (CM) induced chemotaxis of neutrophils (**Figure 13a**). Accordingly, CRC cell lines produced CXCL8, CXCL1 and CXCL2 chemokines both at the mRNA and protein levels (**Figure 13b-d** and data not shown). To verify the involvement of these chemokines, neutrophils were cultured with CM in presence or absence of a small inhibitor molecule of CXCR1/CXCR2 (i.e. *Reparixin*), a selective non-peptide inhibitor of CXCR2 (i.e. SB225002), or the G-coupled receptor inhibitor, pertussis toxin (PTX). All these treatments blocked the chemotactic activity of CM towards neutrophils, showing that CRC cell lines produced soluble molecules with chemotactic activity for neutrophils and acting through CXCR1/2 (**Figure 13e**).

We next investigated whether CM modulate neutrophil lifespan. By FACS analysis, we observed that survival of neutrophils was dramatically increased in presence of CM compared to control medium (**Figure 14a**). On day 3, almost all neutrophils cultured in the control medium were apoptotic (live cells <1%). In contrast, a significant proportion of neutrophils cultured in the presence of CM were live cells ($43.6 \pm 8\%$, $41.9 \pm 9\%$ and $24.15 \pm 4.8\%$ (mean \pm SEM) cultured in CM from SW480, HT29 and SW620, respectively), suggesting that CRC cell lines produced soluble mediators that increased neutrophil survival (**Figure 14a**). To dissect the molecular mechanism for this pro-survival effect, we evaluated the presence of soluble factors known to increase the neutrophil lifespan in CM. We failed to detect G-CSF, IL-1 β and TNF- α (data not shown). In contrast, CRC cell lines produced GM-CSF (**Figure 14b**) and blocking antibody against this growth factor totally blocked the pro-survival effect of CM (**Figure 14c**). In addition, morphological changes in neutrophils, due to cell attachment and spreading and known to occur upon stimulation by inflammatory cytokines and growth factors (Kutsuna et al., 2004), were observed when

cells were cultured in CM and were inhibited in presence of GM-CSF blocking antibody (**Figure 14d**). Therefore, our data suggest that GM-CSF was the main soluble factor produced by CRC cell lines with pro-survival and pro-activating effects for neutrophils.

Neutrophils display a cytostatic activity towards CRC cell lines

We next evaluated whether neutrophils cultured or not with CM had a cytotoxic or cytostatic activity towards CRC cell lines. CM entrained-neutrophils for 30min or naïve neutrophils were co-cultured with CFSE-labeled HT29 cells for 24h at a 20:1 neutrophil to tumor cell ratio. Taking into account the high number of circulating neutrophils, this co-culture ratio is probably an underestimation of the actual *in vivo* ratio, as previously suggested (Granot et al., 2011). We observed that the presence of neutrophils significantly reduced the number of HT29 (**Figure 15a**). A similar effect was observed using either neutrophils entrained in CM or non-entrained neutrophils, suggesting that neutrophils can be stimulated during the co-culture. We next assessed whether HT29 number reduction was a consequence of a cytotoxic or cytostatic activity of neutrophils towards CRC cell lines. Neutrophils did not modulate the percentage of apoptotic HT29 cells, suggesting that they did not display a cytotoxic activity towards HT29 cells (**Figure 15b**). Therefore, we tested the cytostatic activity of neutrophils by measuring HT29 cells proliferation by dilution of CFSE staining. We observed that addition neutrophils reduced the proliferation of HT29 cells (**Figure 15c**). 5-FU, used as positive control, increased the frequency of apoptotic HT29 cells and decreased cell proliferation (**Figure 15a-c**). Collectively, these data suggest that neutrophils display an anti-tumoral activity towards CRC cells due to a direct cytostatic effect.

MATERIALS AND METHODS

Patients

Cancer-tissue specimens from 271 patients who consecutively underwent resective surgery for colorectal cancer were retrieved from previous series (Laghi et al., 2009). For each patient included in this study, demographics and clinical data at diagnosis were available through the institutional intranet. A clinical database was prepared by investigators who were blinded to the results of molecular and immunological phenotype of the cancers. A single pathologist, who was also unaware of the molecular phenotypes, reviewed tissue specimens. Patients who underwent neoadjuvant radiotherapy for rectal cancer were excluded because of the possibility of interference with the assessment of the local immune response. The presence of metastasis at diagnosis was assessed in all patients by combining histopathological findings, surgical records (including intra-operative liver ultrasonography), and perioperative imaging (abdominal CT and chest radiography in all patients). The observation period started immediately after surgery. To monitor postsurgical tumor recurrences, thoraco-abdominal CT, abdominal ultrasonography, and chest radiography were done according to common protocols for surveillance. Chemotherapy treatment was administered and allocated according to adjuvant protocols in use at the time of surgery. The study was approved by the ethical committee of the Humanitas Clinical and Research Center, and written informed consent was obtained by the referring physician at the time of surgery.

Study design

Tissue specimens of patients with colorectal cancer who consecutively underwent radical surgical resection at the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) from January 1997 to November 2006 were retrospectively studied. A clinical retrospective

database containing demographics, clinical, and histopathologic data was assembled from the institutional intranet by the investigators who were blinded to the results of the morphologic analysis assembled. All these variables, together with the values of CD66b⁺ IRA, were tested as predictors of the patients' outcome. The outcome of patients who undergo radical resection of colorectal cancer can be affected by an event defined as cancer-related death of patient (disease specific survival - DSS) or any local tumor recurrences/metachronous distant organ metastases (disease-free survival - DFS). In order to detect or exclude any postsurgical tumor recurrences, patients underwent abdominal ultrasonography, thoraco-abdominal computed tomography and chest radiography, according to common protocols for surveillance. The observation period started immediately after the surgical procedure. The mean follow-up period of the cohort studied was 4.78 years (SD=2.78) for DSS and 4.38 years (SD=2.92) for DFS. The detection of tumor recurrence or death was computed from diagnosis until data were censored on May 30, 2010.

Immunohistochemistry and microsatellite status

Formalin-fixed and paraffin-embedded thin sections of tumor (2µm) were deparaffinised and exposed to antigen unmasking solution in DIVA buffer 1X (Biocare Medical, CA, USA). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min at room temperature. Primary mouse monoclonal anti-CD66b antibody (clone G10F5; BD Pharmingen, CA, USA) was applied for 1 h at room temperature. Reactive sites were identified by exposure to a secondary antibody (HRP rabbit/mouse; MACH 4 Biocare) for 30 min at room temperature. Immunoperoxidase staining was then obtained by using diaminobenzidine (Dako, CA, USA) as chromogen. The slides were finally counterstained with haematoxylin. CD66b⁺ IRA was measured in three randomly selected and non-contiguous microscopic areas encompassing the deep front of tumor invasion and in three randomly selected and non-contiguous microscopic areas in the intratumoral compartment.

Density of CD66⁺ cells was evaluated by a computer-assisted measurement, as previously described (Di Caro et al., 2014; Laghi et al., 2009). With regard of invasive margin, for each selected area, the cancer tissue had to represent approximately 50% of the entire microscopic field. The pathologist who selected the areas of interest was blinded to tumor microsatellite status and to any patients' clinical data. For each selected region, a digital image was captured (**Figure 10b**). Given that an accurate optical count of CD66b⁺ cells was not feasible in the presence of very dense infiltrates or agglomerates, we did not attempt to convert area values to number of cells. For each tumor, the mean of values obtained in three distinct regions was calculated and used for the subsequent analysis of data. In some experiments rabbit polyclonal anti-MPO antibody (Dako) were used with mouse monoclonal anti-CD68 (clone PG-M1; Dako) and mouse monoclonal anti-CD66b antibody. After washed, slides were incubated with the goat anti-rabbit poly-horseradish peroxidase (HRP) and goat anti-mouse poly-alkaline phosphatase (AP). AP reaction was obtained using red chromogen (Warp red Chromogen Kit; Biocare Medical) and HRP reaction was obtained using a green chromogen (Vina Green Chromogen Kit, Biocare Medical). Microsatellite status was determined preliminarily for all cancers included in the study by testing instability at mononucleotide repeats, as previously described (Laghi et al., 2012).

Statistical analysis

Associations between the extent of CD66b⁺ IRA and patients characteristics or tumor features were tested by linear regression analysis. A Cox proportional hazards model was constructed to assess the clinical significance of CD66b⁺ IRA, as well as other clinico-pathological features, for patients' outcome.

The Receiver Operative Characteristic (ROC) curve analysis, which maximizes both sensitivity and specificity of a marker (Heagerty et al., 2000), was used to select the CD66b⁺ IRA cut-off score. Tumors were distributed into two groups designed as “high

CD66b⁺” and “low CD66b⁺” based on the indicated cut-off value of CD66b⁺ IRA. Kaplan-Meier curves of disease specific (DSS) and disease-free survival (DFS) were plotted. In the ROC curve analysis, patients’ outcomes were dichotomized by survival (death vs alive) for DSS and relapse (local failure/distant metastasis vs no local failure/distant metastasis) for DFS, respectively. The detection of tumor recurrence or death was computed from diagnosis until data were censored on May 30, 2010. The log-rank test was used to compare the curves of patient subgroups. A Cox proportional hazard ratio model was used to assess whether CD66b⁺ IRA, as well as other clinicopathological features were independent prognostic factors for patient survival. Mann-Whitney U test, Student’s t test, Spearman’s correlation, Pearson’s correlation, Cusik’s trend test were used as specified. $P \leq 0.05$ was considered significant. For each test, only two-sided and $P \leq 0.05$ was considered significant. Analyses were done using Epi Info version 3.4.3, StatsDirect Statistical software (version 2.5) and GraphPad Prism software (Version 4.1).

Cell cultures and tumor-conditioned media preparation

The human colon tumor cell lines SW480, HT29 and SW620 (ATCC, VA, USA) were cultured and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 U/mL penicillin/streptomycin and 2 mM L-glutamine (Lonza). Conditioned media were obtained and used as follows. Cells were plated at 10-20% confluence in six well tissue cultures. Once cells had reached a confluence of 85-90%, cell culture media were replaced by fresh media containing RPMI 1640 supplemented with 0.5% FBS. After 24 h, the conditioned medium (CM) was collected and filtered at 0.20 μ m. Supernatants were stored at -20°C. All cell lines were routinely checked for mycoplasma contamination.

Neutrophil purification and culture

Leukocytes from peripheral blood of healthy donors were separated from erythrocytes by Dextran sedimentation. Neutrophils were purified by Ficoll-Paque density gradient centrifugation followed by Percoll (65%) density gradient centrifugation, as previously

described (Muzio et al., 1994). These cells were >99% neutrophils as evaluated by flow cytometry analysis by using the following antibodies, anti-CD3, anti-CD14, anti-CD15, anti-CD11b, anti-CD62L, anti-CD66b (all from BD bioscience, CA, USA) and anti-CD11b (eBiosciences, CA, USA). Analysis was performed by using FACS Canto II and FACS Diva software (BD bioscience). Spontaneous activation of neutrophils was analyzed by evaluating CD11b and L-selectin expression by FACS analysis before and after isolation; only L-selectin⁺CD11b^{low} (non activated) neutrophils were used in this study (data not shown).

Quantification of soluble factors in culture supernatants

Production of CXCL8, CXCL1, CXCL2, G-CSF, GM-CSF, TNF- α and IL-1 β by CRC cell lines was analyzed by ELISA (R&D DuoSet ELISA Development System), according to the protocols provided by the manufacturer. A Versamax microplate reader (Molecular Devices Corporation –Biospa) was used to determine sample absorbance at 450 nm.

Cell migration assay

Migration of neutrophils towards CM and CXCL8 was evaluated by using a Boyden chamber (Neuroprobe, CA, USA) assay. Briefly, 30 μ l of CM or control medium (RPMI 1640 supplemented with 0.5% FCS) were added to the lower wells of the chamber. A polyvinylpyrrolidone-free polycarbonate filter (5 μ m pore size; Neuroprobe) was layered onto the wells and covered with a silicon gasket and the top plate. Fifty microliters of cell suspension (7.5×10^4 neutrophils) were seeded in the upper chamber. In some experiments, anti-CXCR1/2 (Reparixin, 1 μ M), anti-CXCR2 (SB225002, 20 nM), PTX (100 ng/ml) or vehicle (DMSO) were added to the cell suspension. The chamber was incubated at 37°C in 5% CO₂ and 95% *air atmosphere* for 60 min. At the end of the incubation, filters were removed and stained with Diff-Quik (Baxter, Rome, Italy). Twenty high power oil-immersion fields were counted for each condition in triplicates.

Apoptosis assay and morphological analysis of neutrophils

Purified neutrophils (10^6 cells/mL) were cultured with SW480, HT29 and SW620 CM with or without mouse monoclonal anti-GM-CSF blocking antibody at 10 μ g/ml (Clone 3209, R&D Systems) or the corresponding control isotype (R&D Systems). For each time point, neutrophils were stained with FITC-conjugated AnnexinV and propidium iodide (PI) according to the manufacturer's instructions (Invitrogen). Live cells were characterized as double negative AnnexinV⁻ PI cells. Analysis was performed by using FACS Canto II and FACS Diva software (BD). After 24 hours of culture, optical images were acquired with Olympus IX53 upright light microscope at 20x magnification.

Cytotoxic and cytostatic assays

HT29 cells (5×10^4 cells) were plated on a 24-well plate for 48 hours and then stained with CFSE (carboxyfluorescein diacetate succinimidyl ester), according to the manufacturer's instruction (Invitrogen). After wash, cells were cultured in RPMI 1640 supplemented with 10% FBS for 4 hours. Neutrophils isolated from healthy donors peripheral blood, were pre-incubated with CM or control medium and added to the HT29 culture at a 20:1 neutrophil to tumor cell ratio. After 24 hours, cells were stained with APC-conjugated Annexin-V and PI according to the manufacturer's instructions (Invitrogen). Analysis was performed by using FACS Canto II and FACS Diva software (BD).

DISCUSSION

Personalized medicine is an important target for clinicians and researchers. In this view, the identification of independent prognostic and predictive markers plays an essential role. Prognostic markers give information on patients' outcome and can be useful to orientate therapeutic decisions. Predictive markers give information as to the likely response to therapeutic regimens.

Colorectal cancer is a complex and heterogeneous disease. Therefore, universal prognostic and predictive markers, to improve patients' follow-up and choose the most adequate treatment protocol for each cancer patient, are still needed. Indeed, the usefulness of the international reference TNM system, currently in use for patients' outcome prediction, has been questioned (Galon et al., 2006).

In clinical research studies on CRC, the power to predict which cancer patient will display a survival advantage from 5-FU-based chemotherapy has been even more difficult than finding consistent prognostic markers. Several candidate molecular markers have been suggested, including BRAF, KRAS or TP53 mutation, microsatellite instability, CpG island methylation status (Brenner et al., 2014). To date, however, none of these markers has been validated in prospective trials. Accordingly, insufficient evidence was declared by current European guidelines for the treatment of CRC to recommend any molecular predictive marker for the response to 5-FU (Brenner et al., 2014; Schmoll et al., 2012).

A link between inflammation and human colorectal cancer is now well established and, while the mechanism was not been completely understood, it is clear that immune and inflammatory cells actively participate in the tumor microenvironment and display both anti-tumor and pro-tumor activities (Grivennikov et al., 2010; Grivennikov et al., 2012; Terzic et al., 2010). A number of evidence have described several roles for cytokines and immune cells in the various steps of colorectal carcinogenesis, inducing initiation,

promotion and progression through metastatic disease. Therefore, efforts have been made to identify new biomarkers related to tumor environment and infiltration of immune and inflammatory cells (Grizzi et al., 2013). In particular, the presence of a type I adaptive immune response and the infiltration of T lymphocytes have been associated with a positive clinical outcome (Galon et al., 2006; Galon et al., 2007).

As a well-known leukocyte subpopulation, neutrophils have long been recognized as a first-line defense against pathogens. Because of their short half-life and terminally differentiated phenotype, the role of neutrophils in tumor development has been considered negligible. However, epidemiological studies and animal models now suggest that neutrophils can be characterized by a surprising plasticity and can be polarized towards distinct phenotypes in response to signals derived from the tumor microenvironment (Fridlender et al., 2009). In addition, recent evidence suggests important roles of neutrophils in modulating tumor behavior and, similarly to their big brother macrophages, also neutrophils infiltrate human tumors (Donskov, 2013).

High density of intra- or peritumoral neutrophils have been related to a worse disease outcome in HCC (n=238), melanoma (n=186), lung cancer (N=632) and head and neck cancer (N=99) (Ilie et al., 2012; Jensen et al., 2012; Kuang et al., 2011; Trellakis et al., 2011a). Along similar lines, tumor-infiltrating neutrophils predicted poor prognosis in a cohort of gastric carcinoma patients (N=115) (Zhao et al., 2012), but were described as a positive prognostic factor in a second cohort of gastric cancer patients (N=273) (Caruso et al., 2002).

With regard to colorectal cancer, few studies examined the prognostic significance of tumor infiltrating neutrophils (**Table 3**). In three of these studies, a higher neutrophil density was associated with better survival (Baeten et al., 2006; Klintrup et al., 2005; Nielsen et al., 1999). In the study of Baeten and colleagues, neutrophils were identified as CD16⁺ cells and significant association between intratumoral CD16⁺ cells and patient

survival was observed (Baeten et al., 2006). In another study, neutrophil infiltration was related to reduced local recurrence rates, but not to survival (Nagtegaal et al., 2001). In addition to the study by Nagtegaal and colleagues, Klintrup et al. reported that an increased neutrophil infiltration at the invasive margin was found to be a prognostic factor, although both studies reported a positive prognostic role of other cell types and concluded that a general non-specific immune response was important in predicting patient survival (Klintrup et al., 2005). However, in all these studies, the prognostic significance of tumor-infiltrating neutrophils was not confirmed by multivariate analysis (Baeten et al., 2006; Klintrup et al., 2005; Nagtegaal et al., 2001; Nielsen et al., 1999). In 2012 Rao and colleagues published the first report describing a high intratumoral neutrophil density as an adverse prognostic factor for patients with CRC (Rao et al., 2012). Indeed, in 229 patients undergoing primary resection for CRC, high intratumoral CD66b⁺ neutrophil count was positively associated with clinical stage. In multivariate survival analysis, high intratumoral neutrophils were described as an independent prognostic factor for adverse overall survival (Rao et al., 2012). In contrast, in 2013, Droeser and colleagues reported that intratumoral MPO⁺ neutrophils were associated with improved survival and confirmed this result by multivariate analysis in a wide cohort of CRC patients (N=1491) (Droeser et al., 2013).

The described discrepancies between these studies can be explained by a number of variables.

First of all, the methodology used to identify neutrophils within human tumors is an important point. Indeed, it is important to note that there was no consensus on the method for the staining of neutrophils in CRC tissues, since hematoxylin-eosin and immunohistochemistry were used in previous studies. Even when immunohistochemistry was used, a wide heterogeneity in the choice of the neutrophil-specific marker was found (CD16, elastase, MPO, CD15, CD66b). In addition, the method of assessment of neutrophil

count is also important, since semi-quantitative or quantitative scales as well as computer aided methods were arbitrarily used in these studies.

In our study, we described that CD66b is a more specific marker for neutrophils in CRC tissues, compared to MPO, and should be used in further studies.

In addition, we took advantage of a computer-aided image analysis system. This method is objective and statistically relevant to quantify the proper threshold values, because of providing detailed and informative data and continuous quantification of immune cell densities.

Moreover, in our study, we analyzed a validated cohort of patients (Laghi et al., 2009) with long follow-up and known adjuvant treatment status. We showed that infiltration of neutrophils in colorectal cancer evolved overtime through a spatiotemporal dynamism. Indeed, neutrophil infiltration increased from stage I to stage III, followed by a decrease in stage IV. This result was in line with previous findings based on tissue microarrays data (Bindea et al., 2013). In addition, infiltration of neutrophils in stage III CRC patients was not spatially homogenous and was found higher in the intratumoral compartment compared to the invasive margin.

By using a rigorous approach based on the detection of neutrophils (CD66b⁺ cells) found in both the invasive margin and the intratumoral compartment, we observed that higher densities of neutrophils were significantly associated with better clinical outcome in patients with CRC (stage I to Stage IV). However, this observation could be biased by the heterogeneity of the population and in particular by the chemotherapy treatment. Indeed, an additional level of heterogeneity among the studies could be due to the patient dataset analyzed. In contrast to the previous studies, we analyzed the prognostic role of neutrophils both for patients treated by surgery alone and for those who received 5-fluorouracyle (5-FU) based chemotherapy. Indeed, investigation of the prognostic significance of clinic-pathologic markers in stage III CRC patients treated with 5-FU revealed that tumor-

infiltrating neutrophil density could be considered as a predictive factor for good response to 5-FU based chemotherapy. Indeed, we observed that infiltration of neutrophils within the intratumoral compartment was significantly associated with better clinical outcome in 5-FU treated patients (HR 0.42; 95% CI 0.21 to 0.83; $p=0.01$) but not in patients treated with surgery alone (HR 1.79; 95% CI 0.64 to 4.99; $p=0.26$).

Our results support the relevance of evaluating patient cohorts with the same adjuvant treatment status when studying the prognostic role of pathologic and molecular markers in disease outcomes. Our findings can be interpreted considering that tumor-infiltrating neutrophils are a novel predictive factor for 5-FU-based chemotherapy response.

However, careful examination is needed when evaluating prognostic markers by using subgroup analyses. Indeed, a differential prognostic power of a marker in distinct patient subgroups does not mean that the prognostic power of the marker differs on the basis of the variable that identifies subgroups (i.e. treatment). To efficiently demonstrate an effect modification of immune cells on chemotherapy treatment in predicting patients' clinical outcome, it should be investigated the existence of an interaction between these two variables at multivariate analysis (Altman et al., 2012). In our study, we found that a statistical interaction between IT CD66b⁺ IRA and 5-FU-based treatment ($p=0.009$), thus further supporting the differential results obtained in patients subgroups.

Previous works have suggested that the tumor immune environment can influence chemotherapy treatment and infiltration of adaptive immune cells was associated with chemotherapy response (Anitei et al., 2014a; Di Caro et al., 2013; Halama et al., 2011; Ma et al., 2013a; Medina-Echeverz et al., 2011). To the best of our knowledge, our study is the first to demonstrate a significant association between infiltration of innate immune cells and chemotherapy response in CRC patients. These data could also explain, at least in part, the discrepancies in results concerning the prognostic value of neutrophil infiltration in CRC

because, in the previous studies, authors do not have taken into account the treatment (Bindea et al., 2013; Droeser et al., 2013; Rao et al., 2012; Roncucci et al., 2008).

Solid tumors are characterized by an inflammatory profile and the presence of infiltrated leukocytes. However, the significance and role of neutrophils in cancer development is still a matter of debate and a dual role for neutrophils in tumor biology has been described (Galdiero et al., 2013a). For instance, neutrophils were involved in genetic instability, which can favor neoplastic transformation and the acquisition of a metastatic phenotype, as well as in tumor angiogenesis (Jablonska et al., 2010; Jaillon et al., 2013; Scapini et al., 2004; Shojaei et al., 2009). In contrast, neutrophils can acquire a cytotoxic phenotype to kill tumor cells and recent evidence suggests that tumor-infiltrating neutrophils can stimulate the T cell-dependent anti-tumoral immunity (Eruslanov et al., 2014; Granot et al., 2011).

Here, we observed that the degree of neutrophil infiltration increased with the clinical stage. Accordingly, we found that CRC cells produce soluble factors able to recruit, prolong the lifespan and activate neutrophils. We found that CRC cells constitutively produced CXCL8, CXCL1 and CXCL2 and, accordingly, conditioned media from CRC cells exerted chemotactic activity towards neutrophils in a CXCR1/2-dependant manner. In addition, we found that CRC cells activated neutrophils and prolonged their survival through the production of GM-CSF. The expression of GM-CSF in CRC tissue was predominantly associated with tumor cells and its role in cancer was debated because this cytokine was involved in the generation of myeloid-derived suppressor cells (Marigo et al., 2010). However, GM-CSF was recently associated with increased survival in patients with colorectal cancer (Nebiker et al., 2014). In the light of our data, this association between GM-CSF and patients' outcome could be related with the capacity of GM-CSF to activate and increase the lifespan of neutrophils.

Mirroring the M1-M2 and Th1-Th2 paradigms, recent evidences have suggested that mouse neutrophils could be polarized toward a proinflammatory phenotype with anti-tumor

activity (N1) or toward a pro-tumor N2 phenotype (Fridlender et al., 2009; Jablonska et al., 2010). Here, we showed that human neutrophils display a cytostatic activity towards CRC cell lines. On the basis of the phenotype observed in CRC cell lines in the presence of neutrophils, one could hypothesize that an induced arrest of both cell death and proliferation occurs, via a mechanism named cellular senescence. Indeed, in the presence of cell damage, damage sensor proteins, such as Ataxia Telangectasia Mutated (ATM) protein, up-regulate effectors of cell cycle arrest (Herbig et al., 2004). A number of stress inducers can activate this DNA damage response (DDR), including oxidants and DNA damaging chemotherapies. For instance, low doses of ROS activate senescence in human fibroblasts (Chen et al., 2000; Herbig et al., 2004). There is now strong evidence that cellular senescence is a potent anticancer mechanism (Braig and Schmitt, 2006; Prieur and Peeper, 2008). However, further investigations are needed to gain deeper inside the mechanisms of the observed cytostatic effects exerted by neutrophils towards CRC cell lines.

A direct link between our *in vitro* data and the clinically significant findings of our study should take into account that the primary tumor was surgically resected. Indeed, the cytostatic activity of neutrophils can be achieved beyond the primary site, as suggested in mouse (Granot et al., 2011). Therefore, we found that the density of neutrophils found in metastatic lymph nodes mirrored their density in the primary tumor, suggesting that neutrophils can exert their cytostatic functions in the metastatic niche.

Even though this study presents some limitations due to the retrospective design and the small number of patients, it highlights the importance of the immune system and its interaction with chemotherapy for orientating the prognosis of localized colorectal cancer, suggesting that a precise evaluation of the local immune response could be useful for predicting prognosis in the context of chemotherapy.

Finally, we demonstrated that innate immunity and neutrophils have predictive significance for chemotherapy response in patients with stage III CRC and exert a direct cytostatic activity towards CRC cells.

FIGURES

Figure 1.

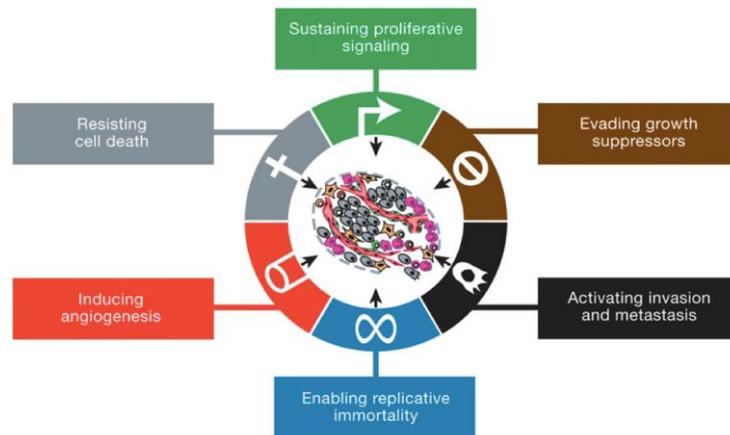


Figure 1. The hallmarks of cancer. This figure represents the six hallmarks originally proposed by Hanahan and Weimberg in 2000. *from Hanahan and Weimberg, 2011*

Figure 2.

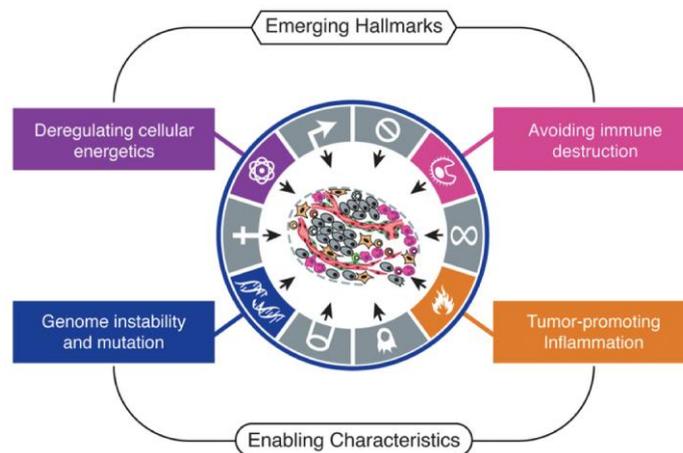


Figure 2. Emerging hallmarks and enabling characteristics. Two additional hallmarks are involved in the pathogenesis of cancers. One involves the ability to modify cellular metabolism and the second allows cancer cells to evade immunological surveillance. In addition, two characteristics favor acquisition of these hallmarks: genomic instability and cancer related inflammation.

from Hanahan and Weimberg, 2011

Figure 3.

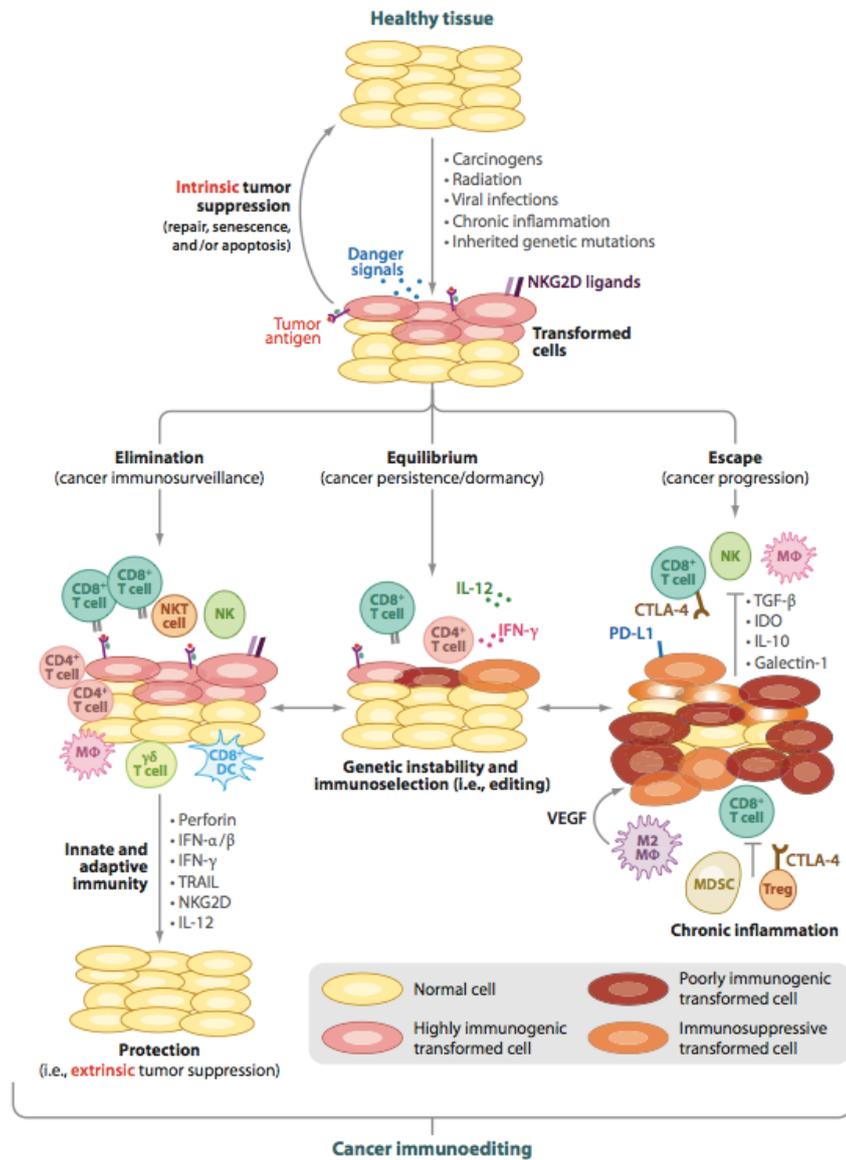


Figure 3. The three phases of immunoediting. Cancer immunoediting results from three processes that occur independently or in sequence to control and shape cancer. In the first phase, elimination, previously known as cancer immunosurveillance, immune cells recognize and destroy transformed cells. If anti-tumor immunity is unable to completely eliminate transformed cells, surviving tumor variants may enter into the equilibrium phase. These variants may acquire further mutations that result in evasion of tumor cell recognition and killing by the immune system. Therefore cancer progresses to clinically detectable malignancy in the escape phase.

from Vesely et al., 2011

Figure 4.

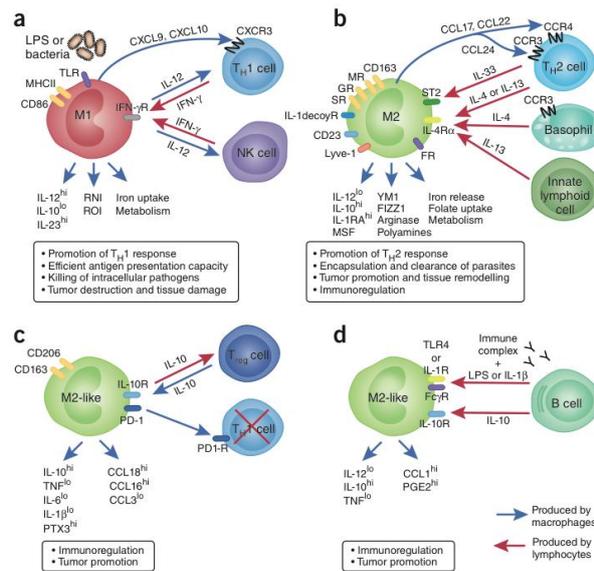


Figure 4. The orchestration of macrophage polarization and their effector functions.

(a) M1-polarized macrophages and their crosstalk with Th1 and NK cells. (b) M2 polarization of macrophages driven by IL-4, IL-13 or IL-33; (c) M2-like macrophages polarized by interaction with Treg cells. (d) M2-like polarization of macrophages by interaction with B cells through antibody-mediated Fc γ R activation or cytokines.
from Biswas and Mantovani, 2010

Figure 5.

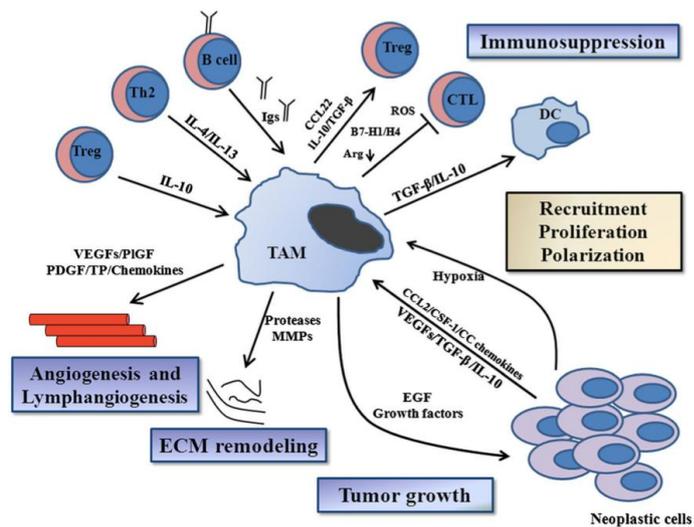


Figure 5. TAMs as key regulators of the tumor-related inflammation. Neoplastic cells recruit macrophages, favoring their polarization toward a pro-tumor phenotype. In turn, TAMs influence many aspects of cancer progression, such as tumor growth, ECM remodeling, angiogenesis and immunosuppression.
from Galdiero et al., 2013b

Figure 6.

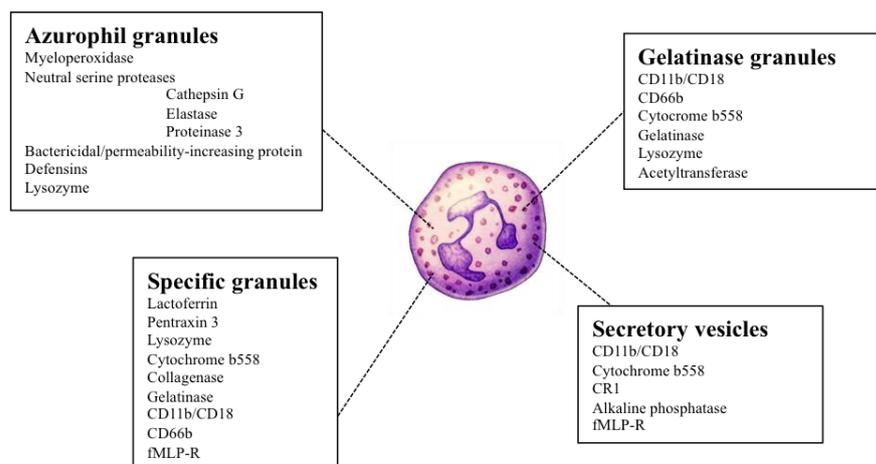


Figure 6. Schematic representation of proteins contained within neutrophil granule subsets.

Modified from Eyles et al., 2006

Figure 7.

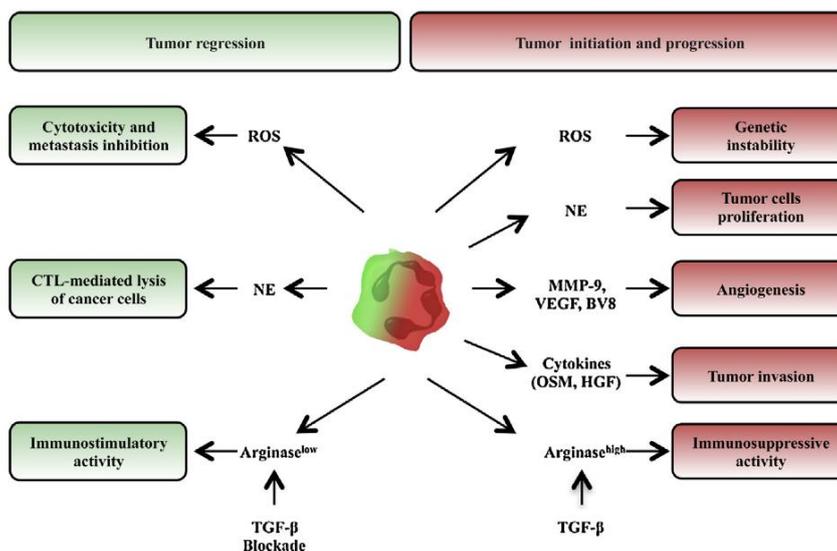


Figure 7. Dual role of TANs in tumor growth and progression. TANs can exert both anti-tumoral and pro-tumoral activities. TANs kill cancer cells through the release of ROS and neutrophil elastase, inhibit metastatic seeding through the release of ROS and potentiate anti-tumoral T cell responses after inhibition of TGF- β . In contrast, TANs favor genetic instability through the release of ROS, promote tumor cell proliferation through elastase, sustain angiogenesis through the release of VEGF, MMP-9 or Bv8, enhance neoplastic cell invasiveness through soluble mediators (e.g. OSM and HGF) and suppress effective anti-tumoral CD8⁺ T-cell immunity through the expression of arginase-1 driven by TGF- β .

from Galdiero et al., 2013a

Figure 8.

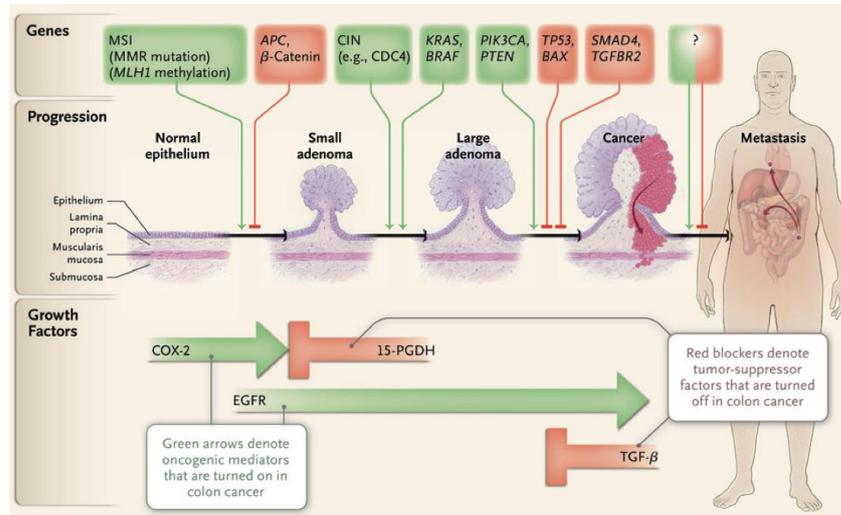


Figure 8. Genes and growth factor pathways that drive the progression of colorectal cancer.

In the progression of colon cancer, genetic alterations target the genes that are identified. The microsatellite instability (MSI) pathway is initiated by MMR gene mutation or by aberrant *MLH1* methylation and is further associated with downstream mutations (i.e. *TGFBR2* and *BAX*). The question mark indicates that genetic or epigenetic changes specific to metastatic progression have not been identified. Key growth factor pathways that are altered during colon neoplasia are shown at the bottom of the diagram. *from Markowitz and Bertagnoli, 2009*

Figure 9.

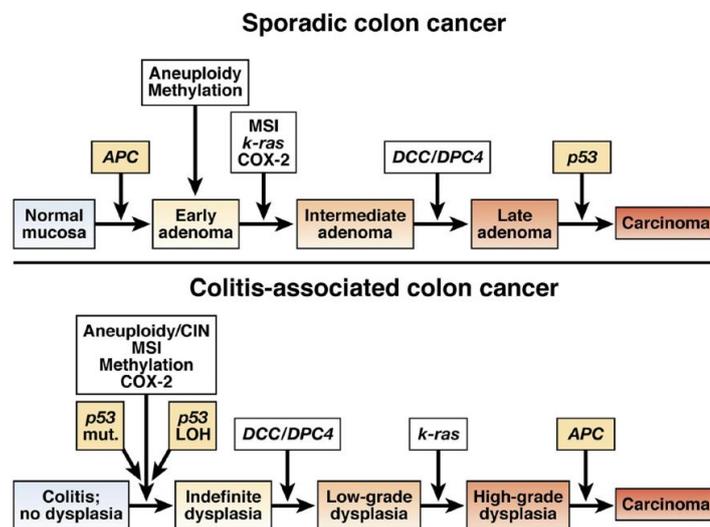


Figure 9. Molecular pathogenesis of sporadic colon cancer (top) and colitis-associated colorectal cancer (bottom). There are similarities between the pathways, including the development of aneuploidy (CIN), MSI, DNA methylation, activation of the oncogene *k-ras*, activation of COX-2, and mutations of *p53*, APC. However, the frequency and sequence of these events differs between the cancers.

from Ullman and Itzkowitz, 2011

Figure 10.

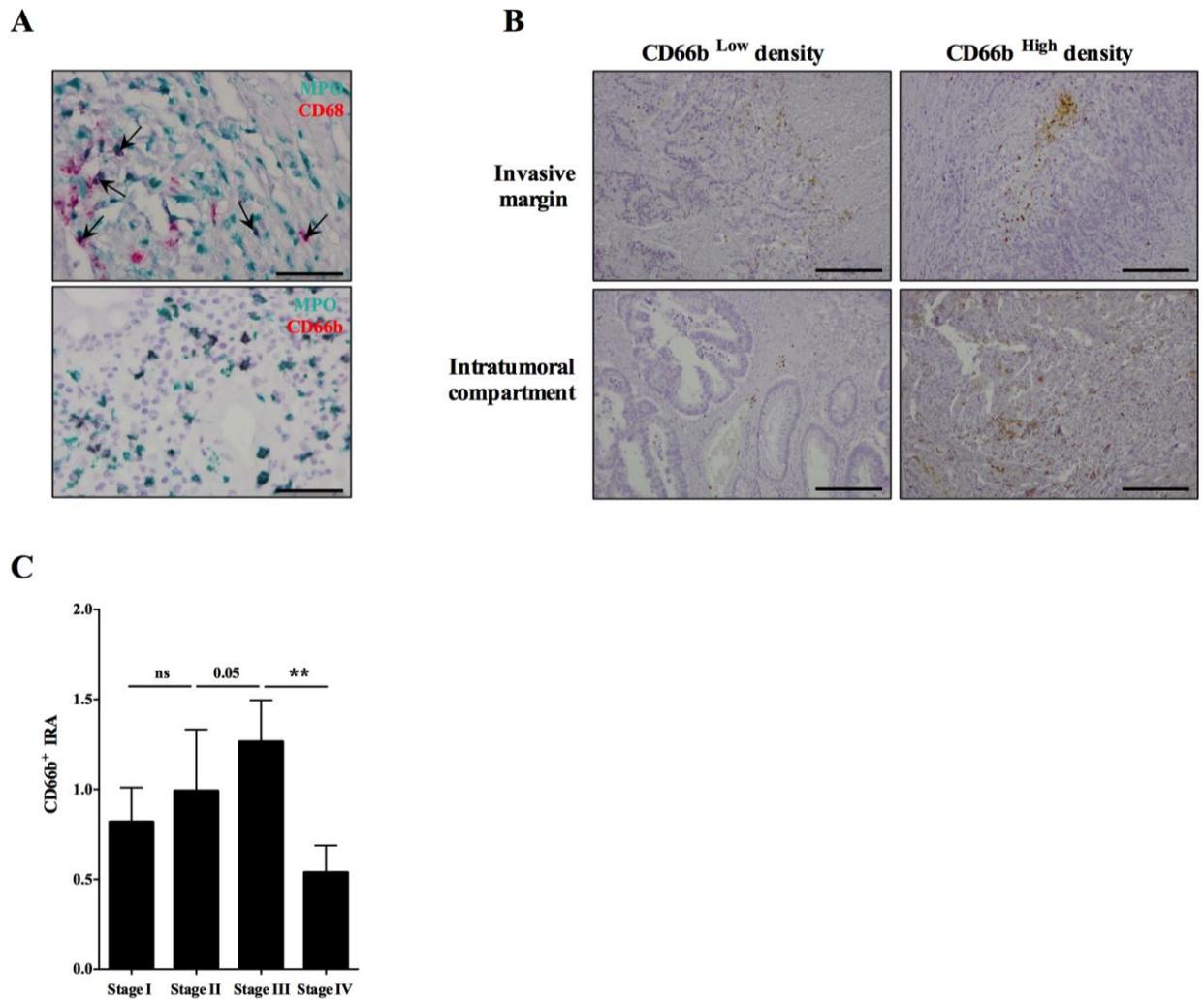


Figure 10. Analysis of neutrophil infiltration in CRC tissue sections.

A. Immunostaining analysis for CD68 (red) and MPO (green) (upper panel) and CD66b (red) and MPO (green) (lower panel) in CRC tissue sections. Double positive cells for MPO and CD68 were indicated by arrows (magnification: 40x). Bar: 50 μ m **B.** Histological analysis of CRC samples stained with monoclonal anti-CD66b antibody. Density of neutrophils was analyzed in the invasive margin of the tumor (upper panel) and in the intratumoral compartment (lower panel) (magnification: 10X). Bar: 200 μ m **C.** CD66b⁺ immunoreactive area found in whole tumor sections (IM and IT) of patients with stage I (n=19), stage II (n=43), stage III (n=35) and stage IV (n=31) CRC.

Figure 11.

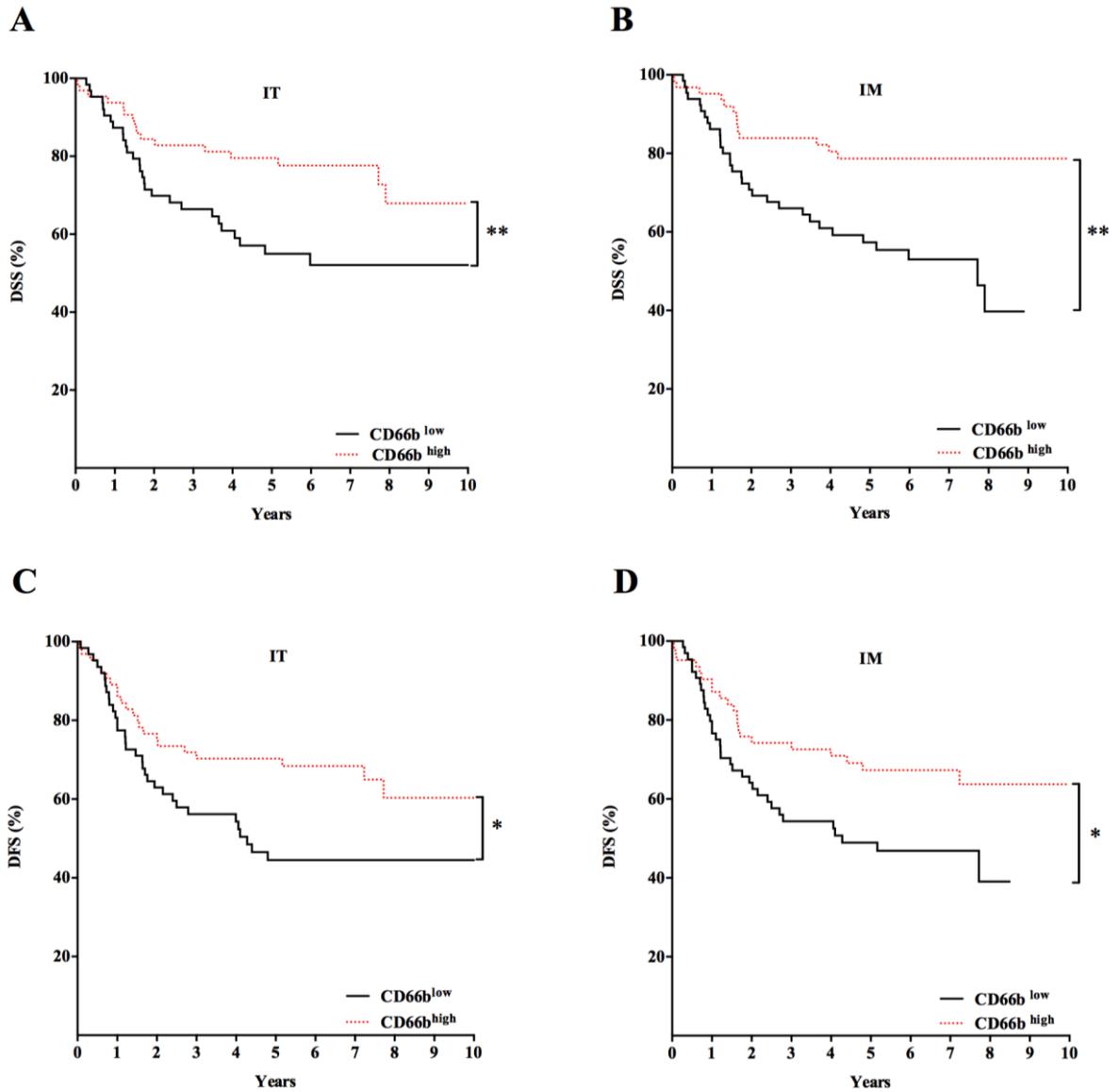


Figure 11. Prognostic significance of CD66b⁺ IRA in patients with Stage I-Stage IV CRC.

A-D. Kaplan-Meier survival curves showed DSS (**A-B**) and DFS (**C-D**) for patients (n=128) with high or low density of neutrophils (CD66b^{high} or CD66b^{low}, respectively) found in the IM or in the IT. ROC curve analysis was employed to define the cut-off values of the CD66b⁺ immunoreactive area.

Figure 12.

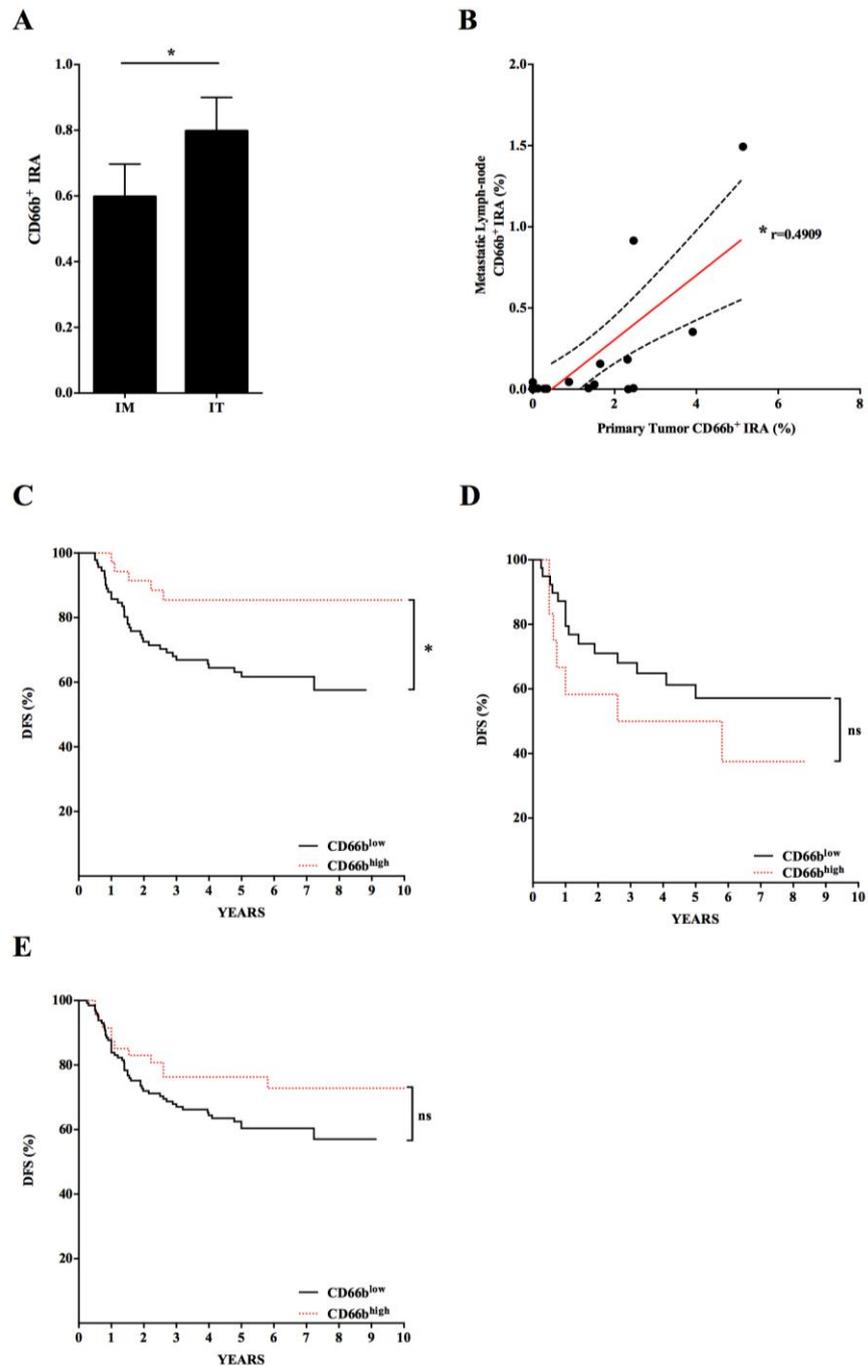


Figure 12: Predictive significance of neutrophil infiltration for 5-FU chemotherapy response

A. CD66b⁺ IRA in the IM and in the IT of tumor sections from patients with stage III CRC (n=178). **B.** Correlation between the CD66b⁺ IRA found in primary tumor and in metastatic lymph nodes in patients with stage III CRC. * $P \leq 0.05$; Spearman's correlation. **C-E.** Kaplan-Meier survival curves showed DFS for stage III CRC patients with high density of neutrophils or low density of neutrophils (CD66b^{high} or CD66b^{low}, respectively) found in the IT. Curves were plotted for 5-FU treated patients (n=126) (**C**), 5-FU non-treated patients (n=52) (**D**) and combined 5-FU treated and non-treated patients (n=178) (**E**).

Figure 13.

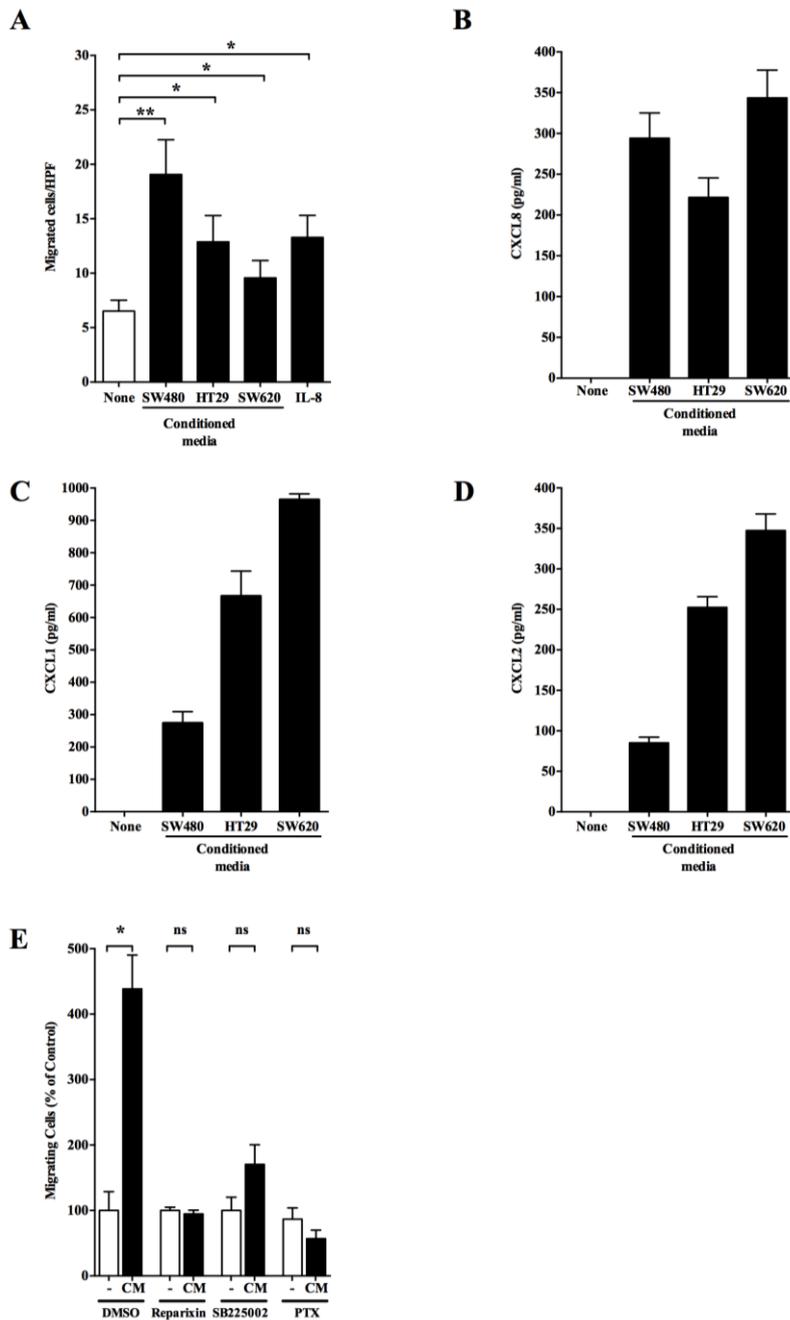
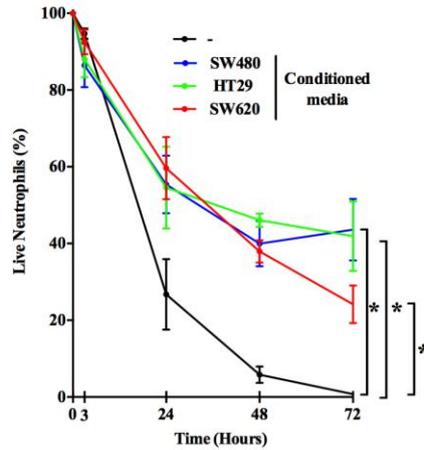


Figure 13. Colorectal cancer cells recruit neutrophils

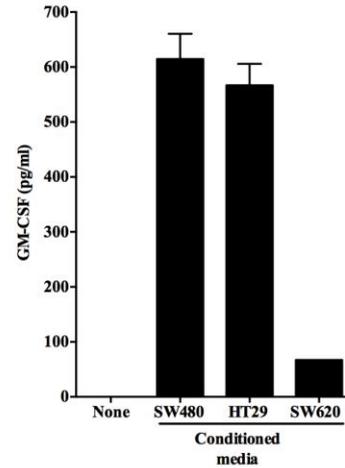
A. Chemotactic activity of CM from CRC cell lines towards human freshly isolated neutrophils was evaluated by using a Boyden chamber. 20 high power oil-immersion fields were counted for each condition in triplicates. Results are mean \pm SEM (n=4). * $P \leq 0.05$; two-tailed Student's *t* test. **B-D.** Production of CXCL8 (B), CXCL1 (C) and CXCL2 (D) by SW480, HT29 and SW620 was evaluated by ELISA in CRC CM. Once cells had reached a confluence of 85-90%, cell culture media were replaced by fresh media and supernatants were harvested after 24 h. Results are mean \pm SEM (n=4). **D.** Chemotactic activity of CM from CRC cells towards freshly isolated neutrophils was analyzed in the presence of *Reparixin* (*i.e.* inhibitor of CXCR1/CXCR2), SB225002 (non-peptide inhibitor of CXCR2) or the G-coupled receptor inhibitor, PTX. Results are mean \pm SEM (n=4). * $P \leq 0.05$; two-tailed Student's *t* test.

Figure 14.

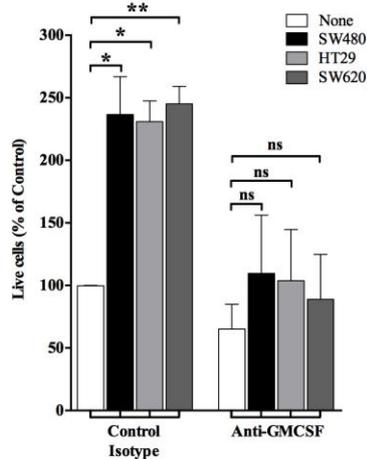
A



B



C



D

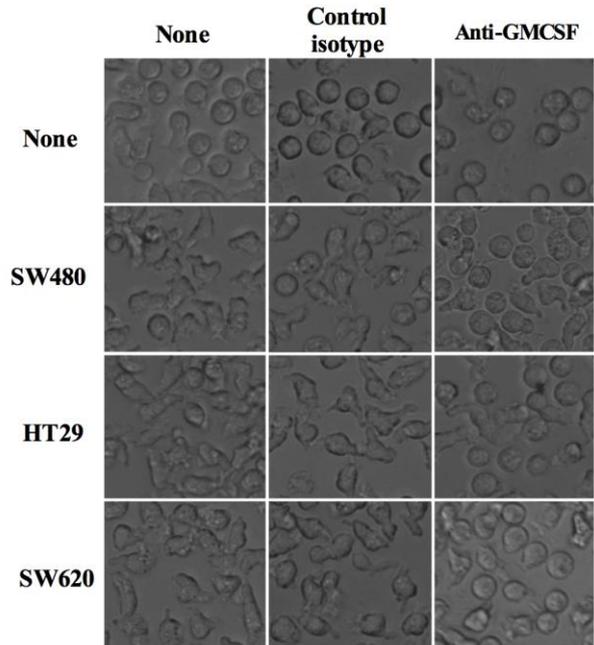


Figure 14. Neutrophil survival and morphological changes.

A. Freshly isolated human neutrophils were cultured in CM from indicated CRC cell lines. Apoptosis was analyzed at different time points by flow cytometry using APC-labeled Annexin V binding and PI. Results are mean \pm SEM (n=4). * $P \leq 0.05$; Cusik's trend test. **B.** Production of GM-CSF by SW480, HT29 and SW620 was evaluated by ELISA in cell supernatants. Once cells had reached a confluence of 85-90%, cell culture media were replaced by fresh media and supernatants were harvested after 24h. Results are mean \pm SEM (n=4). **C-D.** Freshly isolated human neutrophils were cultured in CM from indicated CRC cell lines for 24h and in the presence of blocking monoclonal anti-GMCSF antibody or isotype control. Apoptosis was analyzed by flow cytometry using APC-labeled Annexin V binding and PI (**C**). Results are mean \pm SEM (n=4) ** $P \leq 0.01$; two-tailed Student's *t* test. Cell morphology was assessed by phase-contrast microscopy (magnification: 40X) (**D**). Results shown are representative of four experiments.

Figure 15.

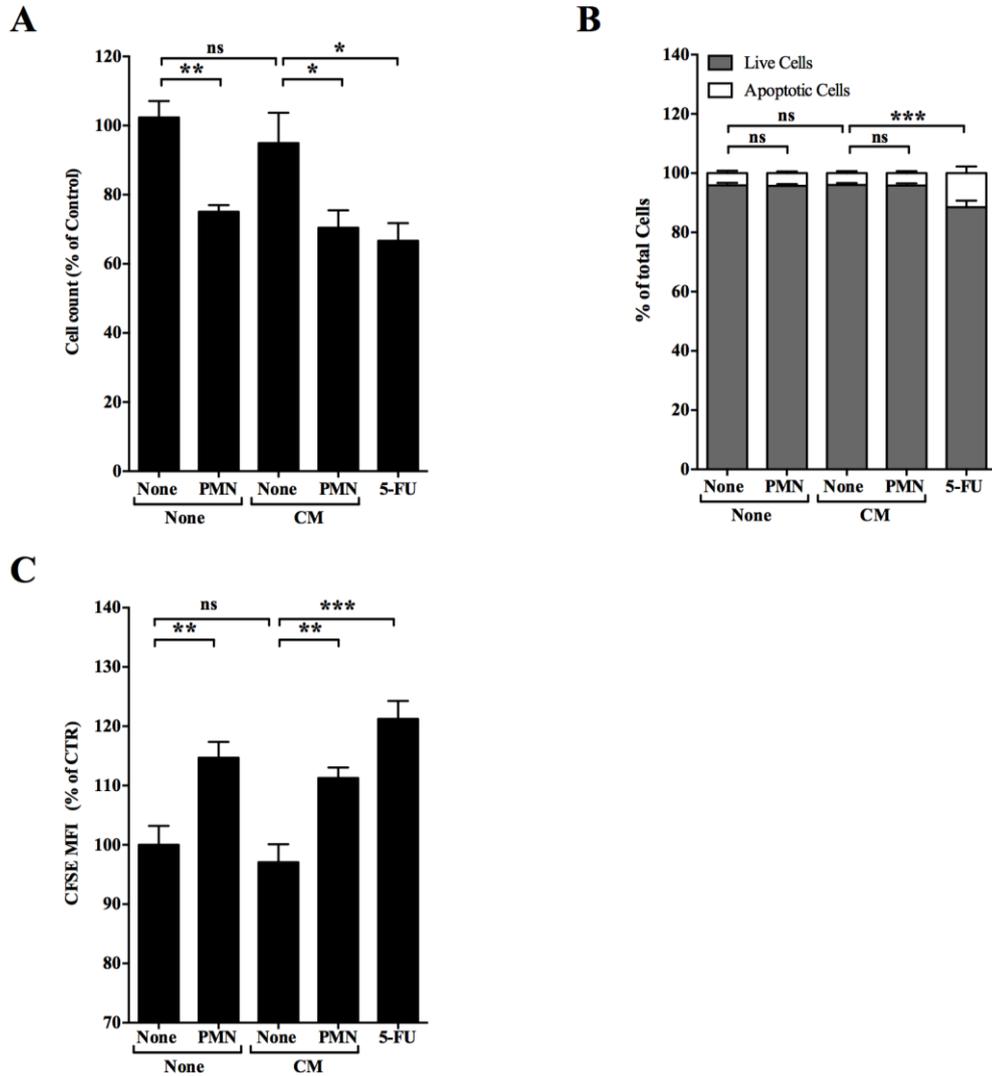


Figure 15. Cytostatic activity of neutrophils

A-C. Freshly isolated neutrophils were pre-incubated with CM or control medium and added to CFSE-labeled HT29 cells at ratio of 20:1. As positive control, HT29 cells were cultured in presence of 5-FU (5 μ M). **A-B.** After 24h, total HT29 cell number (**A**) and percentage of apoptotic HT29 cells (**B**) were evaluated by flow cytometry with APC-conjugated Annexin-V. Results are mean \pm SEM (n=4) * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; two-tailed Student's *t* test. **C.** MFI of CFSE-labeled HT29 cells was analyzed by flow cytometry. Results are mean \pm SEM (n=4) ** $P \leq 0.01$; *** $P \leq 0.001$; two-tailed Student's *t* test.

TABLES

Table 1. Phenotype, characteristics and functions of relevant human T cell subsets.

T cell subset	Phenotype	Characteristic cytokine	Characteristic transcription factors	Functions
Naïve	CD45RA+CCR7+	IL-2		Precursor cells, protection against pathogens
TCM (central memory)	CD45RA-CCR7+	IL-2, IL-21		Secondary expansion, help
TEM (Effector memory)	CCR7-	IFN-g, IL-4, IL-5, IL-17		Protection in tissues, help
TRM (Tissue-resident Memory)	CD103+CD69+	IFN-g		Immediate protection in tissues
TFH (Follicular Helper)	CXCR5+ ICOS+	IL-21	BCL6	B cell help
Th1	CXCR3+	IFN-g	T-bet	Protection against intracellular pathogens
Th2	CRTH2+	IL-4, IL-5, IL-13	GATA3	Protection against extracellular parasites
Th9	?	IL-9	PU.1	Protection against extracellular parasites
Th17	CCR6+CD161+	IL-17, IL-22, IL-26	RORC2	Protection against extracellular bacteria and fungi
Treg	CD25+CD127-	TGF-b	FOXP3	Maintenance of self tolerance
Tr1 (Type 1 regulatory)	CD25-CD127- or CD49b+ LAG3+	IL-10	?	Inhibition of immunopathology

from Geginat et al., 2014

Table 2. Neutrophil-derived cytokines

CXC chemokines	CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12*, CXCL13*
CC chemokines	CCL2, CCL3, CCL4, CCL17, CCL19, CCL20, CCL22
Pro-inflammatory cytokines	IL-1 α , IL-1 β , IL-6 [#] , IL-7, IL-16, IL-17A, IL-17f [#] , IL-18, MIF
Anti-inflammatory cytokines	IL-1RA, IL-4 [#] , IL-10 [#] , TGF- β 1, TGF- β 2
Immunoregulatory cytokines	IFN- α [§] , IFN- β , IFN- γ [#] , IL-12, IL-23, IL-27, IL-21*
Colony-stimulating factors	G-CSF, M-CSF [#] , GM-CSF [#] , IL-3 [#] , SCF ^{#,§}
Angiogenic and fibrogenic factors	HB-EGF, HGF, FGF-2, TGF- α , VEGF, Bv8
TNF superfamily members	APRIL, BAFF, CD30L, CD95L, LIGHT [§] , LT β [§] , RANKL, TNF, TRAIL, CD40L*
Other cytokines	Amphiregulin, BDNF [§] , midkine, NGF [§] , oncostatin M

Cytokines expressed by neutrophils, either spontaneously or following appropriate stimulation, based on gene expression techniques, immunohistochemistry, ELISA or biological assays in vitro or in vivo

*. Reported expressed by "B-helper neutrophils"

#. Controversial data for human neutrophils

§. Studies performed at mRNA level only

Modified from Jaillon et al, 2013

Table 3. Prognostic significance of tumor infiltrating neutrophils in humans.

Ref	Tumor Type	Method/Marker	Location	N	Prognostic Impact
Reid et al., 2011	Pancreas	Hematoxylin- and eosin	Intratumoral	517	Negative
Jensen H et al., 2009	RCC	CD66b	Intratumoral	121	Negative
Li Y-W et al., 2011	HCC	CD66b	Intratumoral	281	Negative
Kuang D-M. et al., 2011	HCC	CD15	Peritumoral and Intratumoral	200	Negative (Peritumoral)
Jensen T. et al., 2011	Melanoma	CD66b	Intratumoral	186	Negative
Ilie M. et al., 2011	NSCLC	MPO, CD66b	Intratumoral	632	NO, Negative
Trellakis S. et al., 2011	HNSCC	MPO, CD66b	NS	99	NO, Negative
Caruso et al., 2002	Gastric	Hematoxylin- and eosin	NS	273	Positive
Zhao J. et al., 2012	Gastric	CD15	Intratumoral	115	Negative
Nielsen et al., 1999	CRC	Hematoxylin- and eosin	Peritumoral	584	Positive
Nagtegaal ID et al., 2001	CRC	Elastase	Peritumoral and Intratumoral	160	Positive (Peritumoral)
Klintrup K et al., 2005	CRC	Hematoxylin- and eosin	Peritumoral and Intratumoral	386	Positive (Peritumoral)
Baeten C.I. et al., 2006	CRC	CD16	Peritumoral and Intratumoral	117	Positive (Intratumoral)
Rao H-L. et al., 2012	CRC	CD66b	Intratumoral	229	Negative
Richards CH. et al., 2012	CRC	Hematoxylin and eosin	Peritumoral	130	NO
Droeser et al., 2013	CRC	MPO, CD15	Intratumoral	1491	Positive

Table 4. Classification of colorectal cancer according to local invasion depth (T), lymph node involvement (N) and presence of distant metastases (M)

Definition	
T stage	
Tx	No information about local tumor infiltration available
Tis	Tumor restricted to mucosa, no infiltration of lamina muscularis mucosa
T1	Infiltration through lamina muscularis mucosa into submucosa, no infiltration of lamina muscularis propria
T2	Infiltration into, but not beyond, lamina muscularis propria
T3	Infiltration into subsierosa or non-peritonealised pericolic or perirectal tissue or both; no infiltration of serosa or neighbouring organs
T4a	Infiltration of the serosa
T4b	Infiltration of the neighbouring tissues or organs
N stage	
Nx	No information about lymph node involvement available
N0	No lymph node involvement
N1a	Cancer cells detectable in 1 regional lymph node
N1b	Cancer cells detectable in 2-3 regional lymph nodes
N1c	Tumor satellites in subserosa or pericolic or perirectal fat tissue, regional lymph nodes not involved
N2a	Cancer cells detectable in 4-6 regional lymph nodes
N2b	Cancer cells detectable in 7 or greater regional lymph nodes
M stage	
Mx	No information about distant metastases
M0	No distant metastases detectable
M1a	Metastases to 1 distant organ or distant lymph node
M1b	Metastases to more than 1 distant organ or set of distant lymph nodes or peritoneal metastases

Adapted from Brenner et al., 2014

Table 5. International Union for Cancer Control (UICC) and the American Joint Committee for Cancer (AJCC) TNM classification of CRC

	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1-T2	N0	M0
Stage II	T3-T4	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
Stage III	Any	N+	M0
IIIA	T1-T2	N1	M0
	T1	N2a	M0
IIIB	T3-T4a	N1	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
Stage IV	Any	Any	M+
IVA	Any	Any	M1a
IVB	Any	Any	M1b

Adapted from Brenner et al., 2014

Table 6. Correlation between the patient demographics and clinicopathologic features and CD66b+ IRA (IM and IT).

	N	CD66b+ IRA IM		CD66b+ IRA IT	
		Median value (IQR)	P value*	Median value (IQR)	P value *
Age at diagnosis (years)					
≤ 65	66	0.27 (0.52-0.07)	...	0.11 (0.36-0.03)	...
> 65	62	0.15 (0.70-0.06)	0.73	0.11 (0.42-0.02)	0.47
Sex					
Male	74	0.18 (0.60-0.07)	...	0.13 (0.35-0.03)	...
Female	54	0.15 (0.58-0.03)	0.92	0.08 (0.42-0.01)	0.30
Microsatellite status					
MSI	8	0.16 (1.28-0.11)	...	0.21 (0.49-0.04)	...
MSS	120	0.18 (0.57-0.06)	0.35	0.10 (0.36-0.02)	0.70
Anatomical site					
Colon	99	0.17 (0.58-0.06)	...	0.1 (0.36-0.02)	...
Rectum	29	0.2 (0.66-0.07)	0.70	0.2 (0.42-0.04)	0.19
Clinical Stage					
Stage I	19	0.39 (0.65-0.07)	...	0.08 (0.42-0.04)	...
Stage II	43	0.16 (0.36-0.08)	0.38	0.13 (0.36-0.01)	0.85
Stage III	35	0.39 (1.31-0.08)	0.97	0.24 (0.46-0.05)	0.77
Stage IV	31	0.07 (0.35-0.03)	0.15	0.08 (0.33-0.01)	0.35
Grade					
G1-G2	110	0.17 (0.54-0.06)	...	0.1 (0.38-0.02)	...
G3	18	0.25 (1.22-0.06)	0.64	0.2 (0.46-0.01)	0.72
Tumor Cell Type					
Adenocarcinoma	119	0.20 (0.58-0.06)	...	0.13 (0.37-0.02)	...
Variants	9	0.11 (0.81-0.06)	0.60	0.08 (0.46-0.03)	0.78
Vascular Invasion					
No	94	0,29 (0,92-0,07)	...	0.13 (0.46-0.02)	...
Yes	34	0,095 (0,35-0,03)	0.06	0.10 (0.27-0.01)	0.26

* Linear regression analysis: % CD66b+IRA was entered as a dependent, continuous variable. Abbreviation: IRA, Immunoreactive area; IM, invasive margin; IT, intratumoral compartment; IQR, interquartile range

Table 7. Univariate and multivariate analysis for DSS and DFS in patients with colorectal cancer

	DSS					DFS			
	Univariate analysis			Multivariate analysis		Univariate analysis		Multivariate analysis	
	N	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at diagnosis (years)^(a)	65.2±12.5	0.98 (0.96-1.00)	0.12			0.99 (0.97-1.01)	0.71		
Sex									
Male	74	1.00 ref				1.00 ref			
Female	54	0.87 (0.53-1.45)	0.60			0.92 (0.56-1.52)	0.75		
Microsatellite status									
MSS	120	1.00 ref	0.05*	NA	NA	1.00 ref	0.01*	NA	NA
MSI	8	0.10				0.06			
Anatomical location									
Colon	99	1.00 ref				1.00 ref			
Rectum	29	1.04 (0.58-1.86)	0.90			0.97 (0.54-1.73)	0.91		
Clinical Stage									
I	19	1.00 ref		1.00 ref		1.00 ref		1.00 ref	
II	43					2.94 (0.63-13.63)			
III	35	3.85 (2.76-5.37)	<0.001	4.55 (2.84-7.28)	<0.0001	8.52 (1.97-36.93)	0.004	2.80 (2.03-3.86)	<0.0001
IV	31					29.18 (6.96-122.34)			
Tumor Grade									
G1-G2	110	1.00 ref				1.00 ref			
G3	18	1.41 (0.72-2.77)	0.32			1.26 (0.64-2.48)	0.50		
Tumor cell type									
Adenocarcinoma	119	1.00 ref				1.00 ref			
Variants	9	1.14 (0.49-2.68)	0.75			1.28 (0.55-2.98)	0.56		
Vascular Invasion									
No	94	1.00 ref		1.00 ref		1.00 ref			
Yes	34	3.27 (1.98-5.4)	<0.001	2.76 (1.47-5.16)	0.001	3.22 (1.95-5.33)	<0.001		
CD66b+ IRA IM									
Low	65	1.00 ref				1.00 ref			
High	63	0.37 (0.19-0.72)	0.003			0.55 (0.32-0.94)	0.030		
CD66b+IRA IT									
Low	63	1.00 ref		1.00 ref		1.00 ref		1.00 ref	
High	65	0.47 (0.25-0.87)	0.002	0.47 (0.25-0.91)	0.03	0.56 (0.32-0.96)	0.03	0.58 (0.33-1.00)	0.05

(a) Age entered as a continuous variable. Abbreviation: IRA, Immunoreactive area; IM, invasive margin; IT, intratumoral compartment; HR: Hazard Ratio; CI, confidence interval

Table 8. Univariate and multivariate analysis of different prognostic factors in 178 Stage III MSS CRC patients

	Univariate analysis			Multivariate analysis	
	N	HR (95% CI)	P value	HR (95% CI)	P value
Age at surgery (years) 178					
< 68	97	1.00 ref			
>=68	81	1.69 (1.02-2.78)	0.04		
Sex					
Male	108	1.00 ref			
Female	70	1.04 (0.62-1.73)	0.87		
Anatomical location					
Colon DX	50	1.00 ref			
Colon SX	75	0.68 (0.36-1.26)	0.22		
Rectum	53	1.09 (0.59-2.02)	0.77		
Tumor Grade					
G1-G2	144	1.00 ref		1.00 ref	
G3	34	2.35 (1.36-4.04)	0.002	2.12 (1.21-3.75)	0.009
Tumor cell type					
Adenocarcinoma	165	1.00 ref			
Variants	13	1.76 (0.80-3.86)	0.16		
Vascular Invasion					
No	123	1.00 ref		1.00 ref	
Yes	55	2.48 (1.50-4.09)	0.0004	1.83 (1.07-3.12)	0.003
Nodal Status					
N1	115	1.00 ref		1.00 ref	
N2	63	2.55 (1.54-4.20)	0.0002	1.96 (1.16-3.30)	0.01
Local Invasion					
T3	153	1.00 ref			
T4	25	1.2 (0.61-2.36)	0.59		
5-FU					
NO	52	1.00 ref			
YES	126	0.61 (0.36-1.02)	0.06		
CD66b+IRA IT					
Low	131	1.00 ref			
High	47	0.61 (0.32-1.15)	0.12		

***INTERACTION between 5-FU and CD66b P=0.009**; HR= 0.17; 95% C.I. 0.05-0.65; † Mean ± Std. Abbreviation: IRA, Immunoreactive area IM, invasive margin; IT, intratumoral compartment

REFERENCES

- Abi Abdallah, D.S., Egan, C.E., Butcher, B.A., and Denkers, E.Y. (2011). Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. *International immunology* 23, 317-326.
- Affara, N.I., Ruffell, B., Medler, T.R., Gunderson, A.J., Johansson, M., Bornstein, S., Bergsland, E., Steinhoff, M., Li, Y., Gong, Q., *et al.* (2014). B cells regulate macrophage phenotype and response to chemotherapy in squamous carcinomas. *Cancer cell* 25, 809-821.
- Ai, S., Cheng, X.W., Inoue, A., Nakamura, K., Okumura, K., Iguchi, A., Murohara, T., and Kuzuya, M. (2007). Angiogenic activity of bFGF and VEGF suppressed by proteolytic cleavage by neutrophil elastase. *Biochemical and biophysical research communications* 364, 395-401.
- Akishima-Fukasawa, Y., Ishikawa, Y., Akasaka, Y., Uzuki, M., Inomata, N., Yokoo, T., Ishii, R., Shimokawa, R., Mukai, K., Kiguchi, H., *et al.* (2011). Histopathological predictors of regional lymph node metastasis at the invasive front in early colorectal cancer. *Histopathology* 59, 470-481.
- Alberts, S.R., Sargent, D.J., Nair, S., Mahoney, M.R., Mooney, M., Thibodeau, S.N., Smyrk, T.C., Sinicrope, F.A., Chan, E., Gill, S., *et al.* (2012). Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial. *JAMA : the journal of the American Medical Association* 307, 1383-1393.
- Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2008). The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunological reviews* 222, 155-161.
- Altman, D.G., McShane, L.M., Sauerbrei, W., and Taube, S.E. (2012). Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK): explanation and elaboration. *PLoS medicine* 9, e1001216.
- Alves-Filho, J.C., Freitas, A., Souto, F.O., Spiller, F., Paula-Neto, H., Silva, J.S., Gazzinelli, R.T., Teixeira, M.M., Ferreira, S.H., and Cunha, F.Q. (2009). Regulation of chemokine receptor by Toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis. *Proceedings of the National Academy of Sciences of the United States of America* 106, 4018-4023.
- Alves-Filho, J.C., Sonogo, F., Souto, F.O., Freitas, A., Verri, W.A., Jr., Auxiliadora-Martins, M., Basile-Filho, A., McKenzie, A.N., Xu, D., Cunha, F.Q., *et al.* (2010). Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nature medicine* 16, 708-712.
- Amado, R.G., Wolf, M., Peeters, M., Van Cutsem, E., Siena, S., Freeman, D.J., Juan, T., Sikorski, R., Suggs, S., Radinsky, R., *et al.* (2008). Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 26, 1626-1634.
- Ammirante, M., Luo, J.L., Grivnenkov, S., Nedospasov, S., and Karin, M. (2010). B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature* 464, 302-305.
- 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.
- Andersson, H., Andersson, B., Eklund, D., Ngoh, E., Persson, A., Svensson, K., Lerm, M., Blomgran, R., and Stendahl, O. (2014). Apoptotic neutrophils augment the inflammatory response to *Mycobacterium tuberculosis* infection in human macrophages. *PloS one* 9, e101514.
- Andre, T., Boni, C., Navarro, M., Tabernero, J., Hickish, T., Topham, C., Bonetti, A., Clingan, P., Bridgewater, J., Rivera, F., *et al.* (2009). Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27, 3109-3116.
- Andreu, P., Johansson, M., Affara, N.I., Pucci, F., Tan, T., Junankar, S., Korets, L., Lam, J., Tawfik, D., DeNardo, D.G., *et al.* (2010). FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer cell* 17, 121-134.

- Anitei, M.G., Zeitoun, G., Mlecnik, B., Marliot, F., Haicheur, N., Todosi, A.M., Kirilovsky, A., Lagorce, C., Bindea, G., Ferariu, D., *et al.* (2014a). Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 20, 1891-1899.
- Anitei, M.G., Zeitoun, G., Mlecnik, B., Marliot, F., Haicheur, N., Todosi, A.M., Kirilovsky, A., Lagorce, C., Bindea, G., Ferariu, D., *et al.* (2014b). Prognostic and predictive values of the immunoscore in patients with rectal cancer. *Clinical Cancer Research* 20, 1891-1899.
- Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., *et al.* (2007). Phenotypic and functional features of human Th17 cells. *The Journal of experimental medicine* 204, 1849-1861.
- Anuradha, R., George, P.J., Hanna, L.E., Chandrasekaran, V., Kumaran, P., Nutman, T.B., and Babu, S. (2013). IL-4-, TGF-beta-, and IL-1-dependent expansion of parasite antigen-specific Th9 cells is associated with clinical pathology in human lymphatic filariasis. *J Immunol* 191, 2466-2473.
- Araki, H., Katayama, N., Yamashita, Y., Mano, H., Fujieda, A., Usui, E., Mitani, H., Ohishi, K., Nishii, K., Masuya, M., *et al.* (2004). Reprogramming of human postmitotic neutrophils into macrophages by growth factors. *Blood* 103, 2973-2980.
- Ardi, V.C., Kupriyanova, T.A., Deryugina, E.I., and Quigley, J.P. (2007). Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 104, 20262-20267.
- Ariel, A., Fredman, G., Sun, Y.P., Kantarci, A., Van Dyke, T.E., Luster, A.D., and Serhan, C.N. (2006). Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression. *Nature immunology* 7, 1209-1216.
- Arita, M., Ohira, T., Sun, Y.P., Elangovan, S., Chiang, N., and Serhan, C.N. (2007). Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J Immunol* 178, 3912-3917.
- Arnaud, J.P., Adloff, M., Schloegel, M., Haegele, P., and Grobde, J.C. (1989). [Progress in evaluating lymphatic spread in cancer of rectum: endoscopic rectal lymphoscintigraphy]. *Journal de chirurgie* 126, 179-182.
- Arnold, D., and Seufferlein, T. (2010). Targeted treatments in colorectal cancer: state of the art and future perspectives. *Gut* 59, 838-858.
- Aruga, A., Aruga, E., Tanigawa, K., Bishop, D.K., Sondak, V.K., and Chang, A.E. (1997). Type 1 versus type 2 cytokine release by Vbeta T cell subpopulations determines in vivo antitumor reactivity: IL-10 mediates a suppressive role. *J Immunol* 159, 664-673.
- Aspord, C., Pedroza-Gonzalez, A., Gallegos, M., Tindle, S., Burton, E.C., Su, D., Marches, F., Banchereau, J., and Palucka, A.K. (2007). Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. *The Journal of experimental medicine* 204, 1037-1047.
- Assi, L.K., Wong, S.H., Ludwig, A., Raza, K., Gordon, C., Salmon, M., Lord, J.M., and Scheel-Toellner, D. (2007). Tumor necrosis factor alpha activates release of B lymphocyte stimulator by neutrophils infiltrating the rheumatoid joint. *Arthritis and rheumatism* 56, 1776-1786.
- Atreya, I., and Neurath, M.F. (2008). Immune cells in colorectal cancer: prognostic relevance and therapeutic strategies. *Expert review of anticancer therapy* 8, 561-572.
- Aust, D.E., Terdiman, J.P., Willenbacher, R.F., Chang, C.G., Molinaro-Clark, A., Baretton, G.B., Loehrs, U., and Waldman, F.M. (2002). The APC/beta-catenin pathway in ulcerative colitis-related colorectal carcinomas: a mutational analysis. *Cancer* 94, 1421-1427.
- Baeten, C.I., Castermans, K., Hillen, H.F., and Griffioen, A.W. (2006). Proliferating endothelial cells and leukocyte infiltration as prognostic markers in colorectal cancer. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 4, 1351-1357.

- Baggiolini, M. (1972). The enzymes of the granules of polymorphonuclear leukocytes and their functions. *Enzyme* 13, 132-160.
- Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nature reviews Cancer* 9, 361-371.
- Balkwill, F., Charles, K.A., and Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer cell* 7, 211-217.
- Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? *Lancet* 357, 539-545.
- Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. *Nature* 392, 245-252.
- Bao, Y., and Cao, X. (2014). The immune potential and immunopathology of cytokine-producing B cell subsets: a comprehensive review. *Journal of autoimmunity* 55, 10-23.
- Barrasa Shaw, A., Lopez-Guerrero, J.A., Calatrava Fons, A., Garcia-Casado, Z., Alapont Olavarrieta, V., Campos Manez, J., and Vazquez Albaladejo, C. (2009). Value of the identification of microsatellite instability in colorectal cancer. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* 11, 465-469.
- Battaglia, M. (2014). Neutrophils and type 1 autoimmune diabetes. *Current opinion in hematology* 21, 8-15.
- Beauvillain, C., Cunin, P., Doni, A., Scotet, M., Jaillon, S., Loiry, M.L., Magistrelli, G., Masternak, K., Chevaller, A., Delneste, Y., *et al.* (2011). CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood* 117, 1196-1204.
- Beauvillain, C., Delneste, Y., Scotet, M., Peres, A., Gascan, H., Guermonprez, P., Barnaba, V., and Jeannin, P. (2007). Neutrophils efficiently cross-prime naive T cells in vivo. *Blood* 110, 2965-2973.
- Becker, C., Fantini, M.C., Schramm, C., Lehr, H.A., Wirtz, S., Nikolaev, A., Burg, J., Strand, S., Kiesslich, R., Huber, S., *et al.* (2004). TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 21, 491-501.
- 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.
- Benatti, P., Gafa, R., Barana, D., Marino, M., Scarselli, A., Pedroni, M., Maestri, I., Guerzoni, L., Roncucci, L., Menigatti, M., *et al.* (2005). Microsatellite instability and colorectal cancer prognosis. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11, 8332-8340.
- Bennouna, S., Bliss, S.K., Curiel, T.J., and Denkers, E.Y. (2003). Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol* 171, 6052-6058.
- Bertagnolli, M.M., Niedzwiecki, D., Compton, C.C., Hahn, H.P., Hall, M., Damas, B., Jewell, S.D., Mayer, R.J., Goldberg, R.M., Saltz, L.B., *et al.* (2009). Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27, 1814-1821.
- Bhatnagar, N., Hong, H.S., Krishnaswamy, J.K., Haghikia, A., Behrens, G.M., Schmidt, R.E., and Jacobs, R. (2010). Cytokine-activated NK cells inhibit PMN apoptosis and preserve their functional capacity. *Blood* 116, 1308-1316.
- 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.

- Bindea, G., Mlecnik, B., Tosolini, M., Kirilovsky, A., Waldner, M., Obenauf, A.C., Angell, H., Fredriksen, T., Lafontaine, L., Berger, A., *et al.* (2013). Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* *39*, 782-795.
- Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature immunology* *11*, 889-896.
- Blankenstein, T., Coulie, P.G., Gilboa, E., and Jaffee, E.M. (2012). The determinants of tumour immunogenicity. *Nature reviews Cancer* *12*, 307-313.
- Bollrath, J., and Greten, F.R. (2009). IKK/NF-kappaB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. *EMBO reports* *10*, 1314-1319.
- Bonavita, E., Gentile, S., Rubino, M., Maina, V., Papait, R., Kunderfranco, P., Greco, C., Feruglio, F., Molgora, M., Laface, I., *et al.* (2015). PTX3 Is an Extrinsic Oncosuppressor Regulating Complement-Dependent Inflammation in Cancer. *Cell* *160*, 700-714.
- 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 (New York, NY)* *237*, 1204-1206.
- Borregaard, N., Sorensen, O.E., and Theilgaard-Monch, K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends in immunology* *28*, 340-345.
- Bosetti, C., Rosato, V., Gallus, S., Cuzick, J., and La Vecchia, C. (2012). Aspirin and cancer risk: a quantitative review to 2011. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* *23*, 1403-1415.
- Bourke, E., Cassetti, A., Villa, A., Fadlon, E., Colotta, F., and Mantovani, A. (2003). IL-1 beta scavenging by the type II IL-1 decoy receptor in human neutrophils. *J Immunol* *170*, 5999-6005.
- Bournazou, I., Pound, J.D., Duffin, R., Bournazos, S., Melville, L.A., Brown, S.B., Rossi, A.G., and Gregory, C.D. (2009). Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *The Journal of clinical investigation* *119*, 20-32.
- Boyle, T., Keegel, T., Bull, F., Heyworth, J., and Fritschi, L. (2012). Physical activity and risks of proximal and distal colon cancers: a systematic review and meta-analysis. *Journal of the National Cancer Institute* *104*, 1548-1561.
- Braber, S., Thio, M., Blokhuis, B.R., Henricks, P.A., Koelink, P.J., Groot Kormelink, T., Bezemer, G.F., Kerstjens, H.A., Postma, D.S., Garssen, J., *et al.* (2012). An association between neutrophils and immunoglobulin free light chains in the pathogenesis of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* *185*, 817-824.
- Braian, C., Hoge, V., and Stendahl, O. (2013). Mycobacterium tuberculosis- induced neutrophil extracellular traps activate human macrophages. *Journal of innate immunity* *5*, 591-602.
- Braig, M., and Schmitt, C.A. (2006). Oncogene-induced senescence: putting the brakes on tumor development. *Cancer research* *66*, 2881-2884.
- 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. *Nature immunology* *15*, 1017-1025.

- Brenner, H., Chang-Claude, J., Seiler, C.M., Rickert, A., and Hoffmeister, M. (2011). Protection from colorectal cancer after colonoscopy: a population-based, case-control study. *Annals of internal medicine* *154*, 22-30.
- Brenner, H., Kloor, M., and Pox, C.P. (2014). Colorectal cancer. *Lancet* *383*, 1490-1502.
- 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 (New York, NY)* *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.
- Bromberg, J., and Wang, T.C. (2009). Inflammation and cancer: IL-6 and STAT3 complete the link. *Cancer cell* *15*, 79-80.
- 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.
- Buckley, C.D., Gilroy, D.W., Serhan, C.N., Stockinger, B., and Tak, P.P. (2013). The resolution of inflammation. *Nature reviews Immunology* *13*, 59-66.
- Bui, J.D., and Schreiber, R.D. (2007). Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Current opinion in immunology* *19*, 203-208.
- Burnet, M. (1957). Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. *British medical journal* *1*, 841-847.
- Burzyn, D., Benoist, C., and Mathis, D. (2013). Regulatory T cells in nonlymphoid tissues. *Nature immunology* *14*, 1007-1013.
- Campbell, E.L., Bruyninckx, W.J., Kelly, C.J., Glover, L.E., McNamee, E.N., Bowers, B.E., Bayless, A.J., Scully, M., Saeedi, B.J., Golden-Mason, L., *et al.* (2014). Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. *Immunity* *40*, 66-77.
- Campbell, M.J., Tonlaar, N.Y., Garwood, E.R., Huo, D., Moore, D.H., Khramtsov, A.I., Au, A., Baehner, F., Chen, Y., Malaka, D.O., *et al.* (2011). Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast cancer research and treatment* *128*, 703-711.
- Carbone, E., Neri, P., Mesuraca, M., Fulciniti, M.T., Otsuki, T., Pende, D., Groh, V., Spies, T., Pollio, G., Cosman, D., *et al.* (2005). HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* *105*, 251-258.
- Caricato, M., Borzomati, D., Ausania, F., Valeri, S., Rosignoli, A., and Coppola, R. (2006). Prognostic factors after surgery for locally recurrent rectal cancer: an overview. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* *32*, 126-132.
- Caruso, R.A., Bellocco, R., Pagano, M., Bertoli, G., Rigoli, L., and Inferrera, C. (2002). Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in northern Italy. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* *15*, 831-837.
- 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.
- Casbon, A.J., Reynaud, D., Park, C., Khuc, E., Gan, D.D., Schepers, K., Passegue, E., and Werb, Z. (2015). Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* *112*, E566-575.

- Cassatella, M.A. (2006). On the production of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) by human neutrophils. *Journal of leukocyte biology* *79*, 1140-1149.
- Cassatella, M.A., Meda, L., Gasperini, S., Calzetti, F., and Bonora, S. (1994). Interleukin 10 (IL-10) upregulates IL-1 receptor antagonist production from lipopolysaccharide-stimulated human polymorphonuclear leukocytes by delaying mRNA degradation. *The Journal of experimental medicine* *179*, 1695-1699.
- Castriconi, R., Dondero, A., Corrias, M.V., Lanino, E., Pende, D., Moretta, L., Bottino, C., and Moretta, A. (2004). Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer research* *64*, 9180-9184.
- Cavaillon, J.M., and Adib-Conquy, M. (2006). Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Critical care* *10*, 233.
- Center, M.M., Jemal, A., and Ward, E. (2009). International trends in colorectal cancer incidence rates. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* *18*, 1688-1694.
- Cerottini, J.P., Caplin, S., Saraga, E., Givel, J.C., and Benhattar, J. (1998). The type of K-ras mutation determines prognosis in colorectal cancer. *American journal of surgery* *175*, 198-202.
- Chacon-Salinas, R., Serafin-Lopez, J., Ramos-Payan, R., Mendez-Aragon, P., Hernandez-Pando, R., Van Soelingen, D., Flores-Romo, L., Estrada-Parra, S., and Estrada-Garcia, I. (2005). Differential pattern of cytokine expression by macrophages infected in vitro with different *Mycobacterium tuberculosis* genotypes. *Clinical and experimental immunology* *140*, 443-449.
- Chakravarti, A., Raquil, M.A., Tessier, P., and Poubelle, P.E. (2009). Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. *Blood* *114*, 1633-1644.
- Chan, D.S., Lau, R., Aune, D., Vieira, R., Greenwood, D.C., Kampman, E., and Norat, T. (2011). Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. *PloS one* *6*, e20456.
- Chang, E.Y., Dorsey, P.B., Johnson, N., Lee, R., Walts, D., Johnson, W., Anadiotis, G., Kiser, K., and Frankhouse, J. (2006). A prospective analysis of microsatellite instability as a molecular marker in colorectal cancer. *American journal of surgery* *191*, 646-651.
- Chang, H.C., Sehra, S., Goswami, R., Yao, W., Yu, Q., Stritesky, G.L., Jabeen, R., McKinley, C., Ahyi, A.N., Han, L., *et al.* (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nature immunology* *11*, 527-534.
- Charles, K.A., Kulbe, H., Soper, R., Escorcio-Correia, M., Lawrence, T., Schultheis, A., Chakravarty, P., Thompson, R.G., Kollias, G., Smyth, J.F., *et al.* (2009). The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *The Journal of clinical investigation* *119*, 3011-3023.
- Cheadle, J.P., and Sampson, J.R. (2003). Exposing the MYth about base excision repair and human inherited disease. *Human molecular genetics* *12 Spec No 2*, R159-165.
- Chen, Q.M., Liu, J., and Merrett, J.B. (2000). Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts. *The Biochemical journal* *347*, 543-551.
- Chen, W., Masterman, K.A., Basta, S., Haeryfar, S.M., Dimopoulos, N., Knowles, B., Bennink, J.R., and Yewdell, J.W. (2004). Cross-priming of CD8+ T cells by viral and tumor antigens is a robust phenomenon. *European journal of immunology* *34*, 194-199.
- Chiang, N., Fredman, G., Backhed, F., Oh, S.F., Vickery, T., Schmidt, B.A., and Serhan, C.N. (2012). Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature* *484*, 524-528.
- Cho, J.H., Fraser, I.P., Fukase, K., Kusumoto, S., Fujimoto, Y., Stahl, G.L., and Ezekowitz, R.A. (2005). Human peptidoglycan recognition protein S is an effector of neutrophil-mediated innate immunity. *Blood* *106*, 2551-2558.

- Chung, A.S., Wu, X., Zhuang, G., Ngu, H., Kasman, I., Zhang, J., Vernes, J.M., Jiang, Z., Meng, Y.G., Peale, F.V., *et al.* (2013). An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. *Nature medicine* *19*, 1114-1123.
- Colotta, F., Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* *30*, 1073-1081.
- 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*.
- Cortez-Retamozo, V., Etzrodt, M., Newton, A., Rauch, P.J., Chudnovskiy, A., Berger, C., Ryan, R.J., Iwamoto, Y., Marinelli, B., Gorbato, R., *et al.* (2012). Origins of tumor-associated macrophages and neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* *109*, 2491-2496.
- Cortez-Retamozo, V., Etzrodt, M., Newton, A., Ryan, R., Pucci, F., Sio, S.W., Kuswanto, W., Rauch, P.J., Chudnovskiy, A., Iwamoto, Y., *et al.* (2013). Angiotensin II Drives the Production of Tumor-Promoting Macrophages. *Immunity* *38*, 296-308.
- Corti, A., Franzini, M., Cianchetti, S., Bergamini, G., Lorenzini, E., Melotti, P., Paolicchi, A., Paggiaro, P., and Pompella, A. (2012). Contribution by polymorphonuclear granulocytes to elevated gamma-glutamyltransferase in cystic fibrosis sputum. *PloS one* *7*, e34772.
- Costantini, C., Calzetti, F., Perbellini, O., Micheletti, A., Scarponi, C., Lonardi, S., Pelletier, M., Schakel, K., Pizzolo, G., Facchetti, F., *et al.* (2011). Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NK-derived IFN{gamma}: role of CD18, ICAM-1, and ICAM-3. *Blood* *117*, 1677-1686.
- Costantini, C., and Cassatella, M.A. (2011). The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity. *Journal of leukocyte biology* *89*, 221-233.
- Cross, A., Moots, R.J., and Edwards, S.W. (2008). The dual effects of TNFalpha on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. *Blood* *111*, 878-884.
- Crotty, S. (2011). Follicular helper CD4 T cells (TFH). *Annual review of immunology* *29*, 621-663.
- Dalli, J., and Serhan, C.N. (2012). Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood* *120*, e60-72.
- Daurkin, I., Eruslanov, E., Stoffs, T., Perrin, G.Q., Algood, C., Gilbert, S.M., Rosser, C.J., Su, L.M., Vieweg, J., and Kusmartsev, S. (2011). Tumor-associated macrophages mediate immunosuppression in the renal cancer microenvironment by activating the 15-lipoxygenase-2 pathway. *Cancer research* *71*, 6400-6409.
- Davey, M.S., Lin, C.Y., Roberts, G.W., Heuston, S., Brown, A.C., Chess, J.A., Toleman, M.A., Gahan, C.G., Hill, C., Parish, T., *et al.* (2011a). Human neutrophil clearance of bacterial pathogens triggers anti-microbial gammadelta T cell responses in early infection. *PLoS pathogens* *7*, e1002040.
- Davey, M.S., Tamassia, N., Rossato, M., Bazzoni, F., Calzetti, F., Bruderek, K., Sironi, M., Zimmer, L., Bottazzi, B., Mantovani, A., *et al.* (2011b). Failure to detect production of IL-10 by activated human neutrophils. *Nature immunology* *12*, 1017-1018; author reply 1018-1020.
- Davies, L.C., Rosas, M., Smith, P.J., Fraser, D.J., Jones, S.A., and Taylor, P.R. (2011). A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *European journal of immunology* *41*, 2155-2164.
- de Gramont, A., Van Cutsem, E., Schmoll, H.J., Taberero, J., Clarke, S., Moore, M.J., Cunningham, D., Cartwright, T.H., Hecht, J.R., Rivera, F., *et al.* (2012). Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (AVANT): a phase 3 randomised controlled trial. *The lancet oncology* *13*, 1225-1233.

- De Larco, J.E., Wuertz, B.R., and Furcht, L.T. (2004). The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. *Clinical cancer research : an official journal of the American Association for Cancer Research* *10*, 4895-4900.
- De Monte, L., Reni, M., Tassi, E., Clavenna, D., Papa, I., Recalde, H., Braga, M., Di Carlo, V., Doglioni, C., and Protti, M.P. (2011). Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. *The Journal of experimental medicine* *208*, 469-478.
- 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. *Nature immunology* *11*, 1039-1046.
- de Visser, K.E., Korets, L.V., and Coussens, L.M. (2005). De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer cell* *7*, 411-423.
- Deban, L., Russo, R.C., Sironi, M., Moalli, F., Scanziani, M., Zambelli, V., Cuccovillo, I., Bastone, A., Gobbi, M., Valentino, S., *et al.* (2010). Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nature immunology* *11*, 328-334.
- Demetri, G.D., and Griffin, J.D. (1991). Granulocyte colony-stimulating factor and its receptor. *Blood* *78*, 2791-2808.
- DeNardo, D.G., Andreu, P., and Coussens, L.M. (2010). Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer metastasis reviews* *29*, 309-316.
- DeNardo, D.G., Barreto, J.B., Andreu, P., Vasquez, L., Tawfik, D., Kolhatkar, N., and Coussens, L.M. (2009). CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer cell* *16*, 91-102.
- Di Caro, G., Bergomas, F., Grizzi, F., Doni, A., Bianchi, P., Malesci, A., Laghi, L., Allavena, P., Mantovani, A., and Marchesi, F. (2014). Occurrence of tertiary lymphoid tissue is associated with T-cell infiltration and predicts better prognosis in early-stage colorectal cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research* *20*, 2147-2158.
- Di Caro, G., Marchesi, F., Laghi, L., and Grizzi, F. (2013). Immune cells: plastic players along colorectal cancer progression. *Journal of cellular and molecular medicine* *17*, 1088-1095.
- Di Nicolantonio, F., Martini, M., Molinari, F., Sartore-Bianchi, A., Arena, S., Saletti, P., De Dosso, S., Mazzucchelli, L., Frattini, M., Siena, S., *et al.* (2008). Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *26*, 5705-5712.
- Donskov, F. (2013). Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *Seminars in cancer biology*.
- Droeser, R.A., Hirt, C., Eppenberger-Castori, S., Zlobec, I., Viehl, C.T., Frey, D.M., Nebiker, C.A., Rosso, R., Zuber, M., Amicarella, F., *et al.* (2013). High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. *PloS one* *8*, e64814.
- Dudas, J. (2015). Supportive and rejective functions of tumor stroma on tumor cell growth, survival, and invasivity: the cancer evolution. *Frontiers in oncology* *5*, 44.
- Dumitru, C.A., Bankfalvi, A., Gu, X., Eberhardt, W.E., Zeidler, R., Lang, S., and Brandau, S. (2013). Neutrophils Activate Tumoral CORTACTIN to Enhance Progression of Orohypharynx Carcinoma. *Frontiers in immunology* *4*, 33.
- Dumitru, C.A., Fechner, M.K., Hoffmann, T.K., Lang, S., and Brandau, S. (2012). A novel p38-MAPK signaling axis modulates neutrophil biology in head and neck cancer. *Journal of leukocyte biology* *91*, 591-598.
- Dumitru, C.A., Gholaman, H., Trellakis, S., Bruderek, K., Dominas, N., Gu, X., Bankfalvi, A., Whiteside, T.L., Lang, S., and Brandau, S. (2011). Tumor-derived macrophage migration inhibitory factor modulates the biology of head and neck cancer cells via neutrophil activation. *International journal of cancer Journal international du cancer* *129*, 859-869.

- Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., and Schreiber, R.D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology* 3, 991-998.
- Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England journal of medicine* 315, 1650-1659.
- Dyugovskaya, L., Berger, S., Polyakov, A., and Lavie, L. (2014). The development of giant phagocytes in long-term neutrophil cultures. *Journal of leukocyte biology* 96, 511-521.
- Eaden, J., Abrams, K., Ekbom, A., Jackson, E., and Mayberry, J. (2000). Colorectal cancer prevention in ulcerative colitis: a case-control study. *Alimentary pharmacology & therapeutics* 14, 145-153.
- 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.
- Edin, S., Wikberg, M.L., Oldenborg, P.A., and Palmqvist, R. (2013). Macrophages: Good guys in colorectal cancer. *Oncoimmunology* 2, e23038.
- Ehrlich, P. (1909). Ueber den jetzigen stand der Karzinomforschung. *Ned Trijdschr Geneesk* 5, 273-290.
- Eken, C., Gasser, O., Zenhausern, G., Oehri, I., Hess, C., and Schifferli, J.A. (2008). Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J Immunol* 180, 817-824.
- Engel, A.M., Svane, I.M., Mouritsen, S., Rygaard, J., Clausen, J., and Werdelin, O. (1996). Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 104, 629-639.
- Engel, A.M., Svane, I.M., Rygaard, J., and Werdelin, O. (1997). MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice. *Scandinavian journal of immunology* 45, 463-470.
- Erreni, M., Mantovani, A., and Allavena, P. (2011). Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* 4, 141-154.
- Eruslanov, E.B., Bhojnarwala, 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.
- Eyles, J.L., Roberts, A.W., Metcalf, D., and Wicks, I.P. (2006). Granulocyte colony-stimulating factor and neutrophils--forgotten mediators of inflammatory disease. *Nature clinical practice Rheumatology* 2, 500-510.
- Fallik, D., Borrini, F., Boige, V., Viguier, J., Jacob, S., Miquel, C., Sabourin, J.C., Ducreux, M., and Praz, F. (2003). Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer. *Cancer research* 63, 5738-5744.
- Fedirko, V., Tramacere, I., Bagnardi, V., Rota, M., Scotti, L., Islami, F., Negri, E., Straif, K., Romieu, I., La Vecchia, C., *et al.* (2011). Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 22, 1958-1972.
- Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C., and Parkin, D.M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer Journal international du cancer* 127, 2893-2917.
- Ferrone, S., and Marincola, F.M. (1995). Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunology today* 16, 487-494.

- Filardy, A.A., Pires, D.R., Nunes, M.P., Takiya, C.M., Freire-de-Lima, C.G., Ribeiro-Gomes, F.L., and DosReis, G.A. (2010). Proinflammatory clearance of apoptotic neutrophils induces an IL-12(low)IL-10(high) regulatory phenotype in macrophages. *J Immunol* *185*, 2044-2050.
- Filep, J.G., Zouki, C., Petasis, N.A., Hachicha, M., and Serhan, C.N. (1999). Anti-inflammatory actions of lipoxin A(4) stable analogs are demonstrable in human whole blood: modulation of leukocyte adhesion molecules and inhibition of neutrophil-endothelial interactions. *Blood* *94*, 4132-4142.
- Floriani, I., Torri, V., Rulli, E., Garavaglia, D., Compagnoni, A., Salvolini, L., and Giovagnoni, A. (2010). Performance of imaging modalities in diagnosis of liver metastases from colorectal cancer: a systematic review and meta-analysis. *Journal of magnetic resonance imaging : JMRI* *31*, 19-31.
- Forsell, J., Oberg, A., Henriksson, M.L., Stenling, R., Jung, A., and Palmqvist, R. (2007). High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* *13*, 1472-1479.
- Fossati, G., Ricevuti, G., Edwards, S.W., Walker, C., Dalton, A., and Rossi, M.L. (1999). Neutrophil infiltration into human gliomas. *Acta neuropathologica* *98*, 349-354.
- Fox, S., Leitch, A.E., Duffin, R., Haslett, C., and Rossi, A.G. (2010). Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *Journal of innate immunity* *2*, 216-227.
- 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.
- Fridlender, Z.G., Sun, J., Mishalian, I., Singhal, S., Cheng, G., Kapoor, V., Horng, W., Fridlender, G., Bayuh, R., Worthen, G.S., *et al.* (2012). Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PloS one* *7*, e31524.
- Fridman, W.H., Pages, F., Sautes-Fridman, C., and Galon, J. (2012). The immune contexture in human tumours: impact on clinical outcome. *Nature reviews Cancer* *12*, 298-306.
- 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., Wroblewski, S.K., Wakefield, T.W., Hartwig, J.H., and Wagner, D.D. (2010). Extracellular DNA traps promote thrombosis. *Proceedings of the National Academy of Sciences of the United States of America* *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.
- Gabrivovich, D.I., Ostrand-Rosenberg, S., and Bronte, V. (2012). Coordinated regulation of myeloid cells by tumours. *Nature reviews Immunology* *12*, 253-268.
- Gai, X.D., Song, Y., Li, C., Lei, Y.M., and Yang, B. (2013). Potential role of plasmacytoid dendritic cells for FOXP3+ regulatory T cell development in human colorectal cancer and tumor draining lymph node. *Pathology, research and practice* *209*, 774-778.
- Galdiero, M.R., Bonavita, E., Barajon, I., Garlanda, C., Mantovani, A., and Jaillon, S. (2013a). Tumor associated macrophages and neutrophils in cancer. *Immunobiology* *218*, 1402-1410.
- Galdiero, M.R., Garlanda, C., Jaillon, S., Marone, G., and Mantovani, A. (2013b). Tumor associated macrophages and neutrophils in tumor progression. *Journal of cellular physiology* *228*, 1404-1412.
- 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.

- Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M., Camus, M., Berger, A., Wind, P., *et al.* (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science (New York, NY)* *313*, 1960-1964.
- Galon, J., Fridman, W.H., and Pages, F. (2007). The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer research* *67*, 1883-1886.
- Galon, J., Mlecnik, B., Bindea, G., Angell, H.K., Berger, A., Lagorce, C., Lugli, A., Zlobec, I., Hartmann, A., Bifulco, C., *et al.* (2014). Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *The Journal of pathology* *232*, 199-209.
- Garcia-Romo, G.S., Caielli, S., Vega, B., Connolly, J., Allantaz, F., Xu, Z., Punaro, M., Baisch, J., Guiducci, C., Coffman, R.L., *et al.* (2011). Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Science translational medicine* *3*, 73ra20.
- Gerosa, F., Paganin, C., Peritt, D., Paiola, F., Scupoli, M.T., Aste-Amezaga, M., Frank, I., and Trinchieri, G. (1996). Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. *The Journal of experimental medicine* *183*, 2559-2569.
- Gill, S., Loprinzi, C.L., Sargent, D.J., Thome, S.D., Alberts, S.R., Haller, D.G., Benedetti, J., Francini, G., Shepherd, L.E., Francois Seitz, J., *et al.* (2004). Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: who benefits and by how much? *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *22*, 1797-1806.
- Glatman Zaretsky, A., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., and Pearce, E.J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *The Journal of experimental medicine* *206*, 991-999.
- Gocheva, V., Wang, H.W., Gadea, B.B., Shree, T., Hunter, K.E., Garfall, A.L., Berman, T., and Joyce, J.A. (2010). IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes & development* *24*, 241-255.
- Goss, K.H., and Groden, J. (2000). Biology of the adenomatous polyposis coli tumor suppressor. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *18*, 1967-1979.
- Grady, W.M., and Pritchard, C.C. (2014). Molecular alterations and biomarkers in colorectal cancer. *Toxicologic pathology* *42*, 124-139.
- Graham, L.M., Gupta, V., Schafer, G., Reid, D.M., Kimberg, M., Dennehy, K.M., Hornsell, W.G., Guler, R., Campanero-Rhodes, M.A., Palma, A.S., *et al.* (2012). The C-type lectin receptor CLECSF8 (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase. *The Journal of biological chemistry* *287*, 25964-25974.
- 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. *The Journal of experimental medicine* *207*, 923-931.
- Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* *118*, 285-296.
- Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, inflammation, and cancer. *Cell* *140*, 883-899.
- Grivennikov, S.I., Wang, K., Mucida, D., Stewart, C.A., Schnabl, B., Jauch, D., Taniguchi, K., Yu, G.Y., Osterreicher, C.H., Hung, K.E., *et al.* (2012). Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* *491*, 254-258.
- Grizzi, F., Bianchi, P., Malesci, A., and Laghi, L. (2013). Prognostic value of innate and adaptive immunity in colorectal cancer. *World journal of gastroenterology : WJG* *19*, 174-184.
- Grosse-Steffen, T., Giese, T., Giese, N., Longerich, T., Schirmacher, P., Hansch, G.M., and Gaida, M.M. (2012). Epithelial-to-mesenchymal transition in pancreatic ductal adenocarcinoma and

- pancreatic tumor cell lines: the role of neutrophils and neutrophil-derived elastase. *Clinical & developmental immunology* 2012, 720768.
- Guerder, S., Joncker, N., Mahiddine, K., and Serre, L. (2013). Dendritic cells in tolerance and autoimmune diabetes. *Current opinion in immunology* 25, 670-675.
- Gungor, N., Knaapen, A.M., Munnia, A., Peluso, M., Haenen, G.R., Chiu, R.K., Godschalk, R.W., and van Schooten, F.J. (2010). Genotoxic effects of neutrophils and hypochlorous acid. *Mutagenesis* 25, 149-154.
- Hagemann, T., Wilson, J., Kulbe, H., Li, N.F., Leinster, D.A., Charles, K., Klemm, F., Pukrop, T., Binder, C., and Balkwill, F.R. (2005). Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK. *J Immunol* 175, 1197-1205.
- 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. *Proceedings of the National Academy of Sciences of the United States of America* 107, 9813-9818.
- Halama, N., Michel, S., Kloor, M., Zoernig, I., Benner, A., Spille, A., Pommerencke, T., von Knebel, D.M., Folprecht, G., Lubber, B., *et al.* (2011). Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer research* 71, 5670-5677.
- Half, E., Bercovich, D., and Rozen, P. (2009). Familial adenomatous polyposis. *Orphanet journal of rare diseases* 4, 22.
- Haller, D.G., Tabernero, J., Maroun, J., de Braud, F., Price, T., Van Cutsem, E., Hill, M., Gilberg, F., Rittweger, K., and Schmoll, H.J. (2011). Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 29, 1465-1471.
- Halvorsen, E.C., Mahmoud, S.M., and Bennewith, K.L. (2014). Emerging roles of regulatory T cells in tumour progression and metastasis. *Cancer metastasis reviews* 33, 1025-1041.
- 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.
- Haringman, J.J., Gerlag, D.M., Zwinderman, A.H., Smeets, T.J., Kraan, M.C., Baeten, D., McInnes, I.B., Bresnihan, B., and Tak, P.P. (2005). Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Annals of the rheumatic diseases* 64, 834-838.
- Harris, D.P., Haynes, L., Sayles, P.C., Duso, D.K., Eaton, S.M., Lepak, N.M., Johnson, L.L., Swain, S.L., and Lund, F.E. (2000). Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nature immunology* 1, 475-482.
- Hayashi, F., Means, T.K., and Luster, A.D. (2003). Toll-like receptors stimulate human neutrophil function. *Blood* 102, 2660-2669.
- Heagerty, P.J., Lumley, T., and Pepe, M.S. (2000). Time-dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics* 56, 337-344.
- Heinemann, V., von Weikersthal, L.F., Decker, T., Kiani, A., Vehling-Kaiser, U., Al-Batran, S.E., Heintges, T., Lerchenmuller, C., Kahl, C., Seipelt, G., *et al.* (2014). FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *The lancet oncology* 15, 1065-1075.
- Heinimann, K. (2013). Toward a Molecular Classification of Colorectal Cancer: The Role of Microsatellite Instability Status. *Frontiers in oncology* 3, 272.
- Herbig, U., Jobling, W.A., Chen, B.P., Chen, D.J., and Sedivy, J.M. (2004). Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Molecular cell* 14, 501-513.

- Hewish, M., Lord, C.J., Martin, S.A., Cunningham, D., and Ashworth, A. (2010). Mismatch repair deficient colorectal cancer in the era of personalized treatment. *Nature reviews Clinical oncology* 7, 197-208.
- Himmel, M.E., Crome, S.Q., Ivison, S., Piccirillo, C., Steiner, T.S., and Levings, M.K. (2011). Human CD4+ FOXP3+ regulatory T cells produce CXCL8 and recruit neutrophils. *European journal of immunology* 41, 306-312.
- Hirt, C., Eppenberger-Castori, S., Sconocchia, G., Iezzi, G., Tornillo, L., Terracciano, L., Spagnoli, G.C., and Droeser, R.A. (2013). Colorectal carcinoma infiltration by myeloperoxidase-expressing neutrophil granulocytes is associated with favorable prognosis. *Oncoimmunology* 2, e25990.
- 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. *Nature medicine* 16, 219-223.
- <http://globocan.iarc.fr>.
- Huh, S.J., Liang, S., Sharma, A., Dong, C., and Robertson, G.P. (2010). Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. *Cancer research* 70, 6071-6082.
- Hurwitz, A.A., and Watkins, S.K. (2012). Immune suppression in the tumor microenvironment: a role for dendritic cell-mediated tolerization of T cells. *Cancer immunology, immunotherapy : CII* 61, 289-293.
- Hutter, C.M., Chang-Claude, J., Slattery, M.L., Pflugeisen, B.M., Lin, Y., Duggan, D., Nan, H., Lemire, M., Rangoj, J., Figueiredo, J.C., *et al.* (2012). Characterization of gene-environment interactions for colorectal cancer susceptibility loci. *Cancer research* 72, 2036-2044.
- Hwang, I., and Nguyen, N. (2015). Mechanisms of tumor-induced T cell immune suppression and therapeutics to counter those effects. *Archives of pharmacal research*.
- Ijichi, H., Chytil, A., Gorska, A.E., Aakre, M.E., Bierie, B., Tada, M., Mohri, D., Miyabayashi, K., Asaoka, Y., Maeda, S., *et al.* (2011). Inhibiting Cxcr2 disrupts tumor-stromal interactions and improves survival in a mouse model of pancreatic ductal adenocarcinoma. *The Journal of clinical investigation* 121, 4106-4117.
- Ilie, M., Hofman, V., Ortholan, C., Bonnetaud, C., Coelle, C., Mouroux, J., and Hofman, P. (2012). Predictive clinical outcome of the intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio in patients with resectable nonsmall cell lung cancer. *Cancer* 118, 1726-1737.
- Imai, Y., Kubota, Y., Yamamoto, S., Tsuji, K., Shimatani, M., Shibatani, N., Takamido, S., Matsushita, M., and Okazaki, K. (2005). Neutrophils enhance invasion activity of human cholangiocellular carcinoma and hepatocellular carcinoma cells: an in vitro study. *Journal of gastroenterology and hepatology* 20, 287-293.
- 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.
- Jacobson, N.G., Szabo, S.J., Weber-Nordt, R.M., Zhong, Z., Schreiber, R.D., Darnell, J.E., Jr., and Murphy, K.M. (1995). Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *The Journal of experimental medicine* 181, 1755-1762.
- Jaeger, B.N., Donadieu, J., Cognet, C., Bernat, C., Ordonez-Rueda, D., Barlogis, V., Mahlaoui, N., Fenis, A., Narni-Mancinelli, E., Beaupain, B., *et al.* (2012). Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. *The Journal of experimental medicine* 209, 565-580.
- 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., Jeannin, P., Hamon, Y., Fremaux, I., Doni, A., Bottazzi, B., Blanchard, S., Subra, J.F., Chevaller, A., Mantovani, A., *et al.* (2009). Endogenous PTX3 translocates at the membrane of late

- apoptotic human neutrophils and is involved in their engulfment by macrophages. *Cell death and differentiation* 16, 465-474.
- 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. *The Journal of experimental medicine* 204, 793-804.
- Jamieson, T., Clarke, M., Steele, C.W., Samuel, M.S., Neumann, J., Jung, A., Huels, D., Olson, M.F., Das, S., Nibbs, R.J., *et al.* (2012). Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. *The Journal of clinical investigation* 122, 3127-3144.
- Jasperson, K.W., Tuohy, T.M., Neklason, D.W., and Burt, R.W. (2010). Hereditary and familial colon cancer. *Gastroenterology* 138, 2044-2058.
- Jass, J.R. (1986). Lymphocytic infiltration and survival in rectal cancer. *Journal of clinical pathology* 39, 585-589.
- Jenkins, S.J., Ruckerl, D., Cook, P.C., Jones, L.H., Finkelman, F.D., van Rooijen, N., MacDonald, A.S., and Allen, J.E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science (New York, NY)* 332, 1284-1288.
- Jensen, H.K., Donskov, F., Marcussen, N., Nordmark, M., Lundbeck, F., and von der Maase, H. (2009). Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27, 4709-4717.
- Jensen, T.O., Schmidt, H., Moller, H.J., Donskov, F., Hoyer, M., Sjoegren, P., Christensen, I.J., and Steiniche, T. (2012). Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer* 118, 2476-2485.
- Jess, T., Rungoe, C., and Peyrin-Biroulet, L. (2012). Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 10, 639-645.
- Jiang, Y., Ben, Q., Shen, H., Lu, W., Zhang, Y., and Zhu, J. (2011). Diabetes mellitus and incidence and mortality of colorectal cancer: a systematic review and meta-analysis of cohort studies. *European journal of epidemiology* 26, 863-876.
- Jouanguy, E., Doffinger, R., Dupuis, S., Pallier, A., Altare, F., and Casanova, J.L. (1999). IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Current opinion in immunology* 11, 346-351.
- Joyce, J.A., and Pollard, J.W. (2009). Microenvironmental regulation of metastasis. *Nature reviews Cancer* 9, 239-252.
- Kaler, P., Galea, V., Augenlicht, L., and Klampfer, L. (2010). Tumor associated macrophages protect colon cancer cells from TRAIL-induced apoptosis through IL-1beta-dependent stabilization of Snail in tumor cells. *PloS one* 5, e11700.
- Kang, T.W., Yevesa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A., *et al.* (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* 479, 547-551.
- 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. *Proceedings of the National Academy of Sciences of the United States of America* 104, 6388-6393.
- Kaplan, D.H., Shankaran, V., Dighe, A.S., Stockert, E., Aguet, M., Old, L.J., and Schreiber, R.D. (1998). Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proceedings of the National Academy of Sciences of the United States of America* 95, 7556-7561.

- Kapsenberg, M.L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. *Nature reviews Immunology* 3, 984-993.
- Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. *Nature reviews Cancer* 2, 301-310.
- Kashyap, D.R., Wang, M., Liu, L.H., Boons, G.J., Gupta, D., and Dziarski, R. (2011). Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems. *Nature medicine* 17, 676-683.
- 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.
- Keane, M.P., Belperio, J.A., Xue, Y.Y., Burdick, M.D., and Strieter, R.M. (2004). Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. *J Immunol* 172, 2853-2860.
- Keeley, E.C., Mehrad, B., and Strieter, R.M. (2010). CXC chemokines in cancer angiogenesis and metastases. *Advances in cancer research* 106, 91-111.
- Kemp, T.J., Ludwig, A.T., Earel, J.K., Moore, J.M., Vanoosten, R.L., Moses, B., Leidal, K., Nauseef, W.M., and Griffith, T.S. (2005). Neutrophil stimulation with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) results in the release of functional soluble TRAIL/Apo-2L. *Blood* 106, 3474-3482.
- Kennedy, A.D., Willment, J.A., Dorward, D.W., Williams, D.L., Brown, G.D., and DeLeo, F.R. (2007). Dectin-1 promotes fungicidal activity of human neutrophils. *European journal of immunology* 37, 467-478.
- Kerrigan, A.M., Dennehy, K.M., Mourao-Sa, D., Faro-Trindade, I., Willment, J.A., Taylor, P.R., Eble, J.A., Reis e Sousa, C., and Brown, G.D. (2009). CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils. *J Immunol* 182, 4150-4157.
- Kessenbrock, K., Krumbholz, M., Schonermarck, U., Back, W., Gross, W.L., Werb, Z., Grone, H.J., Brinkmann, V., and Jenne, D.E. (2009). Netting neutrophils in autoimmune small-vessel vasculitis. *Nature medicine* 15, 623-625.
- Khandpur, R., Carmona-Rivera, C., Vivekanandan-Giri, A., Gizinski, A., Yalavarthi, S., Knight, J.S., Friday, S., Li, S., Patel, R.M., Subramanian, V., *et al.* (2013). NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Science translational medicine* 5, 178ra140.
- Kinzler, K.W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 386, 761, 763.
- Klintrup, K., Makinen, J.M., Kauppila, S., Vare, P.O., Melkko, J., Tuominen, H., Tuppurainen, K., Makela, J., Karttunen, T.J., and Makinen, M.J. (2005). Inflammation and prognosis in colorectal cancer. *Eur J Cancer* 41, 2645-2654.
- Koch, M.A., Tucker-Heard, G., Perdue, N.R., Killebrew, J.R., Urdahl, K.B., and Campbell, D.J. (2009). The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nature immunology* 10, 595-602.
- Korn, T., Oukka, M., Kuchroo, V., and Bettelli, E. (2007). Th17 cells: effector T cells with inflammatory properties. *Seminars in immunology* 19, 362-371.
- Koukourakis, M.I., Giatromanolaki, A., Kakolyris, S., O'Byrne, K.J., Apostolikas, N., Skarlatos, J., Gatter, K.C., and Harris, A.L. (1998). Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. *British journal of cancer* 77, 1696-1703.

- Krishnamoorthy, S., Recchiuti, A., Chiang, N., Yacoubian, S., Lee, C.H., Yang, R., Petasis, N.A., and Serhan, C.N. (2010). Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proceedings of the National Academy of Sciences of the United States of America* *107*, 1660-1665.
- Kristensen, V.N., Vaske, C.J., Ursini-Siegel, J., Van Loo, P., Nordgard, S.H., Sachidanandam, R., Sorlie, T., Warnberg, F., Haakensen, V.D., Helland, A., *et al.* (2012). Integrated molecular profiles of invasive breast tumors and ductal carcinoma in situ (DCIS) reveal differential vascular and interleukin signaling. *Proceedings of the National Academy of Sciences of the United States of America* *109*, 2802-2807.
- Kuang, D.M., Zhao, Q., Wu, Y., Peng, C., Wang, J., Xu, Z., Yin, X.Y., and Zheng, L. (2011). Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *Journal of hepatology* *54*, 948-955.
- Kutsuna, H., Suzuki, K., Kamata, N., Kato, T., Hato, F., Mizuno, K., Kobayashi, H., Ishii, M., and Kitagawa, S. (2004). Actin reorganization and morphological changes in human neutrophils stimulated by TNF, GM-CSF, and G-CSF: the role of MAP kinases. *American journal of physiology Cell physiology* *286*, C55-64.
- Laghi, L., Bianchi, P., Delconte, G., Celesti, G., Di Caro, G., Pedroni, M., Chiaravalli, A.M., Jung, B., Capella, C., de Leon, M.P., *et al.* (2012). MSH3 protein expression and nodal status in MLH1-deficient colorectal cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research* *18*, 3142-3153.
- Laghi, L., Bianchi, P., Miranda, E., Balladore, E., Pacetti, V., Grizzi, F., Allavena, P., Torri, V., Repici, A., Santoro, A., *et al.* (2009). CD3+ cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. *The lancet oncology* *10*, 877-884.
- Lakatos, P.L., and Lakatos, L. (2008). Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies. *World journal of gastroenterology : WJG* *14*, 3937-3947.
- Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., Meller, S., Chamilos, G., Sebasigari, R., Ricciari, V., *et al.* (2011). Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Science translational medicine* *3*, 73ra19.
- 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.
- Lazennec, G., and Richmond, A. (2010). Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends in molecular medicine* *16*, 133-144.
- Le, H.T., Tran, V.G., Kim, W., Kim, J., Cho, H.R., and Kwon, B. (2012). IL-33 priming regulates multiple steps of the neutrophil-mediated anti-Candida albicans response by modulating TLR and dectin-1 signals. *J Immunol* *189*, 287-295.
- Lee, J.H., Lim, Y.S., Yeon, J.E., Song, T.J., Yu, S.J., Gwak, G.Y., Kim, K.M., Kim, Y.J., Lee, J.W., and Yoon, J.H. (2015). Adjuvant Immunotherapy with Autologous Cytokine-induced Killer Cells for Hepatocellular Carcinoma. *Gastroenterology*.
- Lee, J.R., Dalton, R.R., Messina, J.L., Sharma, M.D., Smith, D.M., Burgess, R.E., Mazzella, F., Antonia, S.J., Mellor, A.L., and Munn, D.H. (2003). Pattern of recruitment of immunoregulatory antigen-presenting cells in malignant melanoma. *Laboratory investigation; a journal of technical methods and pathology* *83*, 1457-1466.
- Lee, W.B., Kang, J.S., Yan, J.J., Lee, M.S., Jeon, B.Y., Cho, S.N., and Kim, Y.J. (2012). Neutrophils Promote Mycobacterial Trehalose Dimycolate-Induced Lung Inflammation via the Mincle Pathway. *PLoS pathogens* *8*, e1002614.
- Leifler, K.S., Svensson, S., Abrahamsson, A., Bendrik, C., Robertson, J., Gauldie, J., Olsson, A.K., and Dabrosin, C. (2013). Inflammation induced by MMP-9 enhances tumor regression of experimental breast cancer. *J Immunol* *190*, 4420-4430.

- Ley, K. (2014). The second touch hypothesis: T cell activation, homing and polarization. *F1000Research* 3, 37.
- Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7, 678-689.
- Ley, K., Smith, E., and Stark, M.A. (2006). IL-17A-producing neutrophil-regulatory Tn lymphocytes. *Immunologic research* 34, 229-242.
- Liang, P.S., Chen, T.Y., and Giovannucci, E. (2009). Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis. *International journal of cancer Journal international du cancer* 124, 2406-2415.
- 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.
- Lindau, D., Mussard, J., Wagner, B.J., Ribon, M., Ronnefarth, V.M., Quettier, M., Jelcic, I., Boissier, M.C., Rammensee, H.G., and Decker, P. (2013). Primary blood neutrophils express a functional cell surface Toll-like receptor 9. *European journal of immunology* 43, 2101-2113.
- Liu, L., Belkadi, A., Darnall, L., Hu, T., Drescher, C., Cotleur, A.C., Padovani-Claudio, D., He, T., Choi, K., Lane, T.E., *et al.* (2010). CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. *Nature neuroscience* 13, 319-326.
- Liu, X., Ma, B., Malik, A.B., Tang, H., Yang, T., Sun, B., Wang, G., Minshall, R.D., Li, Y., Zhao, Y., *et al.* (2012). Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases. *Nature immunology* 13, 457-464.
- Ljunggren, H.G., and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today* 11, 237-244.
- 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.
- Lund, F.E., and Randall, T.D. (2010). Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nature reviews Immunology* 10, 236-247.
- Lutgens, E., de Muinck, E.D., Kitslaar, P.J., Tordoir, J.H., Wellens, H.J., and Daemen, M.J. (1999). Biphasic pattern of cell turnover characterizes the progression from fatty streaks to ruptured human atherosclerotic plaques. *Cardiovascular research* 41, 473-479.
- Lynch, H.T., Snyder, C.L., Shaw, T.G., Heinen, C.D., and Hitchins, M.P. (2015). Milestones of Lynch syndrome: 1895-2015. *Nature reviews Cancer* 15, 181-194.
- Ma, Y., Adjemian, S., Mattarollo, S.R., Yamazaki, T., Aymeric, L., Yang, H., Portela Catani, J.P., Hannani, D., Duret, H., Steegh, K., *et al.* (2013a). Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity* 38, 729-741.
- Ma, Y., Yang, Y., Wang, F., Zhang, P., Shi, C., Zou, Y., and Qin, H. (2013b). Obesity and risk of colorectal cancer: a systematic review of prospective studies. *PloS one* 8, e53916.
- Ma, Y., Zhang, P., Wang, F., Yang, J., Liu, Z., and Qin, H. (2011). Association between vitamin D and risk of colorectal cancer: a systematic review of prospective studies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 29, 3775-3782.

- Maffia, P.C., Zittermann, S.E., Scimone, M.L., Tateosian, N., Amiano, N., Guerrieri, D., Lutzky, V., Rosso, D., Romeo, H.E., Garcia, V.E., *et al.* (2007). Neutrophil elastase converts human immature dendritic cells into transforming growth factor-beta1-secreting cells and reduces allostimulatory ability. *The American journal of pathology* *171*, 928-937.
- Maggi, E., Parronchi, P., Manetti, R., Simonelli, C., Piccinni, M.P., Ruggi, F.S., De Carli, M., Ricci, M., and Romagnani, S. (1992). Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *J Immunol* *148*, 2142-2147.
- Mahnke, K., Johnson, T.S., Ring, S., and Enk, A.H. (2007). Tolerogenic dendritic cells and regulatory T cells: a two-way relationship. *Journal of dermatological science* *46*, 159-167.
- Maldonado, R.A., and von Andrian, U.H. (2010). How tolerogenic dendritic cells induce regulatory T cells. *Advances in immunology* *108*, 111-165.
- Malesci, A., Laghi, L., Bianchi, P., Delconte, G., Randolph, A., Torri, V., Carnaghi, C., Doci, R., Rosati, R., Montorsi, M., *et al.* (2007). Reduced likelihood of metastases in patients with microsatellite-unstable colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* *13*, 3831-3839.
- Mancino, A., Schioppa, T., Larghi, P., Pasqualini, F., Nebuloni, M., Chen, I.H., Sozzani, S., Austyn, J.M., Mantovani, A., and Sica, A. (2008). Divergent effects of hypoxia on dendritic cell functions. *Blood* *112*, 3723-3734.
- Manel, N., Unutmaz, D., and Littman, D.R. (2008). The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgamma. *Nature immunology* *9*, 641-649.
- 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. (2009). The yin-yang of tumor-associated neutrophils. *Cancer cell* *16*, 173-174.
- Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. *Nature* *454*, 436-444.
- Mantovani, A., Biswas, S.K., Galdiero, M.R., Sica, A., and Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. *The Journal of pathology* *229*, 176-185.
- 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.
- Marigo, I., Bosio, E., Solito, S., Mesa, C., Fernandez, A., Dolcetti, L., Ugel, S., Sonda, N., Biccato, S., Falisi, E., *et al.* (2010). Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity* *32*, 790-802.
- Markowitz, S.D., and Bertagnoli, M.M. (2009). Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* *361*, 2449-2460.
- Mary, J.Y. (1985). Normal human granulopoiesis revisited. II. Bone marrow data. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* *39*, 66-77.
- Mathy, C., Schneider, K., Chen, Y.Y., Varma, M., Terdiman, J.P., and Mahadevan, U. (2003). Gross versus microscopic pancolitis and the occurrence of neoplasia in ulcerative colitis. *Inflammatory bowel diseases* *9*, 351-355.
- Matsui, A., Yokoo, H., Negishi, Y., Endo-Takahashi, Y., Chun, N.A., Kadouchi, I., Suzuki, R., Maruyama, K., Aramaki, Y., Semba, K., *et al.* (2012). CXCL17 expression by tumor cells recruits CD11b+Gr1 high F4/80- cells and promotes tumor progression. *PloS one* *7*, e44080.
- Matsushima, H., Geng, S., Lu, R., Okamoto, T., Yao, Y., Mayuzumi, N., Kotol, P.F., Chojnacki, B.J., Miyazaki, T., Gallo, R.L., *et al.* (2013). Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. *Blood* *121*, 1677-1689.

- Matsushita, H., Vesely, M.D., Koboldt, D.C., Rickert, C.G., Uppaluri, R., Magrini, V.J., Arthur, C.D., White, J.M., Chen, Y.S., Shea, L.K., *et al.* (2012). Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* *482*, 400-404.
- 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., and Kubes, P. (2012). Neutrophils and intravascular immunity in the liver during infection and sterile inflammation. *Toxicologic pathology* *40*, 157-165.
- McDonald, B., McAvoy, E.F., Lam, F., Gill, V., de la Motte, C., Savani, R.C., and Kubes, P. (2008). Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids. *The Journal of experimental medicine* *205*, 915-927.
- McDonald, B., Pittman, K., Menezes, G.B., Hirota, S.A., Slaba, I., Waterhouse, C.C., Beck, P.L., Muruve, D.A., and Kubes, P. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science (New York, NY)* *330*, 362-366.
- Medina-Echeverz, J., Fioravanti, J., Zabala, M., Ardaiz, N., Prieto, J., and Berraondo, P. (2011). Successful colon cancer eradication after chemoimmunotherapy is associated with profound phenotypic change of intratumoral myeloid cells. *J Immunol* *186*, 807-815.
- Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature* *454*, 428-435.
- Megiovanni, A.M., Sanchez, F., Robledo-Sarmiento, M., Morel, C., Gluckman, J.C., and Boudaly, S. (2006). Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *Journal of leukocyte biology* *79*, 977-988.
- Mei, Z., Liu, Y., Liu, C., Cui, A., Liang, Z., Wang, G., Peng, H., Cui, L., and Li, C. (2014). Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. *British journal of cancer* *110*, 1595-1605.
- Melief, C.J. (2008). Cancer immunotherapy by dendritic cells. *Immunity* *29*, 372-383.
- Mescher, M.F., Agarwal, P., Casey, K.A., Hammerbeck, C.D., Xiao, Z., and Curtsinger, J.M. (2007). Molecular basis for checkpoints in the CD8 T cell response: tolerance versus activation. *Seminars in immunology* *19*, 153-161.
- 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.
- Miller, J.S., Soignier, Y., Panoskaltsis-Mortari, A., McNearney, S.A., Yun, G.H., Fautsch, S.K., McKenna, D., Le, C., Defor, T.E., Burns, L.J., *et al.* (2005). Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood* *105*, 3051-3057.
- Mitsuka, K., Kawataki, T., Satoh, E., Asahara, T., Horikoshi, T., and Kinouchi, H. (2013). Expression of indoleamine 2,3-dioxygenase and correlation with pathological malignancy in gliomas. *Neurosurgery* *72*, 1031-1038; discussion 1038-1039.
- Mittendorf, E.A., Alatrash, G., Qiao, N., Wu, Y., Sukhumalchandra, P., St John, L.S., Philips, A.V., Xiao, H., Zhang, M., Ruisaard, K., *et al.* (2012). Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. *Cancer research* *72*, 3153-3162.
- Mizukami, Y., Kono, K., Kawaguchi, Y., Akaike, H., Kamimura, K., Sugai, H., and Fujii, H. (2008). CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *International journal of cancer Journal international du cancer* *122*, 2286-2293.
- Mlecnik, B., Tosolini, M., Charoentong, P., Kirilovsky, A., Bindea, G., Berger, A., Camus, M., Gillard, M., Bruneval, P., Fridman, W.H., *et al.* (2010). Biomolecular network reconstruction

- identifies T-cell homing factors associated with survival in colorectal cancer. *Gastroenterology* *138*, 1429-1440.
- Moalli, F., Doni, A., Deban, L., Zelante, T., Zagarella, S., Bottazzi, B., Romani, L., Mantovani, A., and Garlanda, C. (2010). Role of complement and Fc{gamma} receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus*. *Blood* *116*, 5170-5180.
- Moalli, F., Jaillon, S., Inforzato, A., Sironi, M., Bottazzi, B., Mantovani, A., and Garlanda, C. (2011). Pathogen recognition by the long pentraxin PTX3. *Journal of biomedicine & biotechnology* *2011*, 830421.
- Moerkerk, P., Arends, J.W., van Driel, M., de Bruine, A., de Goeij, A., and ten Kate, J. (1994). Type and number of Ki-ras point mutations relate to stage of human colorectal cancer. *Cancer research* *54*, 3376-3378.
- 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.
- Moreno-Amaral, A.N., Gout, E., Danella-Polli, C., Tabarin, F., Lesavre, P., Pereira-da-Silva, G., Thielens, N.M., and Halbwachs-Mecarelli, L. (2012). M-ficolin and leukosialin (CD43): new partners in neutrophil adhesion. *Journal of leukocyte biology* *91*, 469-474.
- Moretta, A., Biassoni, R., Bottino, C., Pende, D., Vitale, M., Poggi, A., Mingari, M.C., and Moretta, L. (1997). Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. *Immunological reviews* *155*, 105-117.
- Moretta, L., Locatelli, F., Pende, D., Marcenaro, E., Mingari, M.C., and Moretta, A. (2011). Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *Blood* *117*, 764-771.
- Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stange, G., Van den Bossche, J., Mack, M., Pipeleers, D., In't Veld, P., De Baetselier, P., *et al.* (2010). Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer research* *70*, 5728-5739.
- Mucida, D., and Cheroutre, H. (2010). The many face-lifts of CD4 T helper cells. *Advances in immunology* *107*, 139-152.
- Mulder, A.H., Stegeman, C.A., and Kallenberg, C.G. (1995). Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis: a predominant role for the IgG3 subclass of ANCA. *Clinical and experimental immunology* *101*, 227-232.
- Muller, W.A. (2013). Getting leukocytes to the site of inflammation. *Veterinary pathology* *50*, 7-22.
- Musha, H., Ohtani, H., Mizoi, T., Kinouchi, M., Nakayama, T., Shiiba, K., Miyagawa, K., Nagura, H., Yoshie, O., and Sasaki, I. (2005). Selective infiltration of CCR5(+)CXCR3(+) T lymphocytes in human colorectal carcinoma. *International journal of cancer Journal international du cancer* *116*, 949-956.
- 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.
- Nagtegaal, I.D., Marijnen, C.A., Kranenbarg, E.K., Mulder-Stapel, A., Hermans, J., van de Velde, C.J., and van Krieken, J.H. (2001). Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. *BMC cancer* *1*, 7.
- Nagtegaal, I.D., and Quirke, P. (2008). What is the role for the circumferential margin in the modern treatment of rectal cancer? *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *26*, 303-312.
- Nausch, N., and Cerwenka, A. (2008). NKG2D ligands in tumor immunity. *Oncogene* *27*, 5944-5958.

- Nebiker, C.A., Han, J., Eppenberger-Castori, S., Iezzi, G., Hirt, C., Amicarella, F., Cremonesi, E., Huber, X., Padovan, E., Angrisani, B., *et al.* (2014). GM-CSF Production by Tumor Cells Is Associated with Improved Survival in Colorectal Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 20, 3094-3106.
- Nelson, B.H. (2010). CD20+ B cells: the other tumor-infiltrating lymphocytes. *J Immunol* 185, 4977-4982.
- Netea, M.G., Van der Meer, J.W., Suttmuller, R.P., Adema, G.J., and Kullberg, B.J. (2005). From the Th1/Th2 paradigm towards a Toll-like receptor/T-helper bias. *Antimicrobial agents and chemotherapy* 49, 3991-3996.
- Niekel, M.C., Bipat, S., and Stoker, J. (2010). Diagnostic imaging of colorectal liver metastases with CT, MR imaging, FDG PET, and/or FDG PET/CT: a meta-analysis of prospective studies including patients who have not previously undergone treatment. *Radiology* 257, 674-684.
- Nielsen, H.J., Hansen, U., Christensen, I.J., Reimert, C.M., Brunner, N., and Moesgaard, F. (1999). Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. *The Journal of pathology* 189, 487-495.
- Nizzoli, G., Krietsch, J., Weick, A., Steinfeldler, S., Facciotti, F., Gruarin, P., Bianco, A., Steckel, B., Moro, M., Crosti, M., *et al.* (2013). Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. *Blood* 122, 932-942.
- Noel, W., Raes, G., Hassanzadeh Ghassabeh, G., De Baetselier, P., and Beschin, A. (2004). Alternatively activated macrophages during parasite infections. *Trends in parasitology* 20, 126-133.
- Novitskiy, S.V., Ryzhov, S., Zaynagetdinov, R., Goldstein, A.E., Huang, Y., Tikhomirov, O.Y., Blackburn, M.R., Biaggioni, I., Carbone, D.P., Feoktistov, I., *et al.* (2008). Adenosine receptors in regulation of dendritic cell differentiation and function. *Blood* 112, 1822-1831.
- Nozawa, H., Chiu, C., and Hanahan, D. (2006). Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 103, 12493-12498.
- Numasaki, M., Fukushi, J., Ono, M., Narula, S.K., Zavodny, P.J., Kudo, T., Robbins, P.D., Tahara, H., and Lotze, M.T. (2003). Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101, 2620-2627.
- O'Garra, A., and Vieira, P. (2007). T(H)1 cells control themselves by producing interleukin-10. *Nature reviews Immunology* 7, 425-428.
- O'Sullivan, B., and Thomas, R. (2003). CD40 and dendritic cell function. *Critical reviews in immunology* 23, 83-107.
- Oehler, L., Majdic, O., Pickl, W.F., Stockl, J., Riedl, E., Drach, J., Rappersberger, K., Geissler, K., and Knapp, W. (1998). Neutrophil granulocyte-committed cells can be driven to acquire dendritic cell characteristics. *The Journal of experimental medicine* 187, 1019-1028.
- Ogino, S., Nosho, K., Kirkner, G.J., Kawasaki, T., Meyerhardt, J.A., Loda, M., Giovannucci, E.L., and Fuchs, C.S. (2009). CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 58, 90-96.
- Ohtani, H., Jin, Z., Takegawa, S., Nakayama, T., and Yoshie, O. (2009). Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma. *The Journal of pathology* 217, 21-31.
- Ordonez-Rueda, D., Jonsson, F., Mancardi, D.A., Zhao, W., Malzac, A., Liang, Y., Bertosio, E., Grenot, P., Blanquet, V., Sabrautzki, S., *et al.* (2012). A hypomorphic mutation in the Gfi1 transcriptional repressor results in a novel form of neutropenia. *European journal of immunology* 42, 2395-2408.
- Pages, F., Berger, A., Camus, M., Sanchez-Cabo, F., Costes, A., Molidor, R., Mlecnik, B., Kirilovsky, A., Nilsson, M., Damotte, D., *et al.* (2005). Effector memory T cells, early metastasis, and survival in colorectal cancer. *The New England journal of medicine* 353, 2654-2666.

- Palucka, K., and Banchereau, J. (2012). Cancer immunotherapy via dendritic cells. *Nature reviews Cancer* *12*, 265-277.
- Pan, J., Burdick, M.D., Belperio, J.A., Xue, Y.Y., Gerard, C., Sharma, S., Dubinett, S.M., and Strieter, R.M. (2006). CXCR3/CXCR3 ligand biological axis impairs RENCA tumor growth by a mechanism of immunoangiostasis. *J Immunol* *176*, 1456-1464.
- 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.
- Pardoll, D. (2003). Does the immune system see tumors as foreign or self? *Annual review of immunology* *21*, 807-839.
- Park, S.H., Lee, J.H., Lee, S.S., Kim, J.C., Yu, C.S., Kim, H.C., Ye, B.D., Kim, M.J., Kim, A.Y., and Ha, H.K. (2012). CT colonography for detection and characterisation of synchronous proximal colonic lesions in patients with stenosing colorectal cancer. *Gut* *61*, 1716-1722.
- Parsons, M.T., Buchanan, D.D., Thompson, B., Young, J.P., and Spurdle, A.B. (2012). Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant classification. *Journal of medical genetics* *49*, 151-157.
- Pedroza-Gonzalez, A., Xu, K., Wu, T.C., Aspod, C., Tindle, S., Marches, F., Gallegos, M., Burton, E.C., Savino, D., Hori, T., *et al.* (2011). Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation. *The Journal of experimental medicine* *208*, 479-490.
- Peine, M., Rausch, S., Helmstetter, C., Frohlich, A., Hegazy, A.N., Kuhl, A.A., Grevelding, C.G., Hofer, T., Hartmann, S., and Lohning, M. (2013). Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation. *PLoS biology* *11*, e1001633.
- Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., Cosmi, L., Lunardi, C., Annunziato, F., Romagnani, S., *et al.* (2010a). Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood* *115*, 335-343.
- Pelletier, M., Micheletti, A., and Cassatella, M.A. (2010b). Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. *Journal of leukocyte biology* *88*, 1163-1170.
- Perretti, M., and D'Acquisto, F. (2009). Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature reviews Immunology* *9*, 62-70.
- Pliyev, B.K., Kalintseva, M.V., Abdulaeva, S.V., Yarygin, K.N., and Savchenko, V.G. (2014). Neutrophil microparticles modulate cytokine production by natural killer cells. *Cytokine* *65*, 126-129.
- Pollard, J.W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nature reviews Cancer* *4*, 71-78.
- Popat, S., Hubner, R., and Houlston, R.S. (2005). Systematic review of microsatellite instability and colorectal cancer prognosis. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *23*, 609-618.
- Popivanova, B.K., Kitamura, K., Wu, Y., Kondo, T., Kagaya, T., Kaneko, S., Oshima, M., Fujii, C., and Mukaida, N. (2008). Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *The Journal of clinical investigation* *118*, 560-570.
- Prieur, A., and Peeper, D.S. (2008). Cellular senescence in vivo: a barrier to tumorigenesis. *Current opinion in cell biology* *20*, 150-155.
- 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. *Nature immunology* *13*, 170-180.

- Puli, S.R., Bechtold, M.L., Reddy, J.B., Choudhary, A., and Antillon, M.R. (2010). Can endoscopic ultrasound predict early rectal cancers that can be resected endoscopically? A meta-analysis and systematic review. *Digestive diseases and sciences* 55, 1221-1229.
- Puli, S.R., Bechtold, M.L., Reddy, J.B., Choudhary, A., Antillon, M.R., and Brugge, W.R. (2009). How good is endoscopic ultrasound in differentiating various T stages of rectal cancer? Meta-analysis and systematic review. *Annals of surgical oncology* 16, 254-265.
- Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39-51.
- 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.
- Radic, M., and Marion, T.N. (2013). Neutrophil extracellular chromatin traps connect innate immune response to autoimmunity. *Seminars in immunopathology* 35, 465-480.
- Rafaelsen, S.R., and Jakobsen, A. (2011). Contrast-enhanced ultrasound vs multidetector-computed tomography for detecting liver metastases in colorectal cancer: a prospective, blinded, patient-by-patient analysis. *Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland* 13, 420-425.
- Raimondi, G., Turner, M.S., Thomson, A.W., and Morel, P.A. (2007). Naturally occurring regulatory T cells: recent insights in health and disease. *Critical reviews in immunology* 27, 61-95.
- Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K.W., Vogelstein, B., and Velculescu, V.E. (2002). Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 418, 934.
- Rao, H.L., Chen, J.W., Li, M., Xiao, Y.B., Fu, J., Zeng, Y.X., Cai, M.Y., and Xie, D. (2012). Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. *PloS one* 7, e30806.
- Reid, M.D., Basturk, O., Thirabanjasak, D., Hruban, R.H., Klimstra, D.S., Bagci, P., Altinel, D., and Adsay, V. (2011). Tumor-infiltrating neutrophils in pancreatic neoplasia. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 24, 1612-1619.
- Rivino, L., Messi, M., Jarrossay, D., Lanzavecchia, A., Sallusto, F., and Geginat, J. (2004). Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. *The Journal of experimental medicine* 200, 725-735.
- Robbins, C.S., Hilgendorf, I., Weber, G.F., Theurl, I., Iwamoto, Y., Figueiredo, J.L., Gorbatov, R., Sukhova, G.K., Gerhardt, L.M., Smyth, D., *et al.* (2013). Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nature medicine* 19, 1166-1172.
- Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., Corrigan, C., Durham, S.R., and Kay, A.B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *The New England journal of medicine* 326, 298-304.
- Rodriguez, P.C., Ernstoff, M.S., Hernandez, C., Atkins, M., Zabaleta, J., Sierra, R., and Ochoa, A.C. (2009). Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer research* 69, 1553-1560.
- Rollet-Labelle, E., Vaillancourt, M., Marois, L., Newkirk, M.M., Poubelle, P.E., and Naccache, P.H. (2013). Cross-linking of IgGs bound on circulating neutrophils leads to an activation of endothelial cells: possible role of rheumatoid factors in rheumatoid arthritis-associated vascular dysfunction. *Journal of inflammation* 10, 27.
- Romagnani, S. (1997). The Th1/Th2 paradigm. *Immunology today* 18, 263-266.
- Roncarolo, M.G., Bacchetta, R., Bordignon, C., Narula, S., and Levings, M.K. (2001). Type 1 T regulatory cells. *Immunological reviews* 182, 68-79.
- Roncarolo, M.G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., and Levings, M.K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews* 212, 28-50.

- Roncucci, L., Mora, E., Mariani, F., Bursi, S., Pezzi, A., Rossi, G., Pedroni, M., Luppi, D., Santoro, L., Monni, S., *et al.* (2008). Myeloperoxidase-positive cell infiltration in colorectal carcinogenesis as indicator of colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev* *17*, 2291-2297.
- Rorvig, S., Honore, C., Larsson, L.I., Ohlsson, S., Pedersen, C.C., Jacobsen, L.C., Cowland, J.B., Garred, P., and Borregaard, N. (2009). Ficolin-1 is present in a highly mobilizable subset of human neutrophil granules and associates with the cell surface after stimulation with fMLP. *Journal of leukocyte biology* *86*, 1439-1449.
- Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., *et al.* (2002). Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science (New York, NY)* *295*, 2097-2100.
- Saitoh, T., Komano, J., Saitoh, Y., Misawa, T., Takahama, M., Kozaki, T., Uehata, T., Iwasaki, H., Omori, H., Yamaoka, S., *et al.* (2012). Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell host & microbe* *12*, 109-116.
- Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature immunology* *6*, 345-352.
- Salama, P., Phillips, M., Grieu, F., Morris, M., Zeps, N., Joseph, D., Platell, C., and Iacopetta, B. (2009). Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *27*, 186-192.
- Sargent, D.J., Marsoni, S., Monges, G., Thibodeau, S.N., Labianca, R., Hamilton, S.R., French, A.J., Kabat, B., Foster, N.R., Torri, V., *et al.* (2010). Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *28*, 3219-3226.
- Scapini, P., Morini, M., Tecchio, C., Minghelli, S., Di Carlo, E., Tanghetti, E., Albini, A., Lowell, C., Berton, G., Noonan, D.M., *et al.* (2004). CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. *J Immunol* *172*, 5034-5040.
- Scapini, P., Nesi, L., Morini, M., Tanghetti, E., Belleri, M., Noonan, D., Presta, M., Albini, A., and Cassatella, M.A. (2002). Generation of biologically active angiostatin kringle 1-3 by activated human neutrophils. *J Immunol* *168*, 5798-5804.
- 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.
- Schioppa, T., Moore, R., Thompson, R.G., Rosser, E.C., Kulbe, H., Nedospasov, S., Mauri, C., Coussens, L.M., and Balkwill, F.R. (2011). B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* *108*, 10662-10667.
- Schmielau, J., and Finn, O.J. (2001). Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer research* *61*, 4756-4760.
- Schmoll, H.J., Van Cutsem, E., Stein, A., Valentini, V., Glimelius, B., Haustermans, K., Nordlinger, B., van de Velde, C.J., Balmana, J., Regula, J., *et al.* (2012). ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* *23*, 2479-2516.
- Schoppmann, S.F., Birner, P., Stockl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K., and Kerjaschki, D. (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *The American journal of pathology* *161*, 947-956.

- Schreiber, A., Xiao, H., Jennette, J.C., Schneider, W., Luft, F.C., and Kettritz, R. (2009). C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis. *Journal of the American Society of Nephrology : JASN* 20, 289-298.
- Schreiber, R.D., Old, L.J., and Smyth, M.J. (2011). Cancer immunoeediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331, 1565-1570.
- Schubbert, S., Shannon, K., and Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. *Nature reviews Cancer* 7, 295-308.
- Schuster, S., Hurrell, B., and Tacchini-Cottier, F. (2013). Crosstalk between neutrophils and dendritic cells: a context-dependent process. *Journal of leukocyte biology* 94, 671-675.
- Schwartzberg, L.S., Rivera, F., Karthaus, M., Fasola, G., Canon, J.L., Hecht, J.R., Yu, H., Oliner, K.S., and Go, W.Y. (2014). PEAK: a randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 32, 2240-2247.
- 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.
- Senovilla, L., Vacchelli, E., Galon, J., Adjemian, S., Eggermont, A., Fridman, W.H., Sautes-Fridman, C., Ma, Y., Tartour, E., Zitvogel, L., *et al.* (2012). Trial watch: Prognostic and predictive value of the immune infiltrate in cancer. *Oncoimmunology* 1, 1323-1343.
- Serhan, C.N., Chiang, N., and Van Dyke, T.E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature reviews Immunology* 8, 349-361.
- Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J., and Schreiber, R.D. (2001). IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410, 1107-1111.
- Sharpe, A.H., Wherry, E.J., Ahmed, R., and Freeman, G.J. (2007). The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nature immunology* 8, 239-245.
- Shaughnessy, L.M., and Swanson, J.A. (2007). The role of the activated macrophage in clearing *Listeria monocytogenes* infection. *Frontiers in bioscience : a journal and virtual library* 12, 2683-2692.
- Shevach, E.M. (2009). Mechanisms of foxp3⁺ T regulatory cell-mediated suppression. *Immunity* 30, 636-645.
- Shirey, K.A., Pletneva, L.M., Puche, A.C., Keegan, A.D., Prince, G.A., Blanco, J.C., and Vogel, S.N. (2010). Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R α -, TLR4-, and IFN- β -dependent. *Mucosal immunology* 3, 291-300.
- Shojaei, F., Singh, M., Thompson, J.D., and Ferrara, N. (2008). Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. *Proceedings of the National Academy of Sciences of the United States of America* 105, 2640-2645.
- Shojaei, F., Wu, X., Qu, X., Kowanzet, M., Yu, L., Tan, M., Meng, Y.G., and Ferrara, N. (2009). G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. *Proceedings of the National Academy of Sciences of the United States of America* 106, 6742-6747.
- Shojaei, F., Wu, X., Zhong, C., Yu, L., Liang, X.H., Yao, J., Blanchard, D., Bais, C., Peale, F.V., van Bruggen, N., *et al.* (2007). Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450, 825-831.
- Sietses, C., Meijerink, M.R., Meijer, S., and van den Tol, M.P. (2010). The impact of intraoperative ultrasonography on the surgical treatment of patients with colorectal liver metastases. *Surgical endoscopy* 24, 1917-1922.

- Sindrilaru, A., Peters, T., Wieschalka, S., Baican, C., Baican, A., Peter, H., Hainzl, A., Schatz, S., Qi, Y., Schlecht, A., *et al.* (2011). An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *The Journal of clinical investigation* *121*, 985-997.
- Snelgrove, R.J., Jackson, P.L., Hardison, M.T., Noerager, B.D., Kinloch, A., Gaggar, A., Shastry, S., Rowe, S.M., Shim, Y.M., Hussell, T., *et al.* (2010). A critical role for LTA4H in limiting chronic pulmonary neutrophilic inflammation. *Science (New York, NY)* *330*, 90-94.
- Soehnlein, O., and Lindbom, L. (2010). Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* *10*, 427-439.
- Solito, S., Falisi, E., Diaz-Montero, C.M., Doni, A., Pinton, L., Rosato, A., Francescato, S., Basso, G., Zanovello, P., Onicescu, G., *et al.* (2011). A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* *118*, 2254-2265.
- 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.
- Spite, M., Norling, L.V., Summers, L., Yang, R., Cooper, D., Petasis, N.A., Flower, R.J., Perretti, M., and Serhan, C.N. (2009). Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* *461*, 1287-1291.
- Sreeramkumar, V., Adrover, J.M., Ballesteros, I., Cuartero, M.I., Rossaint, J., Bilbao, I., Nacher, M., Pitaval, C., Radovanovic, I., Fukui, Y., *et al.* (2014). Neutrophils scan for activated platelets to initiate inflammation. *Science (New York, NY)* *346*, 1234-1238.
- 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.
- Stephens, L.A., Mottet, C., Mason, D., and Powrie, F. (2001). Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *European journal of immunology* *31*, 1247-1254.
- Stintzing, S. (2014). Management of colorectal cancer. *F1000prime reports* *6*, 108.
- Stutman, O. (1974). Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science* *183*, 534-536.
- Sun, A.M., Li, C.G., Zhang, Y.Q., Lin, S.M., Niu, H.R., and Shi, Y.S. (2015). Hepatocarcinoma cell-derived hepatoma-derived growth factor (HDGF) induces regulatory T cells. *Cytokine* *72*, 31-35.
- 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., Le Moigne, V., Rossato, M., Donini, M., McCartney, S., Calzetti, F., Colonna, M., Bazzoni, F., and Cassatella, M.A. (2008). Activation of an immunoregulatory and antiviral gene expression program in poly(I:C)-transfected human neutrophils. *J Immunol* *181*, 6563-6573.
- 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.
- Tanaka, H., Ito, T., Kyo, T., and Kimura, A. (2007). Treatment with IFNalpha in vivo up-regulates serum-soluble TNF-related apoptosis inducing ligand (sTRAIL) levels and TRAIL mRNA expressions in neutrophils in chronic myelogenous leukemia patients. *European journal of haematology* *78*, 389-398.

- Tang, Y., Katuri, V., Srinivasan, R., Fogt, F., Redman, R., Anand, G., Said, A., Fishbein, T., Zasloff, M., Reddy, E.P., *et al.* (2005). Transforming growth factor-beta suppresses nonmetastatic colon cancer through Smad4 and adaptor protein ELF at an early stage of tumorigenesis. *Cancer research* 65, 4228-4237.
- Taylor, D.P., Burt, R.W., Williams, M.S., Haug, P.J., and Cannon-Albright, L.A. (2010). Population-based family history-specific risks for colorectal cancer: a constellation approach. *Gastroenterology* 138, 877-885.
- Tecchio, C., Huber, V., Scapini, P., Calzetti, F., Margotto, D., Todeschini, G., Pilla, L., Martinelli, G., Pizzolo, G., Rivoltini, L., *et al.* (2004). IFN α -stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. *Blood* 103, 3837-3844.
- Tecchio, C., Scapini, P., Pizzolo, G., and Cassatella, M.A. (2013). On the cytokines produced by human neutrophils in tumors. *Seminars in cancer biology* 23, 159-170.
- Terzic, J., Grivennikov, S., Karin, E., and Karin, M. (2010). Inflammation and colon cancer. *Gastroenterology* 138, 2101-2114 e2105.
- Thiel, A., and Ristimaki, A. (2013). Toward a Molecular Classification of Colorectal Cancer: The Role of BRAF. *Frontiers in oncology* 3, 281.
- Thomas, L. (1959). *Cellular and Humoral Aspects of the Hypersensitive States.* New York: Hoeber-Harper.
- Thompson, A.A., Elks, P.M., Marriott, H.M., Eamsamarng, S., Higgins, K.R., Lewis, A., Williams, L., Parmar, S., Shaw, G., McGrath, E.E., *et al.* (2014). Hypoxia-inducible factor 2 α regulates key neutrophil functions in humans, mice, and zebrafish. *Blood* 123, 366-376.
- Thoren, F.B., Riise, R.E., Ousback, J., Della Chiesa, M., Alsterholm, M., Marcenaro, E., Pesce, S., Prato, C., Cantoni, C., Bylund, J., *et al.* (2012). Human NK Cells induce neutrophil apoptosis via an NKp46- and Fas-dependent mechanism. *J Immunol* 188, 1668-1674.
- Tran, D.Q., Ramsey, H., and Shevach, E.M. (2007). Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood* 110, 2983-2990.
- Trellakis, S., Bruderek, K., Dumitru, C.A., Gholaman, H., Gu, X., Bankfalvi, A., Scherag, A., Hutte, J., Dominas, N., Lehnerdt, G.F., *et al.* (2011a). Polymorphonuclear granulocytes in human head and neck cancer: Enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. *International journal of cancer Journal international du cancer* 129, 2183-2193.
- Trellakis, S., Farjah, H., Bruderek, K., Dumitru, C.A., Hoffmann, T.K., Lang, S., and Brandau, S. (2011b). Peripheral blood neutrophil granulocytes from patients with head and neck squamous cell carcinoma functionally differ from their counterparts in healthy donors. *International journal of immunopathology and pharmacology* 24, 683-693.
- Trevelin, S.C., Alves-Filho, J.C., Sonogo, F., Turato, W., Nascimento, D.C., Souto, F.O., Cunha, T.M., Gazzinelli, R.T., and Cunha, F.Q. (2012). Toll-like receptor 9 activation in neutrophils impairs chemotaxis and reduces sepsis outcome. *Critical care medicine* 40, 2631-2637.
- Trinchieri, G., and Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. *Nature reviews Immunology* 7, 179-190.
- Tsuda, Y., Takahashi, H., Kobayashi, M., Hanafusa, T., Herndon, D.N., and Suzuki, F. (2004). Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. *Immunity* 21, 215-226.
- Twelves, C., Scheithauer, W., McKendrick, J., Seitz, J.F., Van Hazel, G., Wong, A., Diaz-Rubio, E., Gilberg, F., and Cassidy, J. (2012). Capecitabine versus 5-fluorouracil/folinic acid as adjuvant therapy for stage III colon cancer: final results from the X-ACT trial with analysis by age and preliminary evidence of a pharmacodynamic marker of efficacy. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 23, 1190-1197.

- Ullman, T.A., and Itzkowitz, S.H. (2011). Intestinal inflammation and cancer. *Gastroenterology* *140*, 1807-1816.
- Unutmaz, D. (2009). RORC2: the master of human Th17 cell programming. *European journal of immunology* *39*, 1452-1455.
- 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.
- Van Cutsem, E., Labianca, R., Bodoky, G., Barone, C., Aranda, E., Nordlinger, B., Topham, C., Tabernero, J., Andre, T., Sobrero, A.F., *et al.* (2009). Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *27*, 3117-3125.
- van Gijn, W., Marijnen, C.A., Nagtegaal, I.D., Kranenbarg, E.M., Putter, H., Wiggers, T., Rutten, H.J., Pahlman, L., Glimelius, B., and van de Velde, C.J. (2011). Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *The lancet oncology* *12*, 575-582.
- van Gisbergen, K.P., Ludwig, I.S., Geijtenbeek, T.B., and van Kooyk, Y. (2005a). Interactions of DC-SIGN with Mac-1 and CEACAM1 regulate contact between dendritic cells and neutrophils. *FEBS letters* *579*, 6159-6168.
- van Gisbergen, K.P., Sanchez-Hernandez, M., Geijtenbeek, T.B., and van Kooyk, Y. (2005b). Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *The Journal of experimental medicine* *201*, 1281-1292.
- Vasen, H.F., Tomlinson, I., and Castells, A. (2015). Clinical management of hereditary colorectal cancer syndromes. *Nature reviews Gastroenterology & hepatology* *12*, 88-97.
- Vasiljeva, O., Papazoglou, A., Kruger, A., Brodoefel, H., Korovin, M., Deussing, J., Augustin, N., Nielsen, B.S., Almholt, K., Bogyo, M., *et al.* (2006). Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer. *Cancer research* *66*, 5242-5250.
- Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C., and Stockinger, B. (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature immunology* *9*, 1341-1346.
- Vesely, M.D., Kershaw, M.H., Schreiber, R.D., and Smyth, M.J. (2011). Natural innate and adaptive immunity to cancer. *Annual review of immunology* *29*, 235-271.
- Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., and Brossay, L. (2012). Targeting natural killer cells and natural killer T cells in cancer. *Nature reviews Immunology* *12*, 239-252.
- von Andrian, U.H., and Mempel, T.R. (2003). Homing and cellular traffic in lymph nodes. *Nature reviews Immunology* *3*, 867-878.
- 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. *The Journal of experimental medicine* *209*, 819-835.
- Wang, J., and Arase, H. (2014). Regulation of immune responses by neutrophils. *Annals of the New York Academy of Sciences* *1319*, 66-81.
- Wang, S., and Wang, Y. (2013). Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. *Biochimica et biophysica acta* *1829*, 1126-1135.
- Wang, W., Li, X., Zheng, D., Zhang, D., Peng, X., Zhang, X., Ai, F., Wang, X., Ma, J., Xiong, W., *et al.* (2015). Dynamic changes and functions of macrophages and M1/M2 subpopulations during

ulcerative colitis-associated carcinogenesis in an AOM/DSS mouse model. *Mol Med Rep* *11*, 2397-2406.

Wei, S., Zhao, E., Kryczek, I., and Zou, W. (2012). Th17 cells have stem cell-like features and promote long-term immunity. *Oncoimmunology* *1*, 516-519.

Weitzman, S.A., Weitberg, A.B., Clark, E.P., and Stossel, T.P. (1985). Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science (New York, NY)* *227*, 1231-1233.

Wertel, I., Surowka, J., Polak, G., Barczynski, B., Bednarek, W., Jakubowicz-Gil, J., Bojarska-Junak, A., and Kotarski, J. (2015). Macrophage-derived chemokine CCL22 and regulatory T cells in ovarian cancer patients. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*.

Westbrook, A.M., Wei, B., Braun, J., and Schiestl, R.H. (2009). Intestinal mucosal inflammation leads to systemic genotoxicity in mice. *Cancer research* *69*, 4827-4834.

Whiteside, T.L. (2012). What are regulatory T cells (Treg) regulating in cancer and why? *Seminars in cancer biology* *22*, 327-334.

Whiteside, T.L. (2013). Immune responses to cancer: are they potential biomarkers of prognosis? *Frontiers in oncology* *3*, 107.

Willenbacher, R.F., Aust, D.E., Chang, C.G., Zelman, S.J., Ferrell, L.D., Moore, D.H., 2nd, and Waldman, F.M. (1999). Genomic instability is an early event during the progression pathway of ulcerative-colitis-related neoplasia. *The American journal of pathology* *154*, 1825-1830.

Williams, J.P., Stewart, T., Li, B., Mulloy, R., Dimova, D., and Classon, M. (2006). The retinoblastoma protein is required for Ras-induced oncogenic transformation. *Molecular and cellular biology* *26*, 1170-1182.

Wingender, G., Hiss, M., Engel, I., Peukert, K., Ley, K., Haller, H., Kronenberg, M., and von Vietinghoff, S. (2012). Neutrophilic granulocytes modulate invariant NKT cell function in mice and humans. *J Immunol* *188*, 3000-3008.

Wislez, M., Rabbe, N., Marchal, J., Milleron, B., Crestani, B., Mayaud, C., Antoine, M., Soler, P., and Cadranet, J. (2003). Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. *Cancer research* *63*, 1405-1412.

Witko-Sarsat, V., Mocek, J., Bouayad, D., Tamassia, N., Ribeil, J.A., Candalh, C., Davezac, N., Reuter, N., Mouthon, L., Hermine, O., *et al.* (2010). Proliferating cell nuclear antigen acts as a cytoplasmic platform controlling human neutrophil survival. *The Journal of experimental medicine* *207*, 2631-2645.

Woodfin, A., Voisin, M.B., Imhof, B.A., Dejana, E., Engelhardt, B., and Nourshargh, S. (2009). Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood* *113*, 6246-6257.

Wright, H.L., Moots, R.J., and Edwards, S.W. (2014). The multifactorial role of neutrophils in rheumatoid arthritis. *Nature reviews Rheumatology* *10*, 593-601.

Wrzesinski, S.H., Wan, Y.Y., and Flavell, R.A. (2007). Transforming growth factor-beta and the immune response: implications for anticancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* *13*, 5262-5270.

Xie, J., Lotoski, L.C., Chooniedass, R., Su, R.C., Simons, F.E., Liem, J., Becker, A.B., Uzonna, J., and HayGlass, K.T. (2012). Elevated antigen-driven IL-9 responses are prominent in peanut allergic humans. *PLoS one* *7*, e45377.

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. *Nature medicine* *15*, 1318-1321.

- Yago, T., Shao, B., Miner, J.J., Yao, L., Klopocki, A.G., Maeda, K., Coggeshall, K.M., and McEver, R.P. (2010). E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin alphaLbeta2-mediated slow leukocyte rolling. *Blood* *116*, 485-494.
- Yang, L., Froio, R.M., Sciuto, T.E., Dvorak, A.M., Alon, R., and Luscinskas, F.W. (2005). ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood* *106*, 584-592.
- Yothers, G., O'Connell, M.J., Allegra, C.J., Kuebler, J.P., Colangelo, L.H., Petrelli, N.J., and Wolmark, N. (2011). Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* *29*, 3768-3774.
- Youn, J.I., Collazo, M., Shalova, I.N., Biswas, S.K., and Gabrilovich, D.I. (2012). Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *Journal of leukocyte biology* *91*, 167-181.
- Youn, J.I., and Gabrilovich, D.I. (2010). The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *European journal of immunology* *40*, 2969-2975.
- Youn, J.I., Kumar, V., Collazo, M., Nefedova, Y., Condamine, T., Cheng, P., Villagra, A., Antonia, S., McCaffrey, J.C., Fishman, M., *et al.* (2013). Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. *Nature immunology* *14*, 211-220.
- 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., 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.
- Zamarron, B.F., and Chen, W. (2011). Dual roles of immune cells and their factors in cancer development and progression. *International journal of biological sciences* *7*, 651-658.
- Zandman-Goddard, G., and Shoenfeld, Y. (2005). Infections and SLE. *Autoimmunity* *38*, 473-485.
- Zhang, W., Xu, W., and Xiong, S. (2010). Blockade of Notch1 signaling alleviates murine lupus via blunting macrophage activation and M2b polarization. *J Immunol* *184*, 6465-6478.
- 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.
- Zhao, J.J., Pan, K., Wang, W., Chen, J.G., Wu, Y.H., Lv, L., Li, J.J., Chen, Y.B., Wang, D.D., Pan, Q.Z., *et al.* (2012). The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. *PloS one* *7*, e33655.
- Zhou, S.L., Dai, Z., Zhou, Z.J., Wang, X.Y., Yang, G.H., Wang, Z., Huang, X.W., Fan, J., and Zhou, J. (2012). Overexpression of CXCL5 mediates neutrophil infiltration and indicates poor prognosis for hepatocellular carcinoma. *Hepatology* *56*, 2242-2254.
- Zitvogel, L., Galluzzi, L., Smyth, M.J., and Kroemer, G. (2013). Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. *Immunity* *39*, 74-88.
- Zitvogel, L., Tesniere, A., and Kroemer, G. (2006). Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nature reviews Immunology* *6*, 715-727.
- Zou, W. (2006). Regulatory T cells, tumour immunity and immunotherapy. *Nature reviews Immunology* *6*, 295-307.
- Zumwalt, T.J., Arnold, M., Goel, A., and Boland, C.R. (2014). Active secretion of CXCL10 and CCL5 from colorectal cancer microenvironments associates with GranzymeB+ CD8+ T-cell infiltration. *Oncotarget*.