Immunobiology of Neutrophils in human colorectal cancer

Inauguraldissertation

Zur

Erlangung der Würde eines Doktors der Philosophie vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

Der Universität Basel

Von

Valeria Governa

aus **Italien**

Basel, 2018

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof.Jean Pieters, Prof. Giulio Cesare Spagnoli, Prof. Alfred Zippelius

Basel, 21.02.2017

Prof. Dr. Martin Spiess

The Dean of Faculty

During my PhD training I have extensively investigated the biological relevance of neutrophils in human colorectal cancer (CRC).

This thesis consists of a **first chapter** that includes a **general introduction** covering important aspects related to development and functions of tumor infiltrating neutrophils (TANs). The following **two chapters** include a manuscript, currently being revised for publication, focusing on the interplay between neutrophils and CD8+ cells in CRC and a section reporting unpublished results concerning the impact of microbiota on neutrophils interaction with other non-transformed components of the CRC microenvironment. **General discussion and perspectives** are then reported in the final pages**.**

Four publications resulting from my collaboration to additional projects of our research group, addressing the role of immune cells and stromal cells on CRC biology and clinical course are included with a brief introduction in the **appendix**.

Table of Contents

Summary

Colorectal cancer (CRC) represents a major cause of cancer related death in different geographic areas.

Tumor infiltration by a variety of immune cell types has consistently been observed to be associated with favorable prognosis (1). In particular, CRC infiltration by CD8+ T cells expressing memory and activation markers has repeatedly been reported to represent a favorable prognostic marker, although underlying molecular mechanisms and the antigen specificity of these cells are largely unclear. In sharp contrast, tumor infiltration by myeloid cells has classically been associated with poor prognosis in a variety of cancers of different histological origin (2). However, recent reports underline that granulocytes might participate to anti-tumor immune responses in defined cancer types (3-5). In previous studies, our group has observed that CRC infiltration by myeloid cells expressing CD16 and myeloperoxidase (MPO) is also associated with good prognosis (6;7).

Based on this background, I have addressed the role of granulocyte infiltration in CRC immunobiology. By using a clinically annotated tissue microarray (TMA) including over 650 individual CRC, I have explored the prognostic significance of tumor infiltration by CD66b+ granulocytes. I found that CRC infiltration by CD66b+ granulocytes significantly correlates with favorable prognosis. Most importantly however, dense granulocyte infiltration significantly enhances the prognostic significance of CD8+ T cell infiltration in CRC. Taken together these data suggest that a cross-talk of potentially high clinical relevance takes place in CRC. Indeed, immunofluorescence staining of tissue sections from CRC showing evidence of infiltration by lymphocytes and granulocytes indicates that CD8+ and CD66b+ cells are frequently co-localized. Still unclear is whether neutrophils are able to modulate T-cell responsiveness to antigenic challenges (3;8). On the other hand activated T cells have previously been shown to enhance granulocyte survival and functions by the release of cytokines, including TNF-α, IFN-γ and GM-CSF (8).

In additional studies I have extensively characterized phenotypic profiles of CRC infiltrating granulocytes, as compared to granulocytes from peripheral blood or infiltrating healthy mucosa adjacent to CRC.

Functional studies performed by using tumor-derived and peripheral blood neutrophils indicate that these cells are able to efficiently co-stimulate CD8+ T cell activation induced by T-cell receptor (TCR) triggering, as witnessed by increased expression of CD69 activation marker,

proliferation and IFN-γ release. Furthermore, an expansion of cells expressing a "central memory" phenotypic profile was also observed in cultures stimulated in the presence of autologous neutrophils.

Furthermore, importantly, malignant transformation in the intestinal mucosa is associated with early translocation of microorganisms from the gut lumen (9). A variety of bacterial strains including *Bacteroides fragilis* and *Fusobacterium nucleatum* have been found to be highly represented in CRC (10). Capitalizing on these data, I have investigated the potential significance of the interaction with bacterial strains associated with CRC in the elicitation of the anti-tumor effects of neutrophils. I found that interaction with *Fusobacterium nucleatum* rapidly induces apoptosis in neutrophils and abrogates their co-stimulatory capacity. Taken together, my results contribute to the identification of CRC microenvironment as typically characterized by a "ménage à trois", including cancerous cells, the immune system and gut colonizing microorganisms. The nature of the reciprocal interaction of these actors and its outcome are likely to decisively impact on CRC development and progression.

CHAPTER I: Introduction

1. Human colorectal cancer

Colorectal cancer (CRC) was characterized by a relatively low incidence only some decades ago. Nowadays however, it has become a predominant cancer type accounting for approximately 10% of cancer-related mortality and indeed represents the third most common cancer worldwide. CRC affects both sexes, and the majority of the cases occur in highly developed areas, including Australia, New Zealand, Northern America, Europe and Japan. In contrast a lower incidence of CRC is observed in Latin America, Africa and India. The main reasons explaining the higher incidence of CRC in the last decades and in the most industrialized regions of the world may include evolving dietary habits, physical inactivity, excess body weight and ageing populations (11).

Genetic and environmental factors also play a crucial role into CRC incidence and development. Although the majority of CRC cases are sporadic, up to 20% of CRC patients have a positive family history. Most frequently hereditary CRC cancers are associated to the Lynch syndrome, characterized by mutations in one of DNA mismatch-repair genes: *MLH1*, *MSH2*, *MSH6* or *PMS2* (12). The second most common hereditary syndrome is caused by mutation in the adenomatous polyposis coli (APC) genes (13).

Environmental and genetic factors promote the acquisition of hallmark characteristics of cancer in colon epithelial cells (14). Once these hallmarks are acquired, the progressive accumulation of epigenetic and genetic mutation leads to the activation of oncogenes and the neutralization of tumour suppressor genes. Therefore genomic instability may be considered as a trigger of the early neoplastic lesions in colon that could degenerate in cancer (15). Stem-like cells residing in the basis of the colon crypts have been suggested to represent CRC-initiating cells, essential for the generation and maintenance of the tumour.

In the "classical" CRC formation model, cancers arise from an aberrant crypt, and evolve into early adenomas that may progress to advanced adenoma and, finally, colorectal cancers (16). Additionally, epigenetic alterations appear to cooperate with gene mutations driving cancer progression. DNA methylation alterations related to cancer development may include hypermethylation of CpG islands in gene promoters silencing tumour suppressor genes, and hypomethylation of repetitive genetic elements leading to genetic instability and oncogene activation. These molecular alteration frequently depend on the location of the tumour within the gastrointestinal (GI) tract, thus supporting the notion that specific micro environmental

features, including microbioma and stromal cell infiltration, may modulate disease development and progression (17).

Based on molecular characteristics, CRC may be classified into four subgroups including hypermutable microsatellite stable, hypermutable microsatellite unstable, microsatellite stable or chromosome unstable, and CpG island methylator phenotype (CIMP) cancers. The various specific mutations that characterize the different subgroups are still largely under investigation, even though some of them are in common to all groups such as, for instance, those in APC, SMAD family member 4 (SMAD4) and catenin (CTNNB1) genes, KRAS and BRAF oncogenes, TP53 tumour suppressor genes and those involved in the WNT, RAS-RAF-MAPK, TGFβ and PI13-AKT pathway.

Importantly, the development of colorectal cancer is also associated with the inflammatory bowel disease (IBD). Indeed the overall CRC risk for patients with IBD has been estimated between 4- to 20-fold (18) higher than in control subjects, although IBD explains only 1% of CRC in western populations.

1.2 Colorectal cancer prognosis

CRC prognosis has slowly improved during the past decades. Routine staging relies on UICC-tumor node metastasis (TNM) and American Joint Committee on Cancer criteria classification, representing the gold standard of prognostic factors in CRC. This classification still provides the basis for therapeutic decisions, even though the clinical course in individual patients is frequently poorly predictable (19).

Overall, 5-year survival rates for patients with CRC are largely dependent on TNM stage (20) (Figure 1). However, in addition to TNM, a variety of tumour related features have been identified as essential prognostic factors. For instance, venous and lymphatic invasion represent important steps in the formation of metastases and, eventually, macroscopic tumour growth at a secondary site. Indeed, both these features were identified as independent adverse prognostic factors in multivariate analyses (21).

Additional prognostic factors are represented by tumour grade (percentage of gland formation), tumor budding (transition from glandular structures to single cells beyond invasive margin) and tumor border configuration (infiltrative margin) (22-24).

Remarkably however, patients with tumors with identical morphological features display an extensive heterogeneity in terms of clinical outcome. Microsatellite instability analysis gives important contributions regarding prognosis and therapeutic decision making in CRC. Indeed, previous studies have demonstrated that tumours with microsatellite instability hypermutability (MSI-H) show a significantly improved survival time as compared to microsatellite stable (MSS) CRCs. Moreover MSI-H phenotype may predict chemotherapy outcome. For instance, MSI-H CRCs do not appear to benefit from adjuvant therapy with fluorouracil but, instead, show an improved response to irinotecan-based chemotherapy. Finally, MSI is associated with high density tumor-infiltration by lymphocytes, possibly contributing to the improved prognosis of these patients (25-27).

Since local immune cell infiltration has been shown to represent a potent prognostic factor, ongoing studies aim at developing immunoscores as novel instruments of CRC classification (see below).

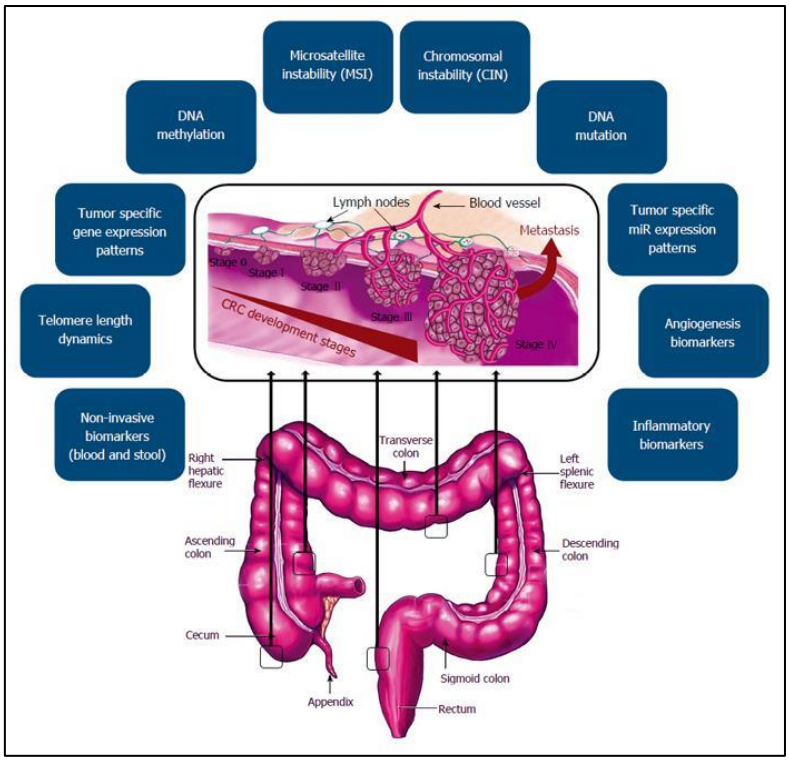


Figure 1. Different classes of molecular and cellular biomarkers in CRC development stages. (Aghagolzadeh P. *et al*., World J Gastroenterol, 2016)

2. The tumour immune contexture

Immune infiltrates are heterogeneous and very diverse in cancers of different histological origin and from patient to patient in cancers of similar origin. A large variety of immune cell types may be observed within tumour microenvironment, including dendritic cells, macrophages, mast cells, natural killer (NK) cells, neutrophils, naive and memory lymphocytes, B cells and T cells, including T helper 1 (T_H1), T_H2, T_H17, regulatory T (Treg), and cytotoxic T cells (28;29).

Immune cells may be located in the center of the tumour, or also in the invasive margins. Based on immune contexture, including density and functional orientation of infiltrating cells (29-31), studies in large annotated collections of human tumours have identified features associated with favorable or severe clinical course. Moreover, the role of chemokines and cytokines involved in shaping the immune contexture has also been investigated at the protein and gene level by bioinformatics tools.

Variable numbers of infiltrating immune cells may be observed in different tumours of the same histological type, and in different locations within and around the tumour (Figure 2). Innate immune system cells, including myeloid cells such as neutrophils and macrophages infiltrating or surrounding tumour beds are frequently detectable both in the core and at the invasive front of the tumour. Immature dendritic cells are preferentially distributed in the tumour core, in contact with tumour cells or in the surrounding stroma, while mature dendritic cells are mainly concentrated in the adjacent tertiary lymphoid structures (TLS), in close contact with naive T cells (29).

Lymphocytes are also not randomly distributed but located in specific areas. NK cells are usually found in the stroma and are not in contact with tumour cells. In contrast T cells, and particularly $CD8^+$ T cells, are mostly located in the invasive margin although they can also be located in tumour core.

Based on immune contexture evaluation, relatively simple scoring systems have been developed, and a high infiltration by CD3+/CD8+/CD45RO+ lymphocytes has consistently been reported to be associated with positive clinical outcomes (32). These data suggest that the interaction between cancer and immune system has significant implications for the clinical outcome and the identification of innovative prognostic markers, possibly also predicting responsiveness to chemo- and radiotherapy.

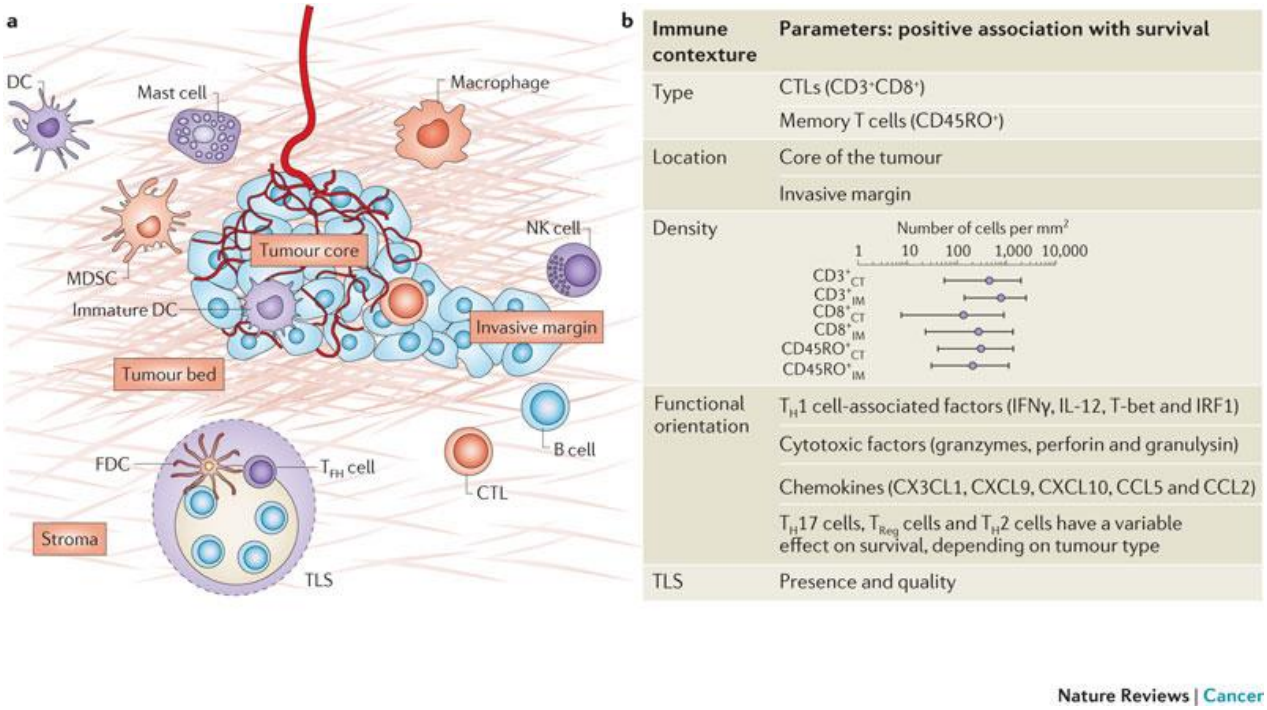


Figure 2. The immune contexture. Tumour anatomy showing features of the immune contexture, including tumour core, the invasive margin, tertiary lymphoid structures (TLS). The distribution of different immune cells is also shown. b | Table depicting the parameters of the immune contexture that predict a good prognosis. CT, core of the tumour; CTL, cytotoxic T lymphocytes; DC, dendritic cells; FDC, follicular dendritic cells; IFNγ, interferon-γ; IL-12, interleukin-12; IM, invasive margin; IRF1, interferon regulatory factor 1; MDSC, myeloid-derived suppressor cells; NK cell, natural killer cells; T_H, T helper cells; T_{Reg} cells, regulatory T cells**.** (Fridman W.H. *et al*., Nature Reviews Cancer, 2012).

Similarly to other cancer types, CRC is infiltrated by an extensive diversity of cells, including cancer associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), adipocytes, pericytes, endothelial cells (33) and immune cells that may change their role temporally and spatially within the tumour tissue (34). However, at difference from other tumors, the microenvironment of colorectal cancer is peculiar since during tumorigenesis microorganisms presented into the gut lumen may translocate through the epithelial barrier and enter in direct contact not only with tumor cells but also with immune cells creating a more complex scenario. To investigate how specific immune cells behave within this tumor scheme may lead to the development of new therapeutic strategies.

2.1 Adaptive immunity and colorectal cancer

Immune cells play important roles in CRC progression and even when the tumour becomes clinically detectable, adaptive immune response may play a crucial role in preventing cancer metastasis and recurrence (35) as observed in other tumour types, as well.

CRC infiltration by various subsets of immune cells of the adaptive immune system, including CD8+CD45RO+, IRF-1+ memory T cells and FoxP3+ regulatory T cells, predicts prolonged patients' survival (36). In particular Galon and his group have analyzed by gene expression and immunohistochemistry type, density and location of tumor associated lymphocytes (TILs) in an extensive numbers of CRC specimens. They identified a prevalent cluster of Th1-related genes whose expression is inversely correlated with tumor recurrence. Moreover, by evaluating the level of CD3+, CD8+, CD45RO+ T cells and granzyme+ cell infiltration, they demonstrated that adaptive immunity promotes patients survival. Because T-cell infiltration is not spatially homogeneous in CRC, attention has been focused on the predictive values of T lymphocytes located in the center of the tumor (CT) and along the invasive margin (IM). The combined analysis of both tumor regions has improved the accuracy of survival predictions, as compared with single region analysis (36).

Cytotoxic T lymphocytes (CTLs) are CD8+ cells representing one of the leading effector cell type in antitumor immunity by recognizing antigens expressed by tumor cells in association with HLA class I determinants (37). Upon triggering of their antigen specific T-cell receptor (TCR), CD8+ clonally expand and differentiate. Once activated, CD8+ cells are able to elicit "killer" lymphocyte functions, resulting in the destruction of tumor cells by either perforin and granzyme release or by Fas ligand-mediated mechanisms. Moreover CTLs also produce

cytokines, such as IFNγ inhibiting the proliferation of tumor cells and promoting their apoptosis.

CD4+ T lymphocytes, responding to antigens restricted by HLA class II determinants, are also important for antitumor immunity. In particular, Th1 CD4+ T cells do produce IFNγ and TNFα and support the proliferation of cytotoxic CD8+ cells by IL-2 release.

Beneficial effects resulting from infiltration by immune cells of the adaptive immune system could persist during the tumor progression leading to a lower risk of metastasis. However, the immune reaction in tumors is much more complex and might include the recruitment of cells playing opposite effects.

2.2 Innate immunity in colorectal cancer

Several types of immunotherapy are taking advantage from the induction or exogenous administration of high numbers of CTLs. However, their use in the treatment of solid tumors is more limited, possibly due to the complexity of the tumor microenvironment which may strongly inhibit the elicitation of the cytotoxic effects of CD8+ cells. Therefore, the generation of tumor-specific CTLs may not suffice for the implementation of clinically effective anticancer immune responses. The investigation on the role of the other components of the tumor microenvironment is therefore essential (35).

Macrophages are considered as one of the most abundant immune populations within the tumor-microenvironment. Although various *in vitro* studies have shown that activated macrophages acquire the capacity to kill tumor cells, for instance by Fas ligand-dependent mechanisms, many studies have suggested that TAMs, conditioned by the tumor microenvironment, may exert pro-tumor functions (38). Indeed a biological peculiarity of the macrophage lineage cells consists in their capacity to express different functional programs in response to various microenvironment stimuli. For instance during early stages of tumor growth, TAMs appear to have tumoricidal and antigen-presenting cells activity, and to produce inflammatory and Th1 cytokines. In this phase TAMs are identified as classical M1-like cells (38). Subsequently, during tumor progression, macrophages may polarize towards an M2-like phenotype, supporting angiogenesis and displaying immunosuppressive effects by producing soluble factors, such as IL-10 (39). Moreover, TGFβ production by TAMs has been shown to be involved in the ephitelial-to-mesenchymal transition (EMT) process frequently associated to metastasis formation (40).

Regarding their prognostic significance, in general terms, high density TAM infiltration has been consistently associated with unfavorable prognosis in a majority of tumor types (41;42). However, interestingly, their role in CRC is still controversial (43;44).

Thus, although TAMs are considered as a promising immunotherapy target, the application of specific treatments to CRC has to wait for a more precise definition of their role.

DC infiltration represents a significant prognostic factor in patients with CRC. In particular they appear to be more abundant during early stages of tumor progression (45). However, on the other hand, driven by tumor microenvironment, DCs may become functionally ineffective and promote tumor escape from immune response by failing to stimulate T lymphocytes.

In addition to TAMs and DCs cells, functions and prognostic significance of CRC infiltration by other cell types of the innate immune system requires a more accurate analysis. Therefore during the last decade our group has started to explore these issues by using a clinically annotated tissue microarray (TMA) including over 1400 cases.

First, we have shown that CRC infiltration by both CD56+NK cells and CD8+ T is associated with prolonged patient survival. In contrast, CRC infiltration by NK cells in combination with CD4+ lymphocytes has no detectable effect on the clinical outcome. These data may suggest a crosstalk between NK and CD8+ cells in the tumor microenvironment and provide a helpful prognostic information (46). Interestingly CRC is not the only tumor type where NK-CD8 interplay impacts the overall survival of patients. For example in head and neck cancers it has been shown that NK cells activated by cetuximab cooperate with dendritic cells to trigger tumor antigen-specific T cell immunity (46). However the functional role of NK cells has not been fully understood and whether CD56+ cells "per se" may represent a potentially positive prognostic marker still has to be elucidated.

Subsequently, we observed that CRC infiltration by CD16+ myeloid cells is associated with favorable outcome. Interestingly, our flow-cytometry analysis indicates that CD16+ cells infiltrating CRC biopsy do not express CD56 and HLA-DR molecules. Therefore, we could speculate that NK, DCs and monocytes are not included in this CD16+ cells subset (7), which might rather comprise neutrophilic granulocytes.

Based on these findings, our lab has developed a particular interest regarding the identification of neutrophils in patients with CRC. In initial studies, we observed that CRC infiltration by myeloperoxidase (MPO+) cells represents an independent marker of favorable prognosis.

Although the majority of the MPO+ cells are also CD66b+, we observed that MPO+ cells only represent a subset of CRC infiltrating CD66b+ neutrophils (6).

In subsequent studies we have additionally observed that MPO+ and CD8+ T cells infiltrating CRC do not appear to synergize in determining a more favorable outcome, as compared with cancers showing $MPO^{high}/CD8^{low}$ or $MPO^{low}/CD8^{high}$ infiltrates. It is tempting to speculate that CRC infiltration by these cell types might reflect different phases of immune response to CRC. However this data have contributed to the identification of a subgroup of CRC with a particular severe prognosis, characterized by $MPO^{low}/CD8^{low}$ tumor infiltration. Probably, patients bearing these cancers could be eligible for adjuvant treatments following surgery, irrespective of conventional TNM staging. (47).

The role of neutrophils in CRC has not been explored in detail. Data from other groups suggest that high tumour infiltration by CD66+ neutrophils may correlate with either benign or poor prognosis in patients with CRC. In particular, in a cohort of East Asian patients (n=229) neutrophil infiltration was found to be associated with severe prognosis (48). Different genetic backgrounds in the cohorts of patients under investigation, e.g. from East Asia or Western Europe, could also play a role. Moreover, differences in gut microbiomes of patients from disparate geographic areas might also be involved.

In contrast, most recently, neutrophil infiltration in CRC was reported to be associated with responsiveness to 5-fluorouracil (5FU) treatment (49). Indeed, Jaillon *et al* have shown that CD66b is a reliable marker for the identification of TANs in CRC tissues. Indeed not all MPO+ cells are also CD66b+. Most importantly, they have observed that TAN density dramatically decreases in Stage IV patients as compared to Stage I-III (n=271). High TAN density was proposed to be associated with a favourable prognosis and prognostic significance could be influenced by clinical stage and 5FU-based chemotherapy.

Since clinical significance of CRC infiltration by TAN is still largely unclear and functional mechanisms remain to be elucidated, I have addressed these issues in the context of my PhD project.

Within the context of innate immunity, heterogeneous populations of immunosuppressive cells also need to be considered. In particular, myeloid derived suppressor cells (MDSC) have been indicated to be present in tumor and lymphoid tissues (50). These cells are much better characterized in murine models than in humans. In particular, in experimental animals, it has been shown that they may secrete various proangiogenic factors such as VEGF-A, PDGF-B,

FGF, metalloproteinases, and chemokines such as IL-8 within the tumor microenvironment (51;52). However, human MDSCs have not been studied in comparable detail, but rather identified as heterogeneous population of myeloid cells which may include immature granulocytes. Nevertheless MDSCs have been implicated in the resistance to antiangiogenic therapies in CRC (53) and high tumor infiltration has been suggested to be associated with poor prognosis (54;54-56). Therefore further investigation is warranted to identify new markers efficiently discriminating different populations within human MDSCs (57).

2.3 CRC immunotherapy

Immunotherapy is frequently used either alone or in combination with chemotherapy for the treatment of a variety of cancers, including melanoma, lung cancers and hematological malignancies. Its use in CRC is currently being explored in different Institutions. Nowadays, CRC treatment generally consists of chemotherapy, anti-angiogenic treatments and epidermal growth factor receptor (EGFR) inhibitors. However, response to treatment is usually limited to a minority of patients and responding patients may develop resistance to the treatment. Therefore the development of innovative therapeutic approaches is urgently needed to improve clinical outcomes (58).

Immune surveillance has been proposed as a process by which innate and adaptive immune systems together concur in detecting and destroying cancer cells. Immunomodulatory cells, cytokines, stromal cells from tumor microenvironment are involved in immune surveillance of CRC. However, the ability of the cancer cells to escape immune responses of the host has also been clearly documented. CRC cells not only evade immune response but may also manipulate the immune system to promote tumor growth and metastasis (59).

Cytokines, monoclonal Abs, adaptive T cell therapies, peptide, protein or whole tumor cell and dendritic cell vaccines are included among immunotherapeutic strategies that have been clinically evaluated in Colorectal cancer. Even though, so far only therapeutic antibodies such as bevacizumab, aflibercept, cetuximab and panitumumab are FDA approved for CRC treatment (60;61).

Peptide-based T-cell vaccines require identification of peptide epitopes recognized by T cells as tumor associated antigen. In this frame, carcinoembryonic antigen is the most frequently targeted antigen in CRC vaccine trials (62-64). Dendritic cells are potent APCs with a unique ability to present antigen and activate $CD4^+$ and $CD8^+$ T cells. They express all costimulatory molecules required for potent immune response. They are easily obtained from peripheral blood and hence they are widely used in the vaccine therapy trials for CRC treatment.

Immune-checkpoint pathways modulate T-cell responses by influencing communication between T cells and antigen-presenting cells. They may be targeted by therapeutic monoclonal antibodies such as those recognizing programmed cell death-1 (PD1), PDL1 and CTLA-4. Studies with combinations of anti PD1 and anti VEGFR2 in mouse model of CRC resulted in dramatic inhibition of tumor growth (65). In contrast to melanoma, renal cancer, lung cancer, CRC cohorts have shown low response rates to PD-1, PDL-1 interaction blockade.

Overall, mismatch repair deficient tumors are highly infiltrated by CTL, displaying a high PD-1 expression, thus suggesting that the immunotherapeutic interventions involving checkpoint blockade might be selectively effective in this important subset of cancer, and mismatch repair-proficient might be much less responsive (66) (Figure 3).

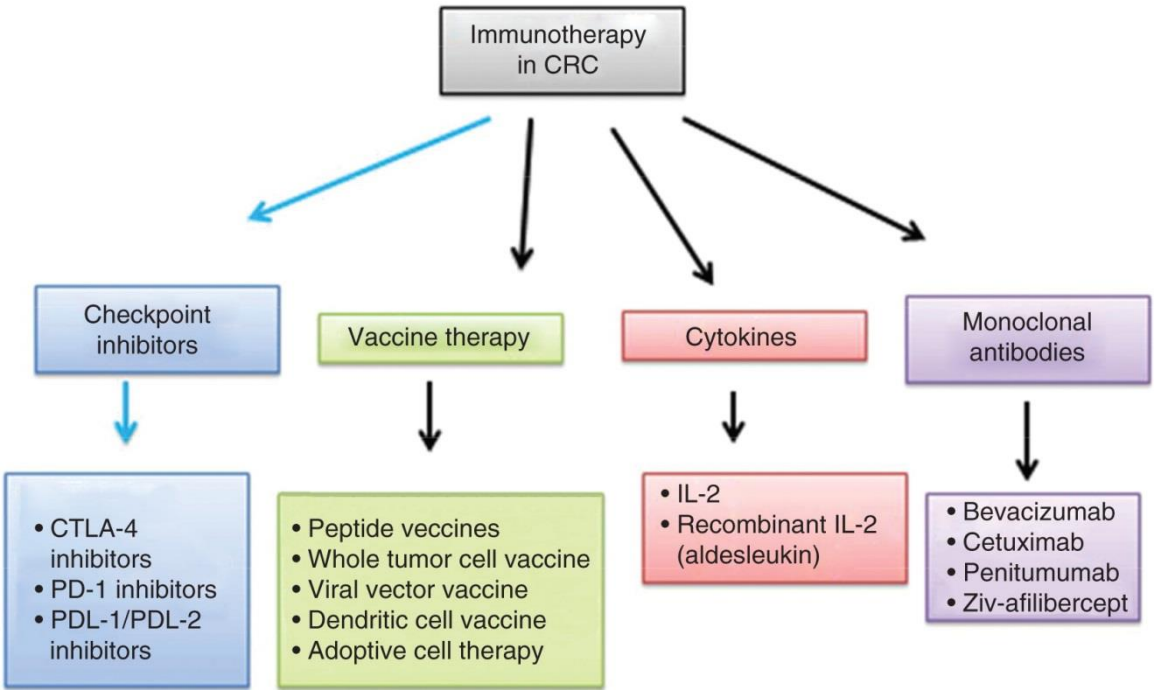


Figure 3. Schematic overview of immunotherapy in colorectal cancer. (Manik A.,*et al* Expert opinion on investigational Drugs, 2014)

3. Neutrophils in cancer

Neutrophils account for 50-70% of all leukocytes in humans and represent a first line defence against a variety of bacterial and fungal infections (67). Chemokines recruit neutrophils to the sites of infection/inflammation where they phagocytize and destroy bacteria by microbicidal activities, including release of reactive oxygen species (ROS), enzymes (MPO), and neutrophils extracellular traps (NET).

In steady-state conditions, the bone marrow devotes about two-thirds of its space to the formation of neutrophils and monocytes (68). During granulopoiesis, neutrophils are generated as lymphoid-primed multipotential progenitors (LMPPs), further differentiating into granulocyte-monocyte myeloid progenitor (GMPs). Subsequently, they start a maturation process including progression through the following steps: myelobast, promyelocyte, myelocyte, metamyelocyte, band neutrophils and, lastly, segmented mature neutrophils (67) (Figure 4). During the different maturation steps, primary, secondary and tertiary granules are formed and stored together with defensive factors and enzymes, such as arginase, elastase and myeloperoxidase, crucial for the protection against infections. At difference with other immune cells, neutrophils are released by the bone marrow as terminally mature cells. However, atypical production of cytokines by tumour or stromal cells may affect the balance of neutrophils retention and release from bone marrow. For instance, in tumour-bearing mice the pressure on the bone morrow can be so intense to induce the release of immature neutrophils and this anomaly has also been noted in tumour patients (69) (Figure 3). The different composition of granules within immature neutrophils leads them to assume functions different from the mature subset and this may have profound consequences on tumour progression. By using density gradient purification it has been shown that different populations of neutrophils with diverse ex vivo properties can circulate within the same tumour-bearing mouse. Still unclear is whether these distinct populations are truly committed to divergent cell destiny or represent cells at diversified stages of maturation (69;70). Tumours share some basic chemotaxis mechanism coordinating neutrophils recruitment to inflammatory sites. For instance, tumour cells, together with stromal cells present in tumour microenvironment, produce diverse chemokines as CXCL8, CXCL1, CXCL5, CXCL2, and cytokines, GM-CSF, G-CSF IL-17 which do attract and activate neutrophils (71;72).

So far, one of the reasons why neutrophils have not been recognized as important player into tumour microenvironment is related to their short life span. Currently it is known that circulating neutrophils life-span is around 7h in human and 8-10h in mice; in animal models

non-circulating neutrophils can survive in tissues for several days (73). However neutrophils may be longer retained in tumour tissues as compared to the spleen suggesting that specific microenvironments may support their survival locally and systemically. It is reasonable to speculate that neutrophils with a longer life span might have more time to synthesize new molecules and perform additional effector functions during tumour development (67).

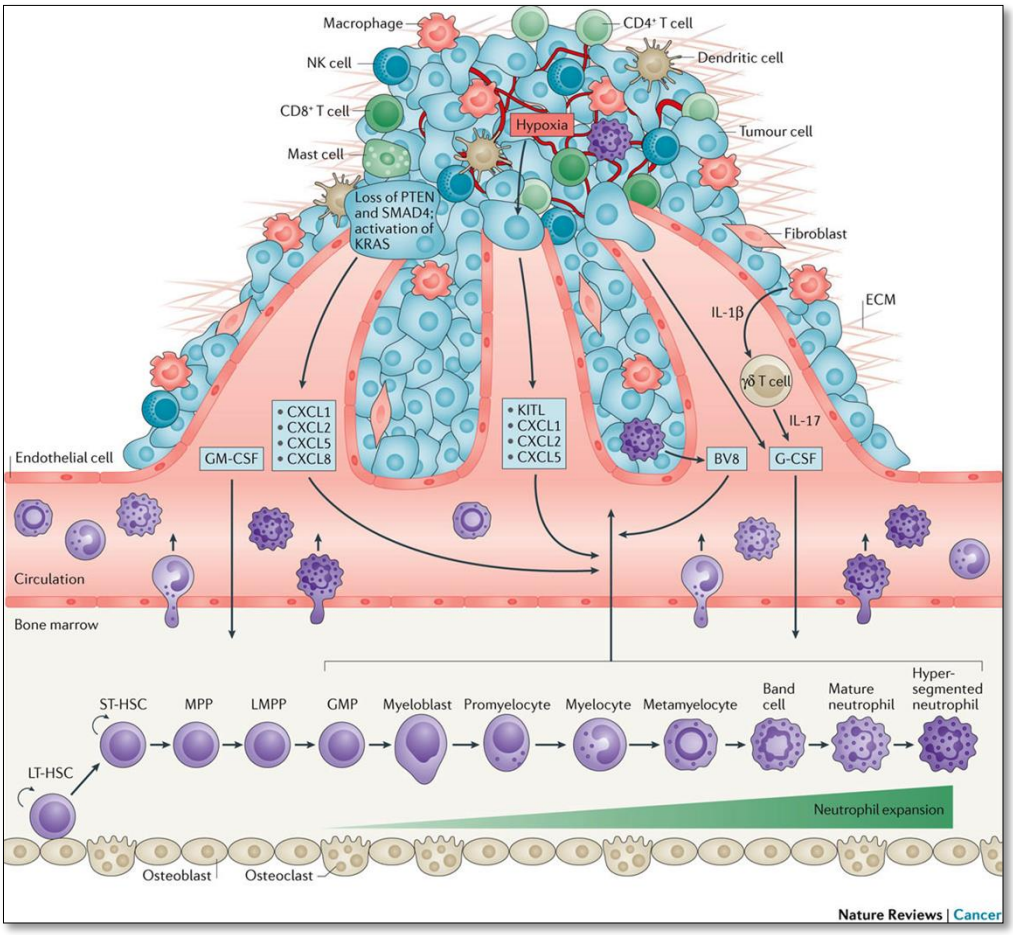


Figure 4. Tumour-induced emergency granulopoiesis. Tumours affect both the development and the release of bone marrow neutrophils. Tumour-induced increases in the levels of granulocyte-colony stimulating factor (G-CSF) and granulocyte–macrophage-colony stimulating factor (GM-CSF) alter haematopoiesis towards production of myeloid cells, thereby increasing the generation of granulocyte–monocyte progenitors (GMPs) and neutrophil progenitors. In addition, tumours interfere with neutrophil retention in the bone marrow by upregulating various cytokines and chemokines. The composition of these mediators depends on the tumour type, mutations and oxygen levels in the tumour. Neutrophil-derived BV8 also induces neutrophil expansion. This pressure on the bone marrow induced by the tumour may result in increased generation and release of immature granulocytes. (Seth B. *et al.*, Nature Reviews Cancer 2016).

Based on this background, many studies have highlighted the importance of tissue-resident neutrophils in oncology (74). In tumor-bearing mice, by analogy with polarized macrophages, tumor-associated neutrophils (TANs) have been classified in N1 and N2 (75). Similarly to M1, N1 are considered anti-tumorigenic ($TNF\alpha^{high}$ $ICAM^{high}$,$CCL3^{high}$, $arginase^{low}$) and, similarly to M2, N2 are considered pro-tumorigenic cells ($TNF\alpha^{low}$ $ICAM^{low}$,$CCL3^{low}$, $arginase^{high}$ $MMP9^{high}$ $VEGF^{high}$ Fas^{low}) (Figure 5). In particular, Fridlender has shown that transforming growth factor-b (TGFβ), can induce the N2 pro-tumor phenotype: indeed upon blocking of TGFβ, CD11b+ /Ly6G+ cells appear to be more segmented, capable to release pro-inflammatory cytokines, and to impair tumour growth by their cytotoxicity ability.

For example, in two murine cancer models (lung carcinoma and mesothelioma) neutrophils were found primarily at an early stage of tumor progression where they elicited their cytotoxic activity against tumor cells by releasing TNFα and H_2O_2. Other mechanism inducing anti-tumorigenic functions by TANs are represented by the production of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (76) and the release of matrix metalloproteinase (MMP-8). Last but not least, neutrophils may modify recruit and activate T cell effector functions that are the main contributors to immune response against tumors (77).

However, TANs may become more pro-tumorigenic during tumor progression. For instance activated neutrophils release arginase 1 (ARG1) able to degrade extracellular arginine, an amino acid playing an essential role in T cell activation. This may trigger immunosuppressive effects similar to those described for G-MDSC. Depletion of TANs in tumor bearing mice has been shown to increase the number of activated CD8+ cells resulting in the generation of smaller tumors (78). This may suggest that TANs can assume a different role based on the tumor microenvironment. However phenotypes and transcription factors regulating this polarization remain largely unknown. Moreover the study proposing N1, N2, terminology, characterized N1 as cells with hypersegmented nucleus and N2 as cells with banded or ring nucleus. Since morphology is a hallmark of neutrophils differentiation, it remains unclear whether N2 cells represent a subset of immature neutrophils or authentically different polarization state, leaving basically unresolved the relationship between maturation and polarization (78). Therefore N1 and N2 classification appears to be reductive, because a polarization could probably exists as a spectrum of different activations stages occurring within the tumor microenvironment (79).

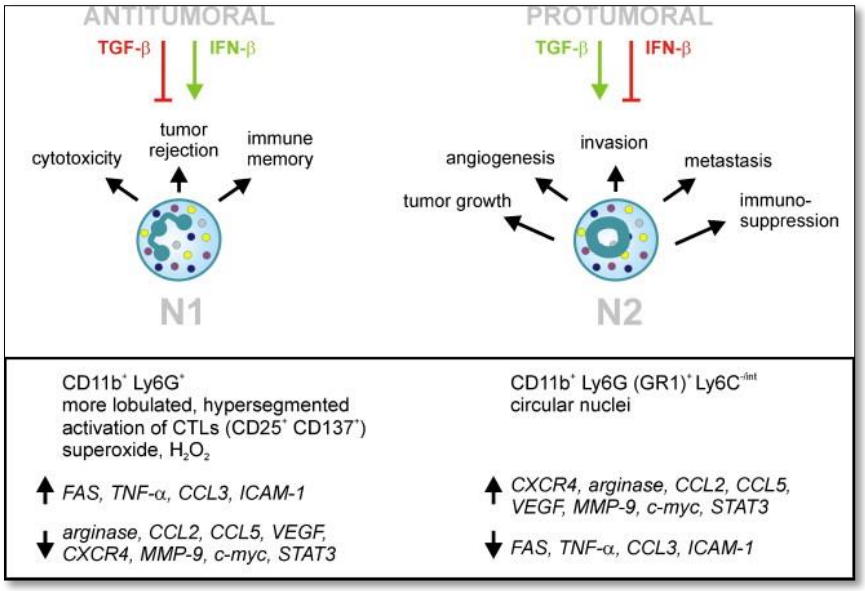


Figure 5. A simplified scheme of tumor-associated neutrophils (TAN) polarization. Neutrophils exert both anti-tumoral and pro-tumoral functions during cancer development and progression. This phenomenon is referred to as "neutrophil polarization" and is influenced by TGF-β and IFN-β cytokines. On this basis, neutrophils may serve as anti-tumoral effector cells by inducing cytotoxicity and by mediating tumor rejection and backing anti-tumoral immune memory response (N1 phenotype). In contrast, neutrophils may also support tumor progression by promoting angiogenesis, invasion, metastasis and immune suppression (N2 phenotype). (Piccard H. *et al*., Critical Reviews in Oncology/Hematology 2012).

A further complication to the picture of neutrophils subtypes is represented by the ongoing debate regarding the relationship between neutrophils and myeloid-derived-suppressor cells (MDSC) because is still unclear whether the latter they do indeed represent a distinct population. There is currently no way to uniquely distinguish MDSCs from neutrophils. However, an accurate comparison of mRNA profiles of naive neutrophils, G-MDSC and TANs has shown that TANs exhibit a completely different transcription programs, including up regulation of chemokines and cytokines genes, as compared to the other two subsets which, in contrast, appear to be more similar to each other (80). Importantly, recently differences in morphology, surface markers, functions and prognostic importance of the different tumor-related circulating neutrophils have been proposed (81) (Figure 6).

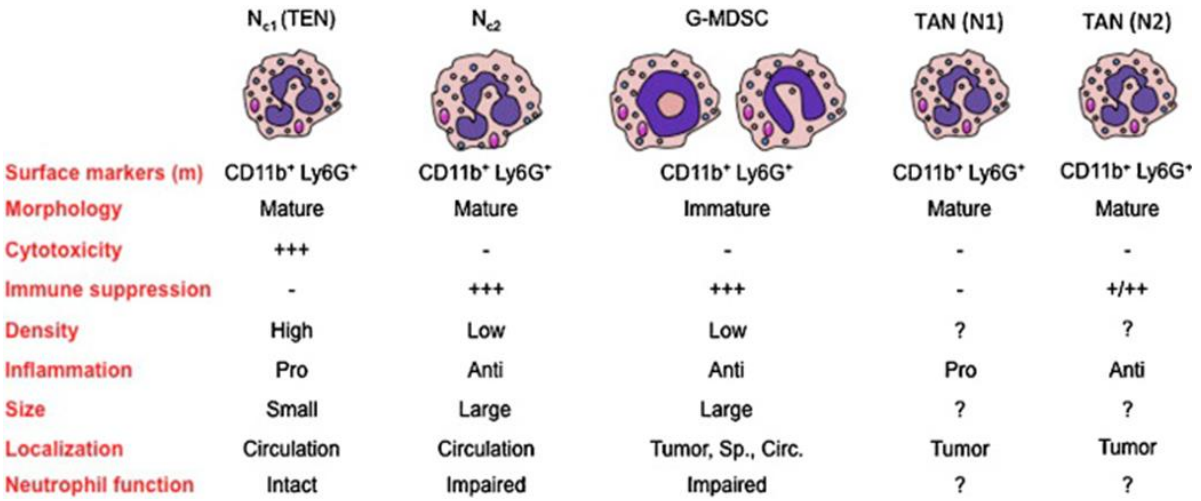


Figure 6. Diversity in the morphology and function of murine circulating neutrophil sub-populations. The characteristics of the neutrophil sub-populations are shown. Nc1 (TEN)—HDN circulating neutrophils, Tumor entrained neutrophils; Nc2—mature LDN circulating neutrophils; G-MDSC—Granulocytic myeloid derived suppressor cells; N1 and N2 TAN—Tumor associated neutrophils. (Inbal M *et al.*, Immunobiology 2016).

Similarly to primary tumor growth, the role of neutrophils is also controversial in metastasis formation. It has been shown that CD11b+ cells are associated with priming of pre-metastatic lung cancer, and enhance the seeding of circulating tumor cells (82) by MMP-9 release.

In fact, recent evidence suggests that neutrophils, upon stimulation by tumor–derived factors may contribute to a variety of steps conductive to cancer metastasis formation. As a matter of fact, neutrophils produce a large numbers of proteins able to support and facilitate tumor cell migration by remodeling extracellular matrix (ECM) through the release, immediately after activation, of a large intracellular pool of MMPs and serine proteases.

Furthermore neutrophils have also been suggested to trigger epithelial-mesenchymal transition (EMT) of tumor cells by releasing TGF-β, a primary inducer of EMT, or through elastase degrading E-cadherin on tumor cells (83). Additional studies show that neutrophils are the major cell type recruited in primary tumors and mobilized to metastatic sites where they express a spectrum of different genes, particularly Bv8, G-CSF and S100A8. Accordingly, treatment with anti-Bv8 reduces lung metastasis formation (84) (Figure 7). Finally, other studies have confirmed the role of neutrophils in fostering metastatic niches and facilitating metastasis by suppressing CD8+ T cells proliferation and activation (85).

Neutrophils may also use neutrophils extracellular traps (NETs) to sequester cancer cells promoting their adhesion to distant organs (86).

On the other hand, neutrophils have also been associated with anti-tumor and anti-metastatic potential in a lung mouse model (87) relying on *cMet* proto-oncogene (hepatocyte growth factor receptor) recruitment (88) (Figure 7).

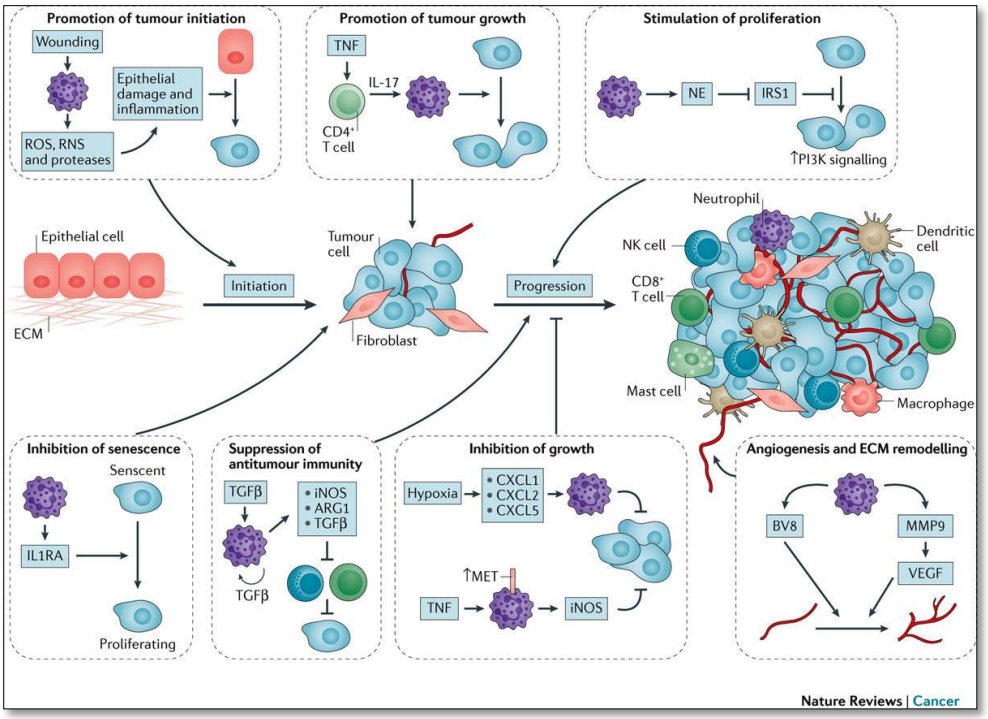


Figure 7. Neutrophil function in tumour initiation and growth. There are several mechanisms by which neutrophils either promote or limit tumorigenesis. Oncogenic transformation may be supported by the production of reactive oxygen species (ROS) and proteases. Promotion of tumour growth may also be mediated by crosstalk between neutrophils that are activated by tumour necrosis factor (TNF) and interleukin (IL)-17-producing CD4+ T cells. In addition to tumour initiation, neutrophils may promote progression of tumour growth by converting senescent cancer cells into proliferating cancer cells by IL-1 receptor antagonist (IL-1RA). Proliferation may be directly stimulated by transfer of neutrophil elastase (NE) to cancer cells. Neutrophils express inducible nitric oxide synthase (iNOS, also known as NOS2) or arginase (ARG1) suppressing CD8+ T cell-mediated anti-tumour immune responses. Immunosuppression can also be accomplished by transforming growth factor-β (TGFβ) activated neutrophils. In contrast, in defined contexts, neutrophils may also limit tumour growth. Upregulation of the hepatocyte growth factor receptor MET on neutrophils by endothelial cell-derived TNF may enhance their cytotoxic potential against cancer cells. Lastly, neutrophils participate in remodelling of the extracellular matrix (ECM) and induce angiogenesis by BV8 production and activation of vascular endothelial growth factor A (VEGFA) by matrix metalloproteinase 9 (MMP9). (Seth B. *et al*., Nature Reviews Cancer 2016).

Despite their major relevance in the immune system, the role of neutrophils in the human tumor microenvironment has been largely neglected and direct analysis of TANs in patients with cancer has started to be addressed only in the last few years.

TANs could originate from either mature or immature cells. Common markers to identify neutrophils in tissues include CD66b (89;90) and CD15 (91). Moreover, TANs may also be identified by expression of functional molecules such as elastase (NE), (92) and myeloperoxidase (MPO) (6;93). The immunohistochemistry represents a valid tool to localize neutrophils in human cancer biopsies, it does not provide any information regarding the maturation and/or activation state of TANs.

The existence of N1 and N2 polarized neutrophils has been postulated based on murine studies, but still unclear is whether a comparable analysis may apply to human cancers. Nevertheless, the clinical relevance of the interaction between neutrophils and cancer has recently begun to emerge (94). In particular, table 1 summarizes data addressing the role of neutrophils in peripheral blood and tumor tissue in clinical studies in different tumor types. It has been shown that TAN density can be correlated with poor prognosis, for instance in bronchoalveolar carcinoma (95) melanoma (96) renal carcinoma, head and neck squamous carcinoma (HNSCC) (97) liver carcinoma (98) and pancreatic adenocarcinoma (99). However, in CRC the potential role of neutrophils appears to be controversial (6).

Table 1. Tumor-Associated Neutrophils as a New Prognostic Factor in Cancer.

First Author	Year	Country of population	Sample size	Histology	Stage	First-antibody	Neutrophils location	Cut-off criteria	Cut-off value	Follow-up time	Hazard ratios	Survival analysis
Rosario Alberto Caruso	2002	Italy	273	Gastric	Ib- IV	- (HE)	Intratumoral	Mean	>10cells/20HPF	NM	Report[a]	OS
Frede Donskov	2006	Denmark	85	RCC	NM	CD66b	Intratumoral	Median	0 cells/mm^2	Median:57m(32–73 m)	Report[a], Author[a]	OS
Hanne Krogh Jensen	2009	Denmark	121	RCC	I-IV	CD66b	Intratumoral	Median	0 cells/HPF	Median:124 m (74–194 m)	Report[a+b+c], Author[a]	RFS, OS, CSS
Sokratis Trellakis	2010	Germany	40	HNC	III -IV	CD66b	Intratumoral	NM	Low: −,+; High: ++,+++	Median:69 m (43–124m)	SR [a]	OS
Dong-Ming Kuang	2010	China	200	HCC	I-III	CD15	Intratumoral, Peritumoral, Stromal	Median	Intratumoral:4; Peritumoral:54; Stromal:10	NM	SR[a+d], Survival curve[a+d]	DFS, OS
Yi-Wei Li	2011	China	281	HCC	I-III	CD66b	Intratumoral Peritumoral	Fixed	Intratumoral:70%, Peritumoral:50%	Median:29 m (1.5–83m)	Report[a+c], Survival curve[a+c]	RFS, OS
Lih-Chyang Chen	2012	China	140	HNC	NM	- (HE)	Intratumoral	Mean	10 neutrophils/ 100 epithelial cells	NM	Survival curve[c]	RFS
Qiang Gao	2012	China	240	HCC	NM	CD66b	Intratumoral	Median	12 cells/ mm^2	NM	Report[c]	RFS
Fang-Ming Gu	2012	China	123	ICC	I/II/IIIa/ IIIc	CD66b	Intratumoral, Peritumoral	Median	86 cells/ mm^2	Median:13m (4–111m)	Report[a]	OS
Marius Ilie	2011	France	632	NSCLC	I – III	CD66b	Intratumoral	Median	49 cells/mm^2	Median:30m (0–112 m)	Survival curve[a]	OS
Trine O. Jensen	2011	Denmark	186	Melanoma	I/II	CD66b	Intratumoral	Fixed	0 cells/2 HPF	Median:12.2y (10.4–14.2 y)	Report[a+b+c]	RFS,OS, CSS
Hui-Lan Rao	2012	China	229	CRC	I-IV	CD66b	Intratumoral	Mean	60 per TMA spot	Average:55.4m Median:60.0m (0.5–98 m)	Report[a], Survival curve[a]	OS
CH Richards	2012	UK	130	CRC	NM	- (HE)	Peritumoral	Median	High: score 2–3, Low: score 0–1	Median:105m (55–163m)	DE[b]	CSS
Jing-jing Zhao	2012	China	115	Gastric	I-IV	CD15	Intratumoral	Median	21.60 cells/HPF	NM	Report[a]	OS
Shao-Lai Zhou	2012	China	323	HCC	NM	CD66b	Intratumoral	NM	Hgih, Low	NM	Report[a+c]	OS, RFS
Andreas Carus	2013	Denmark	101	CC	IB-IIA	CD66b	Intratumoral, Peritumoral, Stromal	Median	Intratumoral:23.2cells/mm^2, Peritumoral:53.1cells/mm^2, Stromal:28.3cells/ mm ^{2}	NM	Author[c], Report[c]	RFS
Andreas Carus	2013	Denmark	335	NSCLC	I–IIIA	CD66b	Intratumoral, Peritumoral, Stromal	Median	Intratumoral:8.7cells/mm^2, Stromal:21.0 cells/ mm^2	NM	Author[a+c]	OS, RFS
Claudia A. Dumitru	2013	Germany	97	HNC	NM	CD66b	Intratumoral	Median	NM	NM	Survival curve[a]	OS
Claudia A. Dumitru	2013	Germany	83	HNC	I-IV	CD66b	Intratumoral	NM	High: medium and strong, Low: negative, weak	NM	Report[a]	OS
Y Ino	2013	Japan	212	PDC	NM	CD66b	Intratumoral	Median	NM	Median:18.8m (2.6–201m)	Report[a+d]	OS, DFS

Abbreviations: Gastric: gastric carcinoma; RCC: renal cell carcinoma; HNC: head and neck carcinoma; HCC: hepatocellular carcinoma; ICC: intrahepatic cholangiocarcinoma; NSCLC: non-small-cell lung cancer; CRC: colorectal carcinomas; CC: cervical cancer; PDC: pancreatic ductal carcinoma; HE: hematoxylin-eosin staining; OS: overall survival; CSS: cancer-specific survival; RFS: recurrence-free survival; DFS: disease-free survival; NM: not mentioned; a: OS; b: CSS; c: RFS; d: DFS; m: months; y: years; DE: data extrapolated; SR: systematic review [36].
doi:10.1371/journal.pone.0098259.t001

(Meixiao Shen *et al*., PLOS ONE, 2014).

On the other hand, the neutrophils/lymphocyte ratio in the peripheral blood of patients with cancer may be considered as an additional prognostic marker. In general terms, the neutrophil-to-lymphocyte ratio (NLR) is elevated in patients with more advanced or aggressive disease. Therefore a high NLR correlates with poor overall survival in many tumors (100;101). However, a high number of neutrophils in peripheral blood may also be associated with good prognosis, as, for instance, in gastric cancer (102). Thus, while the tumor microenvironment promotes the recruitment of neutrophils within the tumor, they might either control cancer progression or enhance it.

Mechanisms underlying the prognostic significance of neutrophils are still far from being fully clarified. Nevertheless, their role has been addressed in detail in human lung cancer. Eruslanov *et al* have shown that neutrophils are able to promote T cell proliferation and interferon-gamma (IFNγ) release in a cell contact dependent manner in the earliest stages of lung cancer, suggesting that TANs are not “per se” immunosuppressive but rather capable of stimulating T cell responses.

Moreover, they have demonstrated that TANs display a different phenotype and a different cytokines/chemokines profile as compared to peripheral blood neutrophils (PBNs). Based on these data, more recently, the same group has additionally identified a peculiar subtype of TANs exhibiting a hybrid phenotype and functional characteristics of neutrophils and antigen presenting cells (APC) such as macrophages and DCs. These APC-like, "hybrid" TANs are superior to canonical TANs in their ability to stimulate an anti-tumor T cell response. Importantly, the APC-like hybrid TANs were able to take up, degrade, and cross-present tumor antigens (103).

In conclusion, neutrophils so far appear to be characterized by conflicting functions with pro- and anti-tumorigenic roles depending on tumor microenvironment and stages, with mechanisms still largely unclear. Thus, while functions and prognostic significance of tumor- associated neutrophils require further investigation and importantly, it is becoming more and more clear that TANs have been to be considered as important players in malignant disease.

3.1 Neutrophils as therapeutic targets in cancer patients

The variable functions of neutrophils in tumorigenesis may represent important therapeutic targets. Ongoing clinical trials in patients with cancer are targeting CXCR1 and CXCR2 to inhibit neutrophils trafficking and activation (104). Another pathway under investigation is represented by the IL-23/IL-17 axis. Neutrophils specific enzymes, known to promote tumor progression such as MMP9 and NE, may also be targeted for therapeutic purposes. Additional therapeutic strategies combine neutrophil targeting with standard treatments (105).

An additional promising therapeutic approach is represented by the use of antitumor monoclonal antibodies (mAbs) to prompt the ADCC potential of neutrophils. Currently, new antibodies are being designed to enhance Fc receptors affinity and induce a stronger antitumor effect. In particular, FcαRI (CD89) appears to be the strongest inducer of ADCC by neutrophils. Therefore a new generation of cancer therapeutic biologicals could include IgA class antibodies to exploit neutrophils cytotoxicity (106). These advances urge the characterization of the polarization of neutrophils in the tumor microenvironment in order to envisage optimal therapeutic conditions.

4. Colorectal cancer and gut microbiome

The gastrointestinal tract is populated by a huge amount of microorganisms. However, while the microbial load is about 10^1 cells per gram of content in the stomach, it raises to 10^{12} cells per gram in the colon. So far, an extensive number of studies have explored the link between microbiota and colorectal carcinogenesis. Alterations of host-microbiome interaction, usually referred to as dysbiosis, rather than infections by specific pathogens, appear to be associated with CRC development (107).

However, still unclear is whether dysbiosis has to be considered causal or consequent to CRC development, and underlying mechanisms have not been elucidated, although immune and inflammatory responses are considered as fundamental factors leading to the alteration of microbial community.

Numerous studies have compared the microbial community composition in patients with CRC and healthy subjects to attempt understanding modifications eventually occurring in the gut. Early events during CRC development may result in the loss of the barrier function of the colonic epithelium, leading to the translocation of microorganisms products and enabling the colonization of the tumour microenvironment by invasive-adherent bacteria (108) (Figure 8).

An extended list of "carcinogenic" bacteria supports the hypothesis that tumorigenesis is driven by mechanisms common to multiple bacterial groups rather than to individual microorganisms. Analysis of fecal samples has shown that *Bacteroides fragilis*, *Enteroccocus feacalis*, and adherent and invasive *Escherichia coli* colonization increase in patients with CRC as compared to the healthy controls. Most importantly, *Fusobacterium nucleatum* appears to be the bacterial strain most reproducibly associated with CRC (Figure 9).

During the multistep development of CRC which is accompanied by morphological (barrier defects) and mutational (APC, K-RAS and p53 mutations) alterations, tumours may provide a selective pressure on bacteria, and particularly on anti-oncomicrobes which may subsequently be largely replaced by commensal gut bacteria as *F.nucleatum* (109).

Intriguingly, two recent studies have shown that the microbial community may impact on efficacy of chemotherapeutic drugs, thereby highlighting the importance of the interaction among microbes, immune system and pharmaceutical interventions (110).

Therefore, understanding the nature of microorganisms interfering with drug efficacy would be of prime importance for cancer therapy. The generation of microbiota signatures could allow

an optimal match of patients with chemotherapeutics drugs potentially leading to a personalized medicine based on the microbiota (111;112). As an example, some lactobacilli contribute to the generation of anti-tumour Th17 cells in cyclophosphamide CTX-treated mice (113). However, the same genus impairs CpG-oligodeoxynucleotides (CpG-ODN) efficacy (114). Furthermore, in murine models additional studies have also demonstrated that gut microbiota impact on DCs activation and anti-tumour T cell responses.

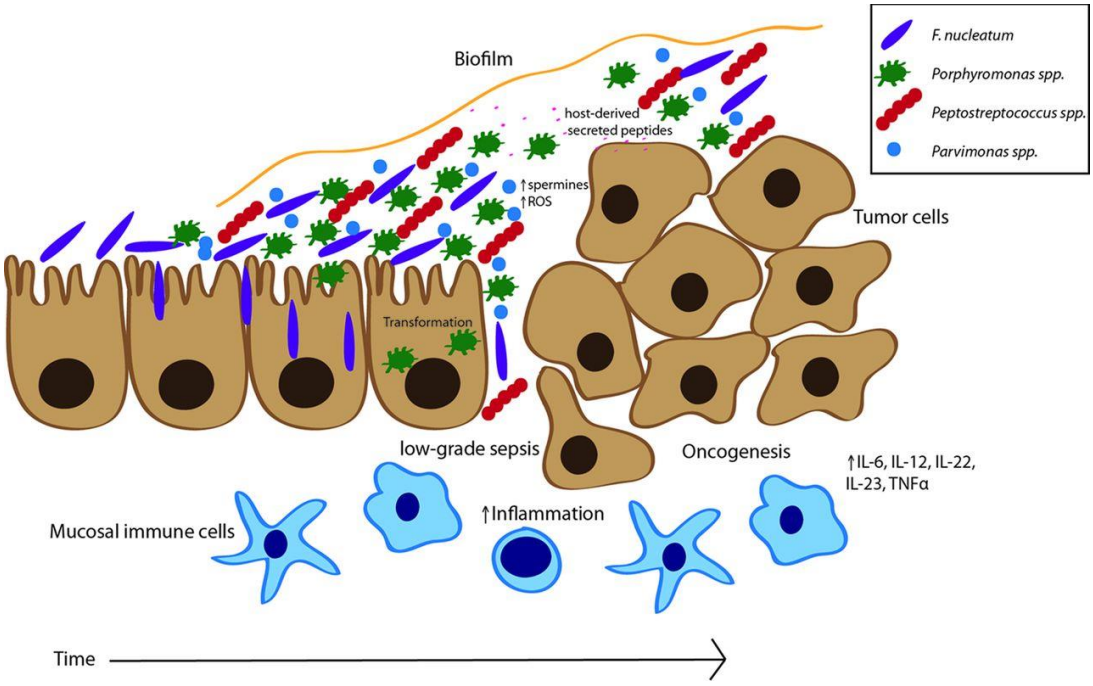


Figure 8. A model of oral microbial activities in colon tumorigenesis. In this model, oral microbes such as *F. nucleatum* colonize gut epithelial surface. *F. nucleatum* may act as a bridging organism, allowing other oral microbes to bind via compatible adhesins. *F. nucleatum* and Porphyromonas can invade epithelial cells, disrupting signaling and promoting transformation. Oral microbes form a biofilm community altering epithelial tight junctions and promoting infiltration and inflammation from mucosal immune cells. Transformation of epithelial cells leads to an oncogenic synergy where host-secreted peptides feed asaccharolytic oral microbes, which in turn produce reactive oxygen species (ROS) and polyspermines, promoting both biofilm formation and continued inflammatory responses potentially promoting the tumor growth. (Kaitlin J, *et al*., Host-Microbe Biology, 2016).

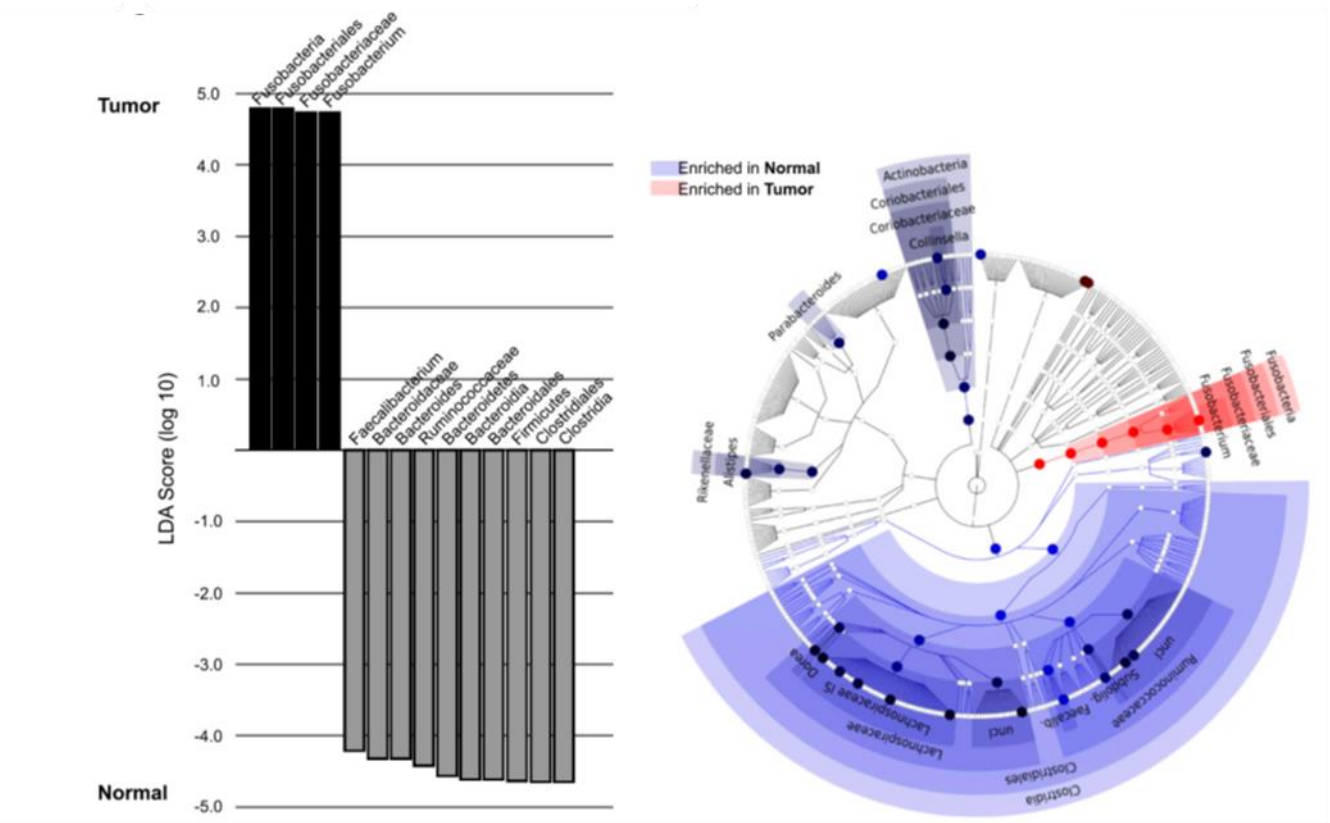


Figure 9. 16s rDNA analysis of the colorectal cancer microbiome. Linear discriminant analysis (LDA) and effect size measurements identify *Fusobacterium* as the most differentially abundant taxon in CRC versus normal mucosa by 16S rDNA sequencing in 95 individuals. Tumor-enriched taxa are indicated with a positive LDA score (black), and taxa enriched in normal tissue have a negative score (gray). Clade data are represented in red for tumor-enriched taxa and blue for taxa enriched in normal tissue. The brightness of each dot is proportional to its effect size. (Kostic AD. *et al*., Genome Research, 2011).

4.1 CRC and Fusobacterium nucleatum

F. nucleatum (*Fn*) is a Gram-negative, opportunistic, obligated anaerobic bacterium. It is normally prevalent in the oral cavity, where it is involved in the pathogenesis of periodontitis. Therefore it is not a primary colonizer of the human gut. The epidemiology of acquisition or colonic colonization by *Fn* is unknown. In particular, whether the oral fusobacteria commonly associated with periodontal disease are in fact related to the *Fn* detected in the colon requires further investigation.

Fn is a well-recognized pro-inflammatory bacterium and it has been found in IBD patients. Moreover, through complementary genomic methods analyzing the microbial associations of CRCs as compared to matched normal tissues, it has been shown that *Fn* might potentially contribute to CRC pathogenesis (115;116). These results were further supported by visualization of excess *Fn* by fluorescent in situ hybridization (FISH) on tumors as compared to corresponding normal colon tissue and by quantitative PCR analysis (118;119)

Although *Fusobacterium* was not associated with defined tumor characteristics, it was found to be more abundant in CRC from Spain as compared to tumors from the United States and Vietnam. This suggests that Fusobacterium colonization may vary regionally, although the reasons for this, including different dietary habits, require further investigation. Recent data have provided experimental support for a tumor-inducing role of *Fn*. Chronic exposure of Apc Min/+ mice to *Fn* strains isolated from patients with IBD induced a modest, but significant, increase of CRC.

More in detail, *Fn* was suggested to impact CRC growth by the activated complex of the FadA adhesin on its surface (117). In vitro colon carcinoma cell lines and tumor xenograft models revealed that FadA binds a select extracellular domain of E-cadherin on tumor cells surface, triggering invasion of the organism and the activation of β-catenin/Wnt signaling promoting tumor growth. Furthermore, evaluation of tumor tissues from adenomas and adenocarcinomas as compared to normal colon tissue from healthy subjects revealed that FadA gene copy number was significantly higher in tumor tissues.

Importantly, *Fn* has been shown to modulate immune responses. For example, recently, a study in 598 CRC cases has been shown that the amount of *Fn* in tumor tissues is inversely associated with CD3+ density (118).

In another study it has been demonstrated that Fap2 protein expressed by *Fn* inhibits tumor cell lysis by interacting with TIGIT receptor and thereby inhibiting the cytotoxic potential of NK and T cells. Based on this we may speculate that colorectal cancer exploits bacterial flora to evade immune evasion (119).

Many bacteria such as *B.fragilis* are considered as oncomicrobes because they are capable of damaging colonic cells and induce their proliferation by releasing genotoxin. Whether *Fn* is able to synthesize genotoxin is still unclear but its peculiar metabolites and the presence of type Fap2 and RadD on its surface have been implicated in immune cell regulation (119).

Finally, *Fn* colonization has been assessed as prognostic biomarker by correlating survival outcomes in CRC patients with bacterial load. A high amount of *Fn* in CRC is associated with poor patient survival (118). This finding supports the hypothesis that *Fn* colonization might promote a more biologically aggressive cancer subtype.

Future investigations are warranted to explore potential influence of tissue colonization *Fn* on the effectiveness of T cell-based immunotherapies.

5. Rationale and aim of the thesis

Colorectal cancer (CRC) remains the third cause of cancer-related mortality worldwide, and new therapeutic strategies are urgently needed. To this purpose, a better understanding of the different components within the tumour microenvironment is crucial.

The immune landscape is of essential relevance in CRC clinical course. In particular, patients bearing tumours which are highly infiltrated by CD8+ and memory T cells have a higher probability of long term survival. In contrast, tumors infiltrated by myeloid cells have frequently been associated with poor prognosis in a variety of cancers of different histological origin. However, our group has previously observed that CRC infiltration by CD16+ myeloid cells, correlates with favorable outcome. Since these cells are HLA-class II- and largely MPO+, a neutrophil nature could reasonably be suspected. Recently, other groups have demonstrated that a high infiltration of CD66+ neutrophils may correlate with benign or poor prognosis in CRC patients.

Based on these discrepancies, the prognostic significance of tumor associated neutrophils (TANs) infiltrating CRC has to be elucidated. Indeed, the biological significance of TANs in human cancer is poorly investigated and a major challenge is to clarify functional mechanisms potentially underlying the prognostic significance of TANs in CRC.

Considering the current lack of understanding of neutrophil functions in tumour immunobiology, in this study we have investigated the prognostic relevance of CD66b cell infiltration in a large cohort of patients with CRC. Furthermore we have addressed the hypothesis of a possible crosstalk between peripheral blood neutrophils (PBN), TANs and CD8+ T cells *in vitro* and of its clinical significance in CRC showing combined infiltration by CD8+ T cells and neutrophils.

Recent reports have documented that the gut microbiome is profoundly altered in CRC patients and specific microbial strains have been found to be associated with malignant transformation. In particular, the Gram-negative bacteria *F. nucleatum* appears to be enriched in CRC. This bacterial strain was shown to induce colitis-independent gut tumorigenesis *in vivo* and to cause the accumulation of myeloid cell subsets, in particular granulocytes, within the tumour mass. The role of these microbial strains in human CRC progression is currently under investigation. Whether specific microbial strains contribute to TANs phenotype and impair their interplay with other immune and stromal cells present in the tumour microenvironment is currently unknown.

Based on this background the main aim of my PhD project is to understand the role of neutrophils in human CRC immunobiology, and how different components of the tumour microenvironment impact on their functionality.

6. Reference

(1) Fridman WH, Pages F, Sautes-Fridman C *et al*. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012;**12**(4):298-306.

(2) Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 2010;**11**(10):889-96.

(3) Eruslanov EB, Bhojnagarwala PS, Quatromoni JG *et al*. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *J Clin Invest* 2014.

(4) Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis* 2012;**33**(5):949-55.

(5) Granot Z, Henke E, Comen EA *et al*. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 2011;**20**(3):300-14.

(6) Droeser RA, Hirt C, Eppenberger-Castori S *et al*. High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. *PLoS One* 2013;**8**(5):e64814.

(7) Sconocchia G, Zlobec I, Lugli A *et al*. Tumor infiltration by FcgammaRIII (CD16)+ myeloid cells is associated with improved survival in patients with colorectal carcinoma. *Int J Cancer* 2011;**128**(11):2663-72.

(8) Pelletier M, Micheletti A, Cassatella MA. Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. *J Leukoc Biol* 2010;**88**(6):1163-70.

(9) Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* 2014;**157**(1):121-41.

(10) Castellarin M, Warren RL, Freeman JD *et al*. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012;**22**(2):299-306.

(11) Kuipers EJ, Grady WM, Lieberman D *et al*. Colorectal cancer. *Nat Rev Dis Primers* 2015;**1**:15065.

(12) Capelle LG, van Grieken NC, Lingsma HF *et al*. Risk and epidemiological time trends of gastric cancer in Lynch syndrome carriers in the Netherlands. *Gastroenterology* 2010;**138**(2):487-92.

(13) Vasen HF, Tomlinson I, Castells A. Clinical management of hereditary colorectal cancer syndromes. *Nat Rev Gastroenterol Hepatol* 2015;**12**(2):88-97.

(14) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;**144**(5):646-74.

(15) Colussi D, Brandi G, Bazzoli F *et al*. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. *Int J Mol Sci* 2013;**14**(8):16365-85.

(16) Fearon ER, Carethers JM. Molecular subtyping of colorectal cancer: time to explore both intertumoral and intratumoral heterogeneity to evaluate patient outcome. *Gastroenterology* 2015;**148**(1):10-3.

(17) Yamauchi M, Lochhead P, Morikawa T *et al*. Colorectal cancer: a tale of two sides or a continuum? *Gut* 2012;**61**(6):794-7.

(18) Janout V, Kollarova H. Epidemiology of colorectal cancer. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2001;**145**(1):5-10.

(19) Sadanandam A, Lyssiotis CA, Homicsko K *et al*. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med* 2013;**19**(5):619-25.

(20) Compton CC, Greene FL. The staging of colorectal cancer: 2004 and beyond. *CA Cancer J Clin* 2004;**54**(6):295-308.

(21) Compton C, Fenoglio-Preiser CM, Pettigrew N *et al*. American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. *Cancer* 2000;**88**(7):1739-57.

(22) Compton CC. Pathology report in colon cancer: what is prognostically important? *Dig Dis* 1999;**17**(2):67-79.

(23) Ueno H, Murphy J, Jass JR *et al*. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002;**40**(2):127-32.

(24) Hase K, Shatney C, Johnson D *et al*. Prognostic value of tumor "budding" in patients with colorectal cancer. *Dis Colon Rectum* 1993;**36**(7):627-35.

(25) Brenner H, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014;**383**(9927):1490-502.

(26) Fallik D, Borrini F, Boige V *et al*. Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer. *Cancer Res* 2003;**63**(18):5738-44.

(27) Bertagnolli MM, Niedzwiecki D, Compton CC *et al*. 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. *J Clin Oncol* 2009;**27**(11):1814-21.

(28) Fridman WH, Dieu-Nosjean MC, Pages F *et al*. The immune microenvironment of human tumors: general significance and clinical impact. *Cancer Microenviron* 2013;**6**(2):117-22.

(29) Fridman WH, Pages F, Sautes-Fridman C *et al*. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012;**12**(4):298-306.

(30) Senovilla L, Vacchelli E, Galon J *et al*. Trial watch: Prognostic and predictive value of the immune infiltrate in cancer. *Oncoimmunology* 2012;**1**(8):1323-43.

(31) Galon J, Angell HK, Bedognetti D *et al*. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* 2013;**39**(1):11-26.

(32) Galon J, Costes A, Sanchez-Cabo F *et al*. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;**313**(5795):1960-4.

(33) Murdoch C, Muthana M, Coffelt SB *et al*. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 2008;**8**(8):618-31.

(34) Peddareddigari VG, Wang D, Dubois RN. The tumor microenvironment in colorectal carcinogenesis. *Cancer Microenviron* 2010;**3**(1):149-66.

(35) Grizzi F, Bianchi P, Malesci A *et al*. Prognostic value of innate and adaptive immunity in colorectal cancer. *World J Gastroenterol* 2013;**19**(2):174-84.

(36) Galon J, Pages F, Marincola FM *et al*. Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med* 2012;**10**:205.

(37) Fridman WH, Teillaud JL, Sautes-Fridman C *et al*. The ultimate goal of curative anti-cancer therapies: inducing an adaptive anti-tumor immune response. *Front Immunol* 2011;**2**:66.

(38) Erreni M, Mantovani A, Allavena P. Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer. *Cancer Microenviron* 2011;**4**(2):141-54.

(39) Mantovani A, Germano G, Marchesi F *et al*. Cancer-promoting tumor-associated macrophages: new vistas and open questions. *Eur J Immunol* 2011;**41**(9):2522-5.

(40) Jing Y, Han Z, Zhang S *et al*. Epithelial-Mesenchymal Transition in tumor microenvironment. *Cell Biosci* 2011;**1**:29.

(41) Hagemann T, Balkwill F, Lawrence T. Inflammation and cancer: a double-edged sword. *Cancer Cell* 2007;**12**(4):300-1.

(42) Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006;**124**(2):263-6.

(43) Ohno S, Inagawa H, Dhar DK *et al*. Role of tumor-associated macrophages (TAM) in advanced gastric carcinoma: the impact on FasL-mediated counterattack. *Anticancer Res* 2005;**25**(1B):463-70.

(44) Ohno S, Inagawa H, Dhar DK *et al*. The degree of macrophage infiltration into the cancer cell nest is a significant predictor of survival in gastric cancer patients. *Anticancer Res* 2003;**23**(6D):5015-22.

(45) Xie ZJ, Jia LM, He YC *et al*. Morphological observation of tumor infiltrating immunocytes in human rectal cancer. *World J Gastroenterol* 2006;**12**(11):1757-60.

(46) Sconocchia G, Eppenberger S, Spagnoli GC *et al*. NK cells and T cells cooperate during the clinical course of colorectal cancer. *Oncoimmunology* 2014;**3**(8):e952197.

(47) Daster S, Eppenberger-Castori S, Hirt C *et al*. Absence of myeloperoxidase and CD8 positive cells in colorectal cancer infiltrates identifies patients with severe prognosis. *Oncoimmunology* 2015;**4**(12):e1050574.

(48) Rao HL, Chen JW, Li M *et al*. Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. *PLoS One* 2012;**7**(1):e30806.

(49) Galdiero MR, Bianchi P, Grizzi F *et al*. Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer. *Int J Cancer* 2016;**139**(2):446-56.

(50) Goedegebuure P, Mitchem JB, Porembka MR *et al*. Myeloid-derived suppressor cells: general characteristics and relevance to clinical management of pancreatic cancer. *Curr Cancer Drug Targets* 2011;**11**(6):734-51.

(51) Ostrand-Rosenberg S, Sinha P, Beury DW *et al*. Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. *Semin Cancer Biol* 2012;**22**(4):275-81.

(52) Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 2009;**182**(8):4499-506.

(53) Ichikawa M, Williams R, Wang L *et al*. S100A8/A9 activate key genes and pathways in colon tumor progression. *Mol Cancer Res* 2011;**9**(2):133-48.

(54) Trellakis S, Farjah H, Bruderek K *et al*. Peripheral blood neutrophil granulocytes from patients with head and neck squamous cell carcinoma functionally differ from their counterparts in healthy donors. *Int J Immunopathol Pharmacol* 2011;**24**(3):683-93.

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

(56) Solito S, Falisi E, Diaz-Montero CM *et al*. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* 2011;**118**(8):2254-65.

(57) Jaillon S, Galdiero MR, Del PD *et al*. Neutrophils in innate and adaptive immunity. *Semin Immunopathol* 2013;**35**(4):377-94.

(58) Amin M, Lockhart AC. The potential role of immunotherapy to treat colorectal cancer. *Expert Opin Investig Drugs* 2015;**24**(3):329-44.

(59) Hoffmann TK, Meidenbauer N, Muller-Berghaus J *et al*. Proinflammatory Cytokines and CD40 Ligand Enhance Cross-Presentation and Cross-Priming Capability of Human Dendritic Cells Internalizing Apoptotic Cancer Cells. *J Immunother (1991)* 2001;**24**(2):162-71.

(60) Douillard JY, Siena S, Cassidy J *et al*. Final results from PRIME: randomized phase III study of panitumumab with FOLFOX4 for first-line treatment of metastatic colorectal cancer. *Ann Oncol* 2014;**25**(7):1346-55.

(61) Douillard JY, Oliner KS, Siena S *et al*. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 2013;**369**(11):1023-34.

(62) Bilusic M, Heery CR, Arlen PM *et al*. Phase I trial of a recombinant yeast-CEA vaccine (GI-6207) in adults with metastatic CEA-expressing carcinoma. *Cancer Immunol Immunother* 2014;**63**(3):225-34.

(63) Morse MA, Niedzwiecki D, Marshall JL *et al*. A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. *Ann Surg* 2013;**258**(6):879-86.

(64) Okuno K, Sugiura F, Itoh K *et al*. Recent advances in active specific cancer vaccine treatment for colorectal cancer. *Curr Pharm Biotechnol* 2012;**13**(8):1439-45.

(65) Yasuda S, Sho M, Yamato I *et al*. Simultaneous blockade of programmed death 1 and vascular endothelial growth factor receptor 2 (VEGFR2) induces synergistic anti-tumour effect in vivo. *Clin Exp Immunol* 2013;**172**(3):500-6.

(66) Llosa NJ, Cruise M, Tam A *et al*. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov* 2015;**5**(1):43-51.

(67) Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer* 2016;**16**(7):431-46.

(68) Borregaard N. Neutrophils, from marrow to microbes. *Immunity* 2010;**33**(5):657-70.

(69) Sagiv JY, Michaeli J, Assi S *et al*. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep* 2015;**10**(4):562-73.

(70) Sagiv JY, Voels S, Granot Z. Isolation and Characterization of Low- vs. High-Density Neutrophils in Cancer. *Methods Mol Biol* 2016;**1458**:179-93.

(71) Dumitru CA, Lang S, Brandau S. Modulation of neutrophil granulocytes in the tumor microenvironment: mechanisms and consequences for tumor progression. *Semin Cancer Biol* 2013;**23**(3):141-8.

(72) Amicarella F, Muraro MG, Hirt C *et al*. Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. *Gut* 2015.

(73) Cheretakis C, Leung R, Sun CX *et al*. Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model. *Blood* 2006;**108**(8):2821-6.

(74) Moses K, Brandau S. Human neutrophils: Their role in cancer and relation to myeloid-derived suppressor cells. *Semin Immunol* 2016;**28**(2):187-96.

(75) Fridlender ZG, Sun J, Kim S *et al*. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009;**16**(3):183-94.

(76) Kemp TJ, Ludwig AT, Earel JK *et al*. Neutrophil stimulation with Mycobacterium bovis bacillus Calmette-Guerin (BCG) results in the release of functional soluble TRAIL/Apo-2L. *Blood* 2005;**106**(10):3474-82.

(77) Delneste Y, Beauvillain C, Jeannin P. [Innate immunity: structure and function of TLRs]. *Med Sci (Paris)* 2007;**23**(1):67-73.

(78) Fridlender ZG, Sun J, Kim S *et al*. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009;**16**(3):183-94.

(79) Sionov RV, Fridlender ZG, Granot Z. The Multifaceted Roles Neutrophils Play in the Tumor Microenvironment. *Cancer Microenviron* 2015;**8**(3):125-58.

(80) Fridlender ZG, Sun J, Mishalian I *et al*. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. *PLoS One* 2012;**7**(2):e31524.

(81) Mishalian I, Granot Z, Fridlender ZG. The diversity of circulating neutrophils in cancer. *Immunobiology* 2016.

(82) Erler JT, Weaver VM. Three-dimensional context regulation of metastasis. *Clin Exp Metastasis* 2009;**26**(1):35-49.

(83) Grosse-Steffen T, Giese T, Giese N *et al*. Epithelial-to-mesenchymal transition in pancreatic ductal adenocarcinoma and pancreatic tumor cell lines: the role of neutrophils and neutrophil-derived elastase. *Clin Dev Immunol* 2012;**2012**:720768.

(84) Kowanetz M, Wu X, Lee J *et al*. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci U S A* 2010;**107**(50):21248-55.

(85) Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Annu Rev Pathol* 2014;**9**:181-218.

(86) Cools-Lartigue J, Spicer J, McDonald B *et al*. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J Clin Invest* 2013.

(87) Granot Z, Henke E, Comen EA *et al*. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 2011;**20**(3):300-14.

(88) Finisguerra V, Di CG, Di MM *et al*. MET is required for the recruitment of anti-tumoural neutrophils. *Nature* 2015;**522**(7556):349-53.

(89) Trellakis S, Farjah H, Bruderek K *et al*. Peripheral blood neutrophil granulocytes from patients with head and neck squamous cell carcinoma functionally differ from their counterparts in healthy donors. *Int J Immunopathol Pharmacol* 2011;**24**(3):683-93.

(90) Jensen TO, Schmidt H, Moller HJ *et al*. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer* 2012;**118**(9):2476-85.

(91) Gregoire M, Guilloton F, Pangault C *et al*. Neutrophils trigger a NF-kappaB dependent polarization of tumor-supportive stromal cells in germinal center B-cell lymphomas. *Oncotarget* 2015;**6**(18):16471-87.

(92) Ibrahim SA, Katara GK, Kulshrestha A *et al*. Breast cancer associated a2 isoform vacuolar ATPase immunomodulates neutrophils: potential role in tumor progression. *Oncotarget* 2015;**6**(32):33033-45.

(93) Fossati G, Ricevuti G, Edwards SW *et al*. Neutrophil infiltration into human gliomas. *Acta Neuropathol* 1999;**98**(4):349-54.

(94) Donskov F. Immunomonitoring and prognostic relevance of neutrophils in clinical trials. *Semin Cancer Biol* 2013;**23**(3):200-7.

(95) Wislez M, Rabbe N, Marchal J *et al*. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. *Cancer Res* 2003;**63**(6):1405-12.

(96) Schmidt H, Bastholt L, Geertsen P *et al*. Elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma: a prognostic model. *Br J Cancer* 2005;**93**(3):273-8.

(97) Jensen HK, Donskov F, Marcussen N *et al*. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol* 2009;**27**(28):4709-17.

(98) Halazun KJ, Marangoni G, Hakeem A *et al*. Elevated preoperative recipient neutrophil-lymphocyte ratio is associated with delayed graft function following kidney transplantation. *Transplant Proc* 2013;**45**(9):3254-7.

(99) Reid MD, Basturk O, Thirabanjasak D *et al*. Tumor-infiltrating neutrophils in pancreatic neoplasia. *Mod Pathol* 2011;**24**(12):1612-9.

(100) Templeton AJ, Pezaro C, Omlin A *et al*. Simple prognostic score for metastatic castration-resistant prostate cancer with incorporation of neutrophil-to-lymphocyte ratio. *Cancer* 2014;**120**(21):3346-52.

(101) Paramanathan A, Saxena A, Morris DL. A systematic review and meta-analysis on the impact of pre-operative neutrophil lymphocyte ratio on long term outcomes after curative intent resection of solid tumours. *Surg Oncol* 2014;**23**(1):31-9.

(102) Caruso RA, Muda AO, Bersiga A *et al*. Morphological evidence of neutrophil-tumor cell phagocytosis (cannibalism) in human gastric adenocarcinomas. *Ultrastruct Pathol* 2002;**26**(5):315-21.

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

(104) Bertini R, Allegretti M, Bizzarri C *et al*. Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. *Proc Natl Acad Sci U S A* 2004;**101**(32):11791-6.

(105) Di MM, Gridelli C, Gallo C *et al*. Chemotherapy-induced neutropenia and treatment efficacy in advanced non-small-cell lung cancer: a pooled analysis of three randomised trials. *Lancet Oncol* 2005;**6**(9):669-77.

(106) Bakema JE, Ganzevles SH, Fluitsma DM *et al*. Targeting FcalphaRI on polymorphonuclear cells induces tumor cell killing through autophagy. *J Immunol* 2011;**187**(2):726-32.

(107) Schwabe RF, Jobin C. The microbiome and cancer. *Nat Rev Cancer* 2013;**13**(11):800-12.

(108) Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. *Nat Rev Microbiol* 2014;**12**(10):661-72.

(109) Bashir A, Miskeen AY, Hazari YM *et al*. Fusobacterium nucleatum, inflammation, and immunity: the fire within human gut. *Tumour Biol* 2016;**37**(3):2805-10.

(110) Viaud S, Daillere R, Yamazaki T *et al*. Why should we need the gut microbiota to respond to cancer therapies? *Oncoimmunology* 2014;**3**(1):e27574.

(111) Arthur JC, Gharaibeh RZ, Muhlbauer M *et al*. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. *Nat Commun* 2014;**5**:4724.

(112) Arthur JC, Perez-Chanona E, Muhlbauer M *et al*. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* 2012;**338**(6103):120-3.

(113) Viaud S, Saccheri F, Mignot G *et al*. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* 2013;**342**(6161):971-6.

(114) Iida N, Dzutsev A, Stewart CA *et al*. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science* 2013;**342**(6161):967-70.

(115) Castellarin M, Warren RL, Freeman JD *et al*. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012;**22**(2):299-306.

(116) Kostic AD, Chun E, Robertson L *et al*. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013;**14**(2):207-15.

(117) Rubinstein MR, Wang X, Liu W *et al*. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. *Cell Host Microbe* 2013;**14**(2):195-206.

(118) Mima K, Nishihara R, Qian ZR *et al*. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* 2015.

(119) Gur C, Ibrahim Y, Isaacson B *et al*. Binding of the Fap2 protein of Fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. *Immunity* 2015;**42**(2):344-55.

CHAPTER II: “The interplay between neutrophils and CD8+ cells improves survival in human colorectal cancer”

V Governa[1, 2], E Trella[1], V Mele[1], L Tornillo[2], F Amicarella[1], E Cremonesi[1], MG Muraro[1], H Xu[1], RA Droeser[1,3], S Däster[1,3], M Bolli[4], R Rosso[5], D Oertli[3], S Eppenberger-Castori[2] , L Terracciano[2], G Iezzi[1] and GC Spagnoli[1,6].

[1]Department of Biomedicine, Basel University Hospital and University of Basel, Basel, CH; [2]Molecular Pathology Division, Institute of Pathology, Basel University Hospital and University of Basel, CH; [3]Department of General Surgery, Basel University Hospital and University of Basel, CH; [4]Department of Visceral Surgery, St. Claraspital, Basel, CH; [5]Department of Visceral Surgery, Ospedale Civico, Lugano, CH.

(in revision)

Abstract

Purpose: Tumor infiltration by different T lymphocyte subsets is known to be associated with favorable prognosis in colorectal cancer (CRC). Still debated is the role of innate immune system. We investigated clinical relevance, phenotypes and functional features of CRC infiltrating CD66b+ neutrophils and their crosstalk with CD8+ T cells.

Experimental design: CD66b+ and CD8+ cell infiltration was analyzed by immunohistochemistry on a tissue microarray including >650 evaluable CRC samples. Phenotypic profiles of tissue infiltrating and peripheral blood CD66b+ cells were evaluated by flow cytometry. CD66b+/ CD8+ cells crosstalk was investigated by in vitro experiments.

Results: CD66b+ cell infiltration in CRC is significantly associated with increased survival. Interestingly, neutrophils frequently co-localize with CD8+ T cells in CRC. Functional studies indicate that although neutrophils are devoid of direct antitumor potential, co-culture with peripheral blood or tumor associated neutrophils (TANs) enhances CD8+ T cell activation, proliferation and cytokine release induced by suboptimal concentrations of anti-CD3 monoclonal antibody (mAb). Moreover, under optimal activation conditions, CD8+ cells initially stimulated in the presence of CD66b+ cells show decreased expression of PD-1 "exhaustion" marker and are significantly less susceptible to apoptosis induced by T- cell receptor triggered re-stimulation. Importantly, combined tumor infiltration by CD66b+ and CD8+ T lymphocytes is associated with significantly better prognosis, as compared to CD8+ T cell infiltration alone.

Conclusions: Neutrophils enhance the responsiveness of CD8+ T cells to TCR triggering. Accordingly, infiltration by neutrophils enhances the prognostic significance of CRC infiltration by CD8+ T cells, suggesting that they might effectively promote antitumor immunity.

1. Introduction

Granulocytes account for 50-70% of all leukocytes in humans. They represent a first line defense against a variety of bacterial and fungal infections (1;2). However, their role in anti-cancer immune responses is debated (1-3). A number of studies suggest that high granulocyte/lymphocyte ratios in peripheral blood are associated with poor prognosis in different malignancies (4). Furthermore, myeloid cells of the granulocytic lineage at different maturation stages have been shown to represent sizeable subsets of myeloid-derived suppressor cells (MDSC), promoting tumor growth and inhibiting cancer specific adaptive responses (5-7).

More recently, the possibility that neutrophils might promote anti-tumor immune responses of potential clinical relevance has started to be explored (2). In particular, the ability of neutrophil to polarize into N1 and N2 functional profiles, similarly to macrophages, has been documented in experimental models (8;9). Furthermore, tumor "educated" neutrophils were shown to elicit anti-metastatic effects (10) and the interaction of hepatocyte growth factor (HGF) with its receptor MET has been suggested to play a key role in the recruitment of neutrophils mediating anti-tumor activities (11). Most interestingly, earlier studies indicated that production of granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF), promoting neutrophil survival and activation by tumor cells, could induce adaptive anti-tumor immune responses and regression of established tumors based on neutrophil-T cell interaction (12;13). Clinical studies have also been pursued. In particular, the ability of early stage lung cancer infiltrating neutrophils to support T cell proliferation and anti-tumor responses has been demonstrated (14;15). However, their prognostic significance was not addressed.

Colorectal cancer (CRC) represents the third cause of cancer-related mortality worldwide (16). TNM staging, routinely used to identify patients eligible for different treatments is frequently ineffective in predicting CRC clinical course (17).

Clinical relevance of the composition of tumor infiltrate in CRC has been extensively investigated in the past decade (18). CRC infiltration by CD8+ and memory T cells has been consistently associated with favorable prognosis (19;20). The specificity of these cells is largely unclear. Recognition of differentiation antigens (21) or neo-antigens (22) expressed by tumor cells has been reported. Alternatively, bystander effects related to T cell responses against

antigens expressed by microbial commensal could also be hypothesized. Interestingly, expression of activation markers by CRC infiltrating lymphocytes was found to correlate with prolonged survival (23).

The role of the innate immune system is still debated. NK cell infiltration is modest and apparently devoid of prognostic significance (24). Although tumor infiltration by myeloid cells has frequently been associated with poor prognosis in a variety of cancers (25), macrophage infiltration, has been suggested to correlate with favorable prognosis in CRC (26).

The role of neutrophils has not been explored in comparable detail. We previously observed that CRC infiltration by CD16+ myeloid cells is associated with favorable outcome (27). Similarly to neutrophils, these cells are HLA-class II- and largely myeloperoxidase (MPO)+ (28). Data from other groups suggest that high tumor infiltration by CD66+ neutrophils may correlate with either benign or poor prognosis in patients with CRC. In particular, in a cohort of East Asian patients (n=229) neutrophil infiltration was found to be associated with severe prognosis (29). Moreover, neutrophil infiltration in lung metastases has been suggested to be associated with severe prognosis following surgical excision (30). In contrast, most recently, neutrophil infiltration in CRC was reported to be associated with responsiveness to 5-fluorouracil (5FU) treatment (31). Thus, potential clinical significance of tumor associated neutrophils (TANs) infiltrating CRC is still unclear and underlying functional mechanisms remain to be elucidated.

Here we have analyzed the prognostic significance of CRC infiltrating CD66b+ neutrophils by using a clinically annotated tissue microarray (TMA) including over 650 cases. In addition, we have comparatively evaluated phenotypes of neutrophils from healthy and cancerous colon tissues and peripheral blood from patients and healthy donors (HD). Their ability to support adaptive immune responses was also specifically addressed. Finally, the prognostic relevance of the association of neutrophils with CD8+ T cells infiltrating CRC microenvironment was explored.

2. Materials and methods

2.1 Tissue Microarray construction

The TMA used in this work was constructed by using >650 non-consecutive, formalin-fixed and paraffin-embedded primary CRC samples, from the tissue biobank of the Institute of Pathology of the University Hospital Basel (Switzerland), as previously described (27;32;33). A semi-automated tissue arrayer was used to transfer punches of a 0.6 mm diameter from tissue blocks onto glass slides. Punches were derived from the center of the tumors and consisted of at least 50% tumor cells. Clinical-pathological data for patients included in the TMA are summarized in Table 1. Use of clinical information was approved by local ethical authorities.

2.2 Immunohistochemistry

TMA slides were incubated with primary antibodies specific for CD8, CD16, MPO (23;27;28) and CD66b (clone G10F5, Biolegend). Secondary stainings and negative controls were performed as described (23;27;28). CRC infiltration by cells expressing defined markers was scored by experienced pathologists.

2.3 Tumor cell lines

Established human CRC cell lines (DLD1, HCT116, SW480, HT29, and SW620) were purchased from European Collection of Authenticated Cell Cultures (ECACC). DLD1 and HCT116 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), GlutaMAX-I, nonessential amino acids (NEAA), 100 mM sodium pyruvate, 10 mM HEPES (all from GIBCO). HT29 cells were cultured in McCoy's 5A medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum, GlutaMAX-I and kanamycin (GIBCO). SW480 and SW620 cells were cultured in L15 medium (Sigma-Aldrich), supplemented with 10% FBS, GlutaMAX-I and Kanamycin (GIBCO). Absence of mycoplasma contamination in tumor cell lines was verified by PCR with mycoplasma-specific primers, prior to experimental procedures.

2.4 Clinical specimen collection and processing

Clinical specimens from consenting patients undergoing surgical treatment at Basel University Hospital, St. Claraspital Basel, and Ospedale Civico, Lugano (all in Switzerland), were obtained according to procedures approved by local ethical commissions. Tumor tissues and

corresponding tumor-free mucosa fragments were embedded in OCT for further histological evaluation or enzymatically digested by using an enzyme cocktail including 2 mg/mL collagenase IV (Worthington Biochemical Corporation) and 0.2 mg/mL DNAse I, (Sigma-Aldrich for 1h at 37°C) to obtain single cell suspensions, as previously detailed (32).

2.5 Neutrophil and lymphocyte isolation

TANs were isolated from tumor cell suspensions by positive selection of CD66b+ cells with antibody coated microbeads according to the manufacturer's instructions (Miltenyi Biotec). The purity as well as the viability of TAN obtained was tumor sample dependent (between 70% to 90%, and about 20%-30% of Annexin V positive cells respectively). Peripheral blood was collected from patients with CRC prior to surgery or from healthy donors, and density-gradient centrifugation was performed. Sedimented fractions containing high-density neutrophils were washed and treated with dextran 4% (T500, Pharmacia) in saline solution and residual erythrocytes in supernatants were lysed by using lysis buffer (Miltenyi Biotec). Peripheral blood neutrophils (PBNs) were further enriched by positively removing contaminating cells, to reach 98% purity using the EasySep Human Neutrophil Enrichment Kit (Stemcell Technologies). Purity of isolated TANs and PBNs was evaluated by flow cytometry upon staining for the neutrophils/myeloid markers CD66b, CD16, myeloperoxidase (MPO), and CD11b.

2.6 CRC/N co-cultures

CRC cells from established cell lines (see above) were cultured in the presence or absence of neutrophils, at different ratios and tumor cell proliferation was assessed by ^{3}H-Thymidine incorporation (^{3}H-TdR). In specific experiments, induction of apoptosis in tumor cells was tested by annexin V/PI staining (BioLegend).

2.7 Flow cytometry

Cell suspensions from CRCs and tumor-free mucosa, and peripheral blood of healthy donors or patients with CRC, were stained with fluorochrome-conjugated antibodies specific for human CD66b, CD16, CD11b, (BioLegend) and CD54, CD62L, CXCR1, CXCR2 (BD Biosciences). Alternatively, cells were fixed and intracellular staining was performed with antibodies specific for MPO (28). Stained cells were analyzed by FACS Calibur flow cytometer (BD Biosciences), using FlowJo software (Tree Star).

2.8 Imagestream

Following CD66b, CD16 and intracellular MPO-specific staining, cells were washed and re-suspended in PBS supplemented with 0.5% FBS and 5mM EDTA, prior to processing through ImageStream, Mark II Imaging Flow Cytometer (Amnis, EMD Millipore). Analysis was performed using IDEAS software (Amnis, EMD Millipore) and neutrophils from CRC tissue, healthy mucosa and peripheral blood were identified based on brightfield morphology, granularity and CD66b expression.

2.9 Neutrophil and CD8+ T cell co-cultures

TANs and PBNs, obtained from HD and CRC patients were co-cultured with autologous peripheral blood CD8+ T lymphocytes isolated by using antibody-coated magnetic beads (Miltenyi Biotec) following gradient centrifugation (34). For co-stimulation experiments, 96-well flat bottom culture plates were coated overnight with anti CD3 mitogenic mAb (TR66, a gift of Dr. Lanzavecchia, Bellinzona, Switzerland), or UCHT-1, (eBiosciences) at sub-optimal concentrations ranging between 0.5 and 5 μg/ml depending from hybridoma and lot. Neutrophils and CD8+ T cells, at a 0,5 10^6/ml concentration each were cultured in RPMI 1640 medium supplemented with GlutaMAX I, HEPES, sodium pyruvate, non-essential amino acids, antibiotics (all from GIBCO) and 5% AB serum (Blood Bank, Kantonsspital Basel), thereafter referred to as complete medium, in the presence of anti-CD28 (1μg/ml, BD Pharmingen). Following 24 hours incubation, expression of CD69 early T cell activation marker, was evaluated by flow cytometry. T cells proliferation was measured by assessing carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) dilution in labelled CD8+ T cells following 72h culture by flow cytometry (34). IFN-γ release in culture supernatants was assessed by using commercial ELISA kits (BD Biosciences). When indicated, co-culture experiments were performed by using trans-well plates (Corning), or in the presence of anti CD11a (BioLegend) or control reagents.

2.10 Immunofluorescence

CRC sections were fixed with Formalin 4% for 15 minutes at room temperature (RT) and blocked with 2% goat serum diluted in PBS containing 0.3% Triton X-100 for one hour at RT. They were then incubated with rabbit polyclonal anti-human CD8 (Abcam) or rabbit polyclonal anti-human CD45RO (BiorByt) and mouse monoclonal anti-human CD66b (Biolegend) for one hour at 37° C. Slides were washed with PBS and incubated for one hour at RT with goat anti-

mouse Alexa Fluor 488 and anti-rabbit 546-conjugated antibodies (Invitrogen). Nuclei were counterstained with 4,6-diamidino-2-phenyldole (DAPI, Invitrogen). Section were examined using Olympus BX61 fluorescence microscope (Olympus) and images were captured with 10x and 20x magnification using a F-View II camera (Olympus) and AnalySiS software (Soft Imaging System GmbH).

2.11 Statistical analysis

Statistical significance of differential expression of activation markers, cytokine release and cell proliferation were analyzed by Student's T and Wilcoxon/Mann-Whitney tests, as appropriate.

Associations with survival were explored using the Cox proportional hazards regression model. Cut-off values used to classify CRC with low or high immune cell infiltration were obtained by regression tree analysis (rpart package). Based on this calculation and on the test evaluability, threshold value for CD66b+ infiltration was set at 10 cells per punch. After dichotomization, Kaplan-Meier curves were plotted, and compared by log rank test.

Kruskal-Wallis and Jonckheere-Terpstra tests were used to determine the association of CD66b+ and CD8+ cell infiltration and clinical-pathological features depending on continuous or discrete nature of the variable. Any missing clinical-pathological information was assumed to be missing at random. Subsequently, CD66b+ and CD8+ cell infiltration data were entered into multivariate Cox regression analysis with clinical-pathological variables and hazard ratios (HR) and 95% confidence intervals (CI) were used to determine prognostic effects on survival time. Several models with additive inclusion of single clinical-pathological data were tested. Only age and gender of the patients did not affect the significant impact of CD66+ and CD8+ cell infiltration. As soon as pT or pN or any other pathological parameters were added to the model, CRC infiltration by CD66+ cells lost its independent prognostic value (data not shown).

Data were analyzed using the Statistical Package Software R (Version 3.2.4, www.r-project.org). *P*-values <0.05 were considered statistically significant.

3. Results

3.1 Prognostic significance of CD66b+ cell infiltration in CRC

In previous studies we had observed that CRC infiltration by MPO+ cells is associated with favorable prognosis (28;35). However, this enzyme is produced by different cells of the myeloid lineage. Therefore, to precisely identify TANs in CRC we stained a TMA including >650 CRC with a mAb recognizing CD66b, a classical neutrophil marker (31). CRC infiltrating CD66b+ cells could be efficiently detected in punches from fixed, paraffin embedded tissues (Fig. 1A-B). In this cohort of patients CRC infiltration by CD66b+ cells was associated with favorable prognosis (Fig. 1C, *P*: <0.001). Additional data on clinical –pathological characterists of these tumors are shown in table 1.

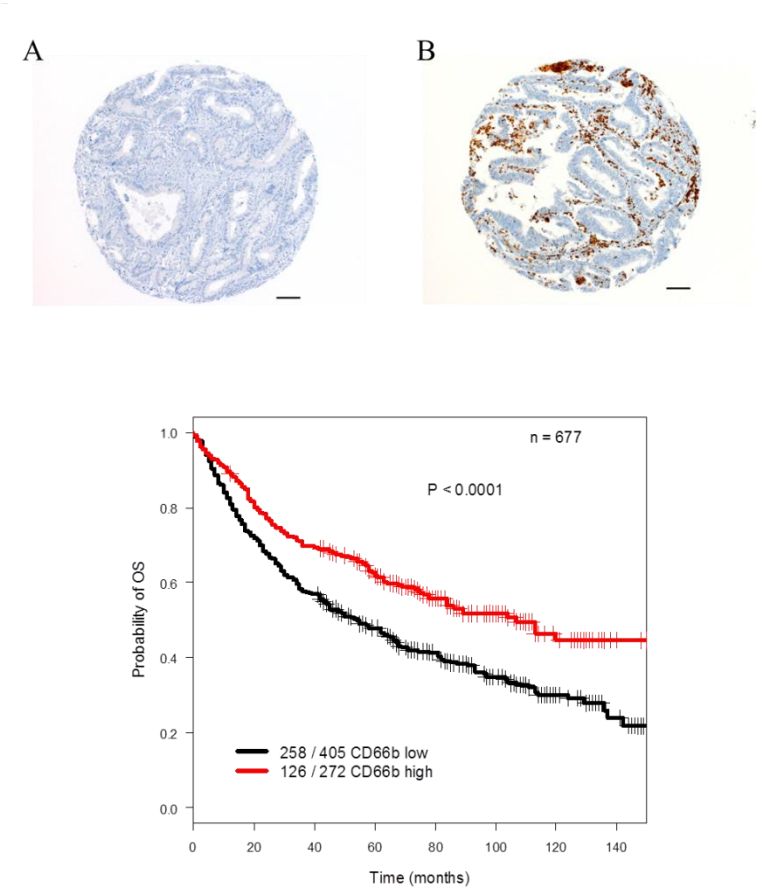


Figure 1. CD66b+ cell infiltration in CRC is associated with favourable prognosis. CRC samples were stained with a CD66b specific mAb. Tumour punches are representative of low **(A)** and high **(B)** density of CRC infiltration by CD66b+cells. Magnification: 10×**,** scale bar: 100 µm. **(C)** Kaplan–Meier curves illustrating overall survival (OS) probability according to CD66b+ cell density. Numbers of deaths/total cases within each category are indicated.

3.2 Phenotypic characterization of tissue infiltrating and peripheral blood CD66b+ cells in patients with CRC

Prompted by data supporting their prognostic significance, we investigated phenotypic profiles of neutrophils infiltrating CRC, adjacent healthy mucosa and autologous peripheral blood.

CRC were characterized by a significantly higher infiltration by CD66b+ cells, as compared with autologous healthy mucosa (Fig. 2A), although wide variations were observed from patient to patient. Imagestream analysis indicated that tumor and mucosa associated neutrophils included CD66b+ cells with variable expression of MPO and CD16 (Fig. 2B).

In order to obtain accurate quantitative data, phenotypic profiles of tissue infiltrating neutrophil were analyzed by flow-cytometry in comparison to PBNs. Representative examples are shown in figure 2C. In keeping with previous reports (35;36), we observed that sizeable percentages of tissue infiltrating CD66b+ neutrophils express MPO and CD16 to lower extents as compared to autologous PBN (Fig. 2C-D). Based on this background, and considering that MPO and CD16 are also expressed by cells other than neutrophils, we explored the relative prognostic significance of the expression of these markers in the TMA under investigation. We observed that CRC infiltration by CD66b+ cells is associated with improved OS also in the absence of a concomitantly high CD16+ or MPO+ cell infiltration (Fig. 2E-F). Furthermore, in the presence of a high CD66b+ cell infiltration, presence or absence of concomitant CD16+ or MPO+ high density infiltration did not significantly modify survival curves (*P*:0.75 and *P*:0.79, respectively).

Expression of other markers was also investigated. CD66b and CD11b are similarly expressed in TANs and PBNs (Fig. 2C and data not shown). CD62L, CXCR1 and CXCR2 are expressed to lower extents in TANs, as compared to autologous PBNs (Figure 2G). This phenotypic profile is shared by neutrophils infiltrating adjacent autologous healthy mucosa (Figure 2D and G). However, higher percentages of TANs express CD54, as compared to autologous PBNs and mucosa infiltrating neutrophils (Fig. 2G).

Notably, expression of all these markers is similar in PBNs from patients with CRC and HD (Supplementary Fig. S1).

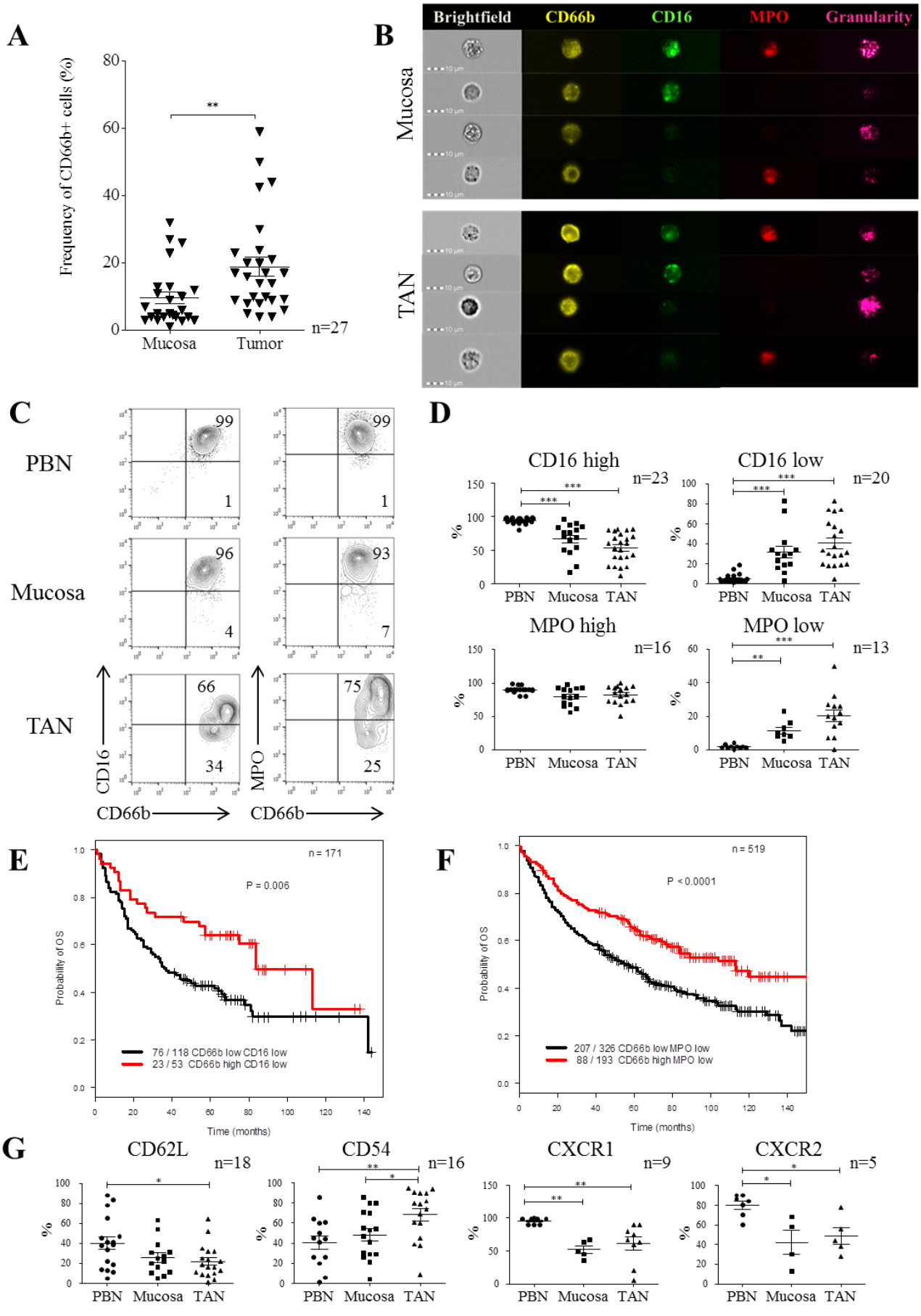
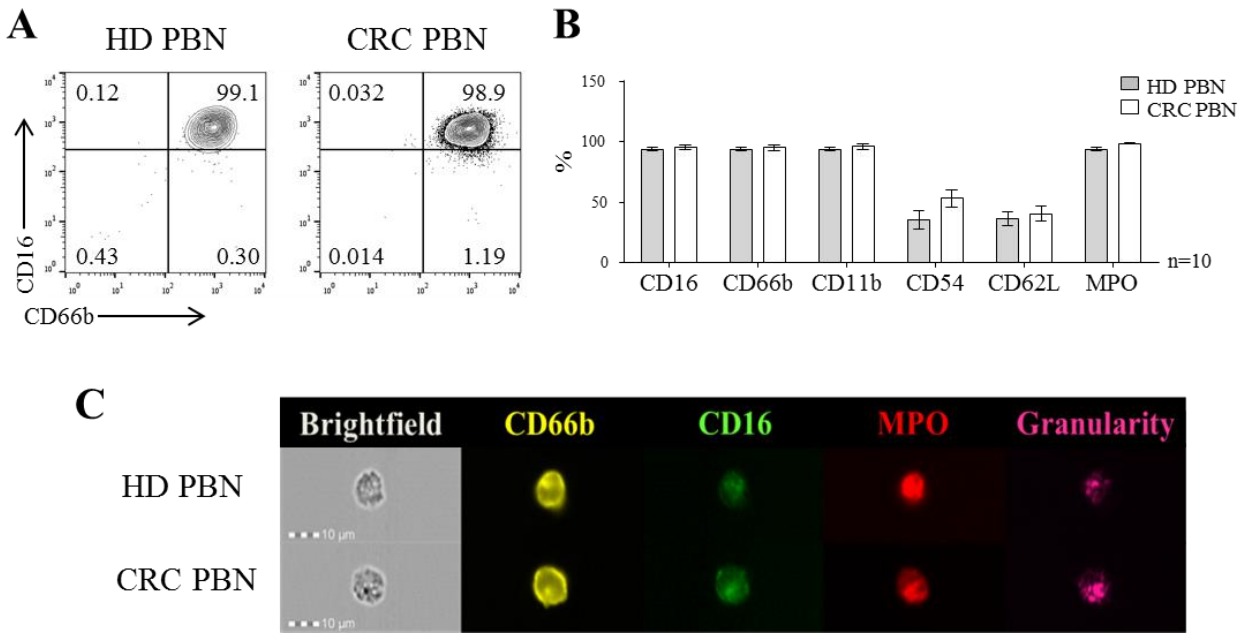

A
Frequency of CD66b+ cells (%)
80
60
40
20
0
**
n=27
Mucosa
Tumor
B
Brightfield
CD66b
CD16
MPO
Granularity
Mucosa
TAN
C
PBN
Mucosa
TAN
99
1
96
4
66
34
93
7
75
25
CD16
MPO
CD66b
CD66b
D
CD16 high
n=23
CD16 low
n=20
MPO high
n=16
MPO low
n=13
%
PBN
Mucosa
TAN
E
n = 171
P = 0.006
Probability of OS
76 / 118 CD66b low CD16 low
23 / 53 CD66b high CD16 low
Time (months)
F
n = 519
P < 0.0001
Probability of OS
207 / 326 CD66b low MPO low
88 / 193 CD66b high MPO low
Time (months)
G
CD62L
n=18
CD54
n=16
CXCR1
n=9
CXCR2
n=5
%
PBN
Mucosa
TAN

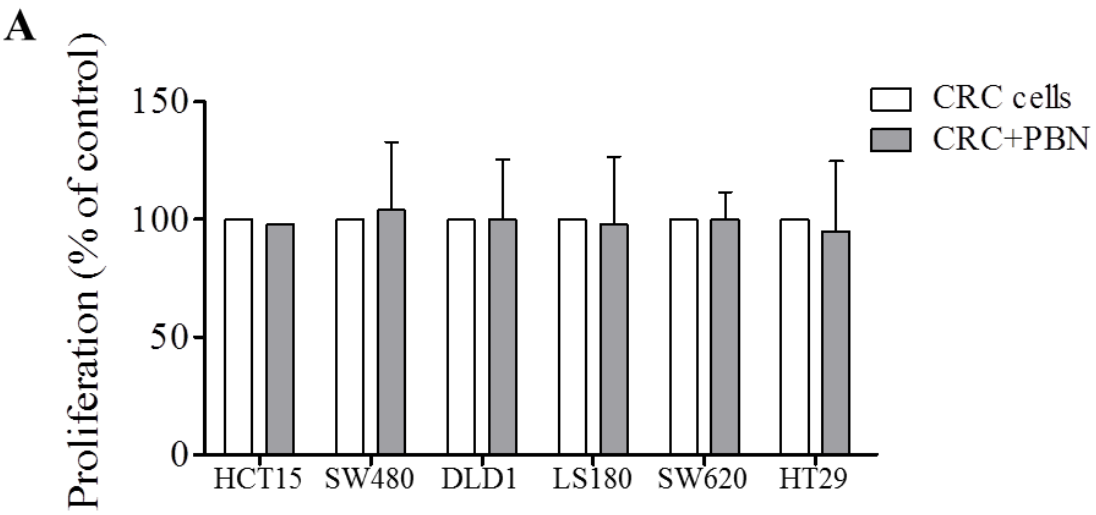
Figure 2. **TANs phenotype.** **(A)** Percentages of CD66b+ cells in CRC tissues and autologous healthy mucosa were determined by flow cytometry within cell suspensions following enzymatic tissue digestion. **(B)** Representative Imagestream pictures of CD66b, CD16 and MPO expression on tumour and autologous healthy mucosa-derived neutrophils. **(C)** Representative flow-cytometry plots of CD16, CD66b and MPO-specific stainings of autologous PBNs, healthy mucosa-derived neutrophils and TAN from a CRC patient. **(D)** Cumulative analysis of percentages of CD16 low/ high and MPO low/ high cells within gated CD66b+ cells from PB, healthy mucosa and tumours. **(E-F)** Kaplan–Meier OS curves designed according to CD66b+ high/low and CD16+ low **(E)** or CD66b+ high/low and MPO+ low **(F)** cell infiltration in CRC. **(G)** Cumulative results showing expression of activation markers and chemokine receptors on PBNs, healthy mucosa-derived neutrophils and TANs, as gated on CD66b+ cells. *= P<0.05; **= P<0.005; ***= P<0.0001.

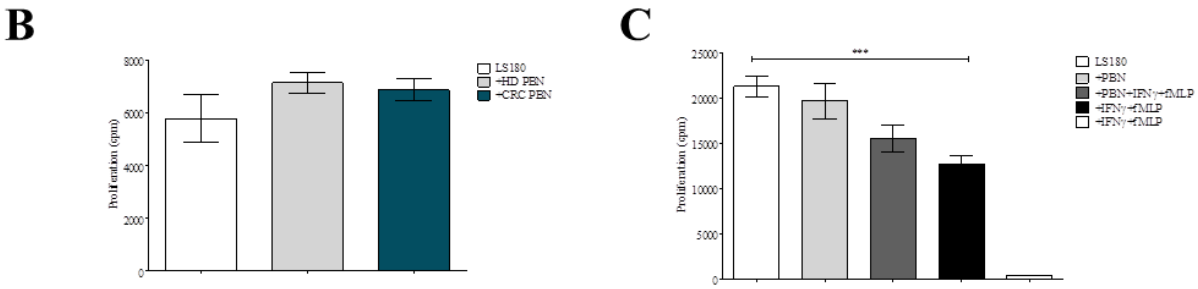


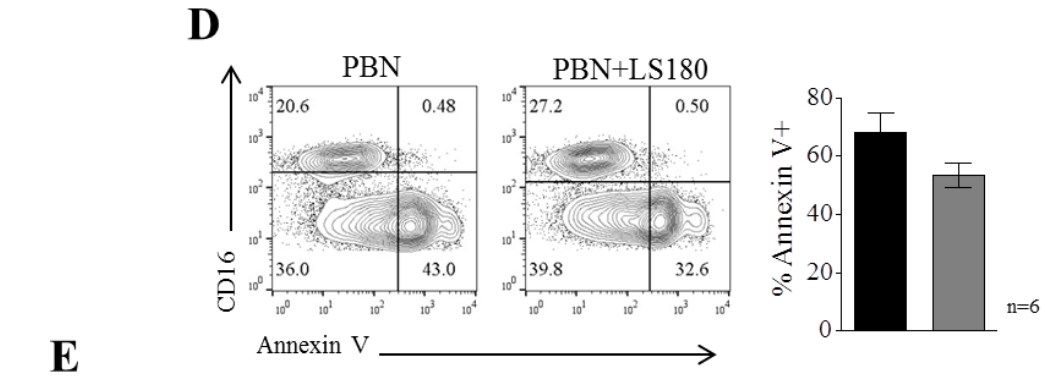
Supplementary figure 1. Phenotypic profiles of PBN from patients with CRC and HD are similar. (A) Representative flow-cytometry plots of CD16 and CD66b expression in PBNs from HD and patients with CRC. **(B)** Percentages of cells positive for the indicated markers in PBNs from HD and patients with CRC. **(C)** Representative pictures from ImageStream analysis of CD66b, CD16 and MPO expression in PBNs from HD and patients with CRC.

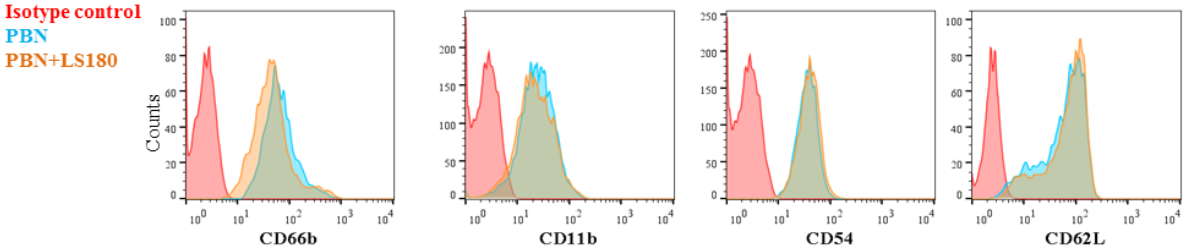
3.3 Neutrophils do not directly inhibit CRC cell proliferation

Data from TMA analysis consistent with an anti-tumor potential of CRC infiltration by neutrophils prompted us to explore possible mechanisms of action. Direct effects on CRC cells were first considered (37). However, short life span, and relatively low numbers of cells recovered from clinical specimens hampered routine use of TANs in these functional assays. Therefore, these experiments were performed by using PBNs from patients with CRC and HD. Co-culture in the presence of granulocytes did not decrease proliferation (supplementary Fig. 2A-B) nor induced apoptosis (supplementary Fig 2C) in a panel of CRC cell lines. Furthermore prior treatment of neutrophils with IFN-γ or N-formyl methionyl-leucyl-phenylalanine (fMLP) did not impact on viability and proliferation potential of co-cultured CRC cells (supplementary Fig. 2C). On the other hand co-culture with a CRC cell line promoted PBNs viability (supplementary Fig. 2D), although without modifying their phenotypic profile (supplementary Fig. 2E).


A
Proliferation (% of control)
150
100
50
0
HCT15
SW480
DLD1
LS180
SW620
HT29
CRC cells
CRC+PBN


B
Proliferation (cpm)
LS180
+HD PBN
+CRC PBN
C
Proliferation (cpm)
***
LS180
+PBN
+PBN+IFNγ+fMLP
+IFNγ+fMLP
+IFNγ+fMLP


D
PBN
PBN+LS180
20.6
0.48
36.0
43.0
27.2
0.50
39.8
32.6
CD16
Annexin V
% Annexin V+
n=6


E
Isotype control
PBN
PBN+LS180
Counts
CD66b
CD11b
CD54
CD62L

Supplementary figure 2. Co-culture with PBNs does not impact on growth of CRC cell lines. (A) PBNs from healthy donors (HD) were co-cultured with 6 different CRC cell lines at a CRC: PBN ratio 1: 20, as described in "materials and methods". After 72h, tumour cells proliferation was measured by 3Hthymidine incorporation. Similar experiment was assessed with PBNs from CRC patients as well **(B). (C)** Proliferative responses of LS180 cells cultured in the absence or presence of HD PBN (N: T ratio = 1:20*)*, with or without IFN-γ and fMLP as assessed by ^{3}H-Thymidine incorporation after 72h. A decreased CRC proliferation was observed in the presence of IFN- γ and fMLP in the absence of N, the effect of these stimuli on PBN/CRC co-cultures remains unclear. **(D)** LS180 Viability was assessed by AnnexinV staining in presence of PBN from HD. **(E)** PBNs from HD were co-cultured with LS180 CRC cell line at a CRC: PBN ratio 1: 20 and after 24h, PBNs viability was verified by Annexin V staining and the expression of their lineage and markers of activation were also measured.

3.4 Neutrophil/CD8+ lymphocyte cross-talk: effects on TCR triggered T cell activation

Alternative mechanisms of action underlying favorable prognostic significance of TANs in CRC might be related to their ability to exert indirect anti-tumor effects, mediated by other cell subsets. CRC infiltration by CD8+ T cells has widely been reported (18) to associate with favorable prognosis, although their antigen specificity is largely unclear (19). Cytokines released by activated T cells, including GM-CSF and IFN-γ are able to activate neutrophils and to prolong their survival (38). More recently, neutrophils infiltrating early stage lung cancers, but not their peripheral blood counterpart, were shown to promote T cell response to anti CD3 triggering (14;15).

Initial studies suggested that CD66b+ granulocytes frequently co-localize with CD8+ and CD45RO+ T lymphocytes within tumor tissues (Fig. 3A-B). Based on this finding, we tested the ability of TANs derived from enzyme digested CRC specimens to modulate responses of autologous peripheral blood CD8+ T cells to anti CD3 triggering. Addition of TANs to CD8+ lymphocyte cultures resulted in a significantly increased expression of CD69 early activation marker induced by suboptimal concentrations of anti CD3 mAb in the presence of anti CD28 mAb was observed. More importantly, IFN-γ release in these cultures was significantly enhanced (*P*: 0.01) (Fig. 3C and supplementary Fig. 3A-B).

Consistent with data from experiments with TANs, we observed that interaction with PBNs from patients and HD resulted in significant increases in CD69 expression and IFN-γ release by autologous CD8+ lymphocytes upon stimulation with suboptimal concentrations of anti CD3 mAb and anti CD28 mAb (Fig. 3D-E). T cells proliferation, as assessed by CFSE dilution at 72 hours, was also significantly enhanced (Fig. 3E and supplementary Fig. 5A). In contrast, these co-stimulatory effects were undetectable in T cells activated with optimal mitogenic concentrations of anti CD3 mAb (supplementary Fig. S4A).

Neutrophil-mediated co-stimulation critically required cell-to-cell contact since it was not observed in experiments performed in trans-well plates (Fig. 4A, supplementary Fig. S3B). Furthermore, blocking of CD11a on CD8+ T cells, preventing binding to CD54/ICAM-1 expressed by neutrophils, significantly (*P*=0.015) inhibited elicitation of co-stimulatory functions (Fig. 4B and supplementary Fig. 3C). Notably, CD54/ICAM-1 expression appeared to be up-regulated in neutrophils upon culture in the presence of resting and activated CD8+ T

cells (Fig. 4C). Furthermore, co-culture with activated CD8+ T cells improved neutrophil viability (Fig. 4D).

These data indicate that untreated granulocytes are able to co-stimulate CD8+ T cells, and that their effects are best detectable under sub-optimal activation conditions.

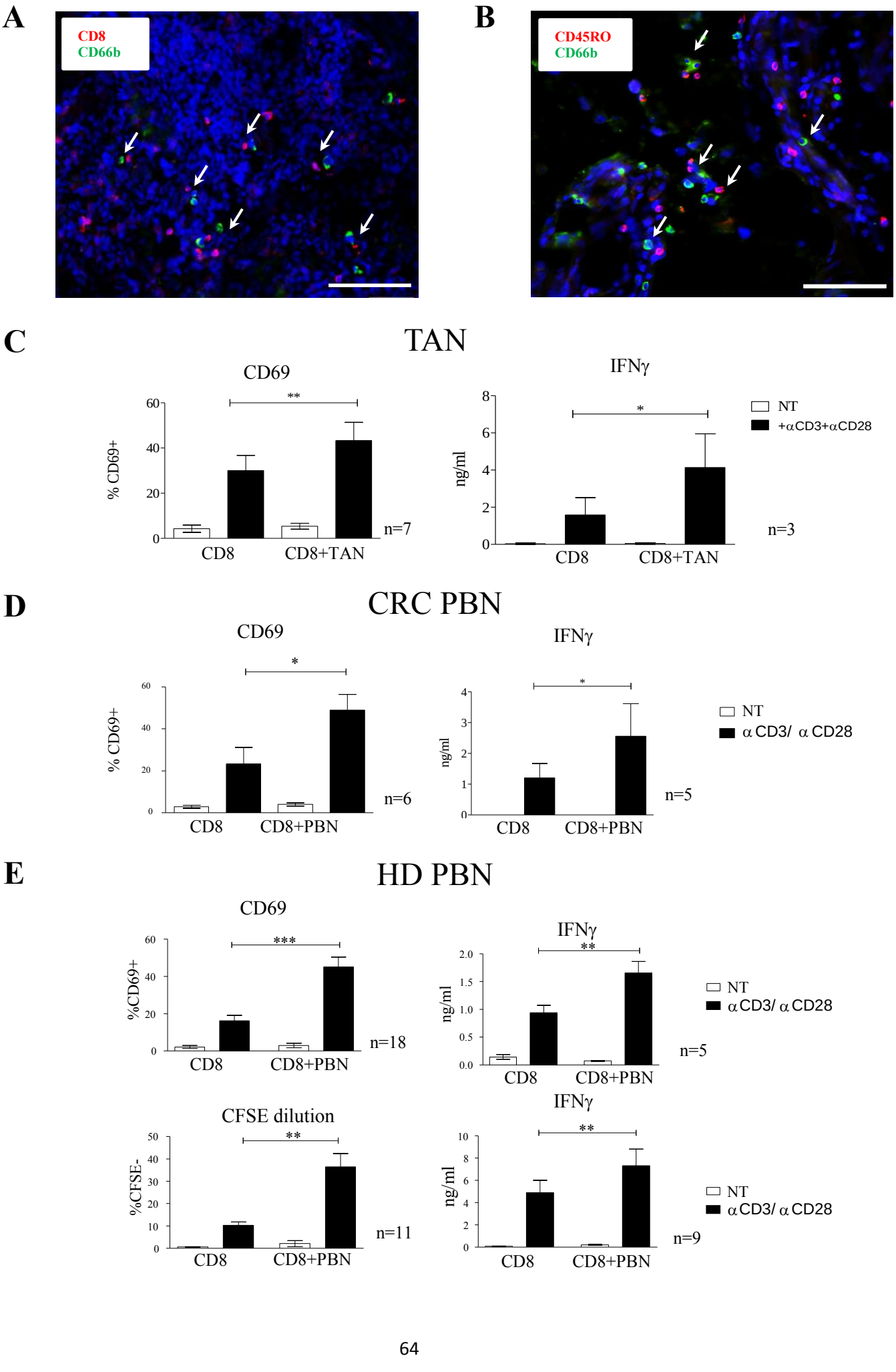
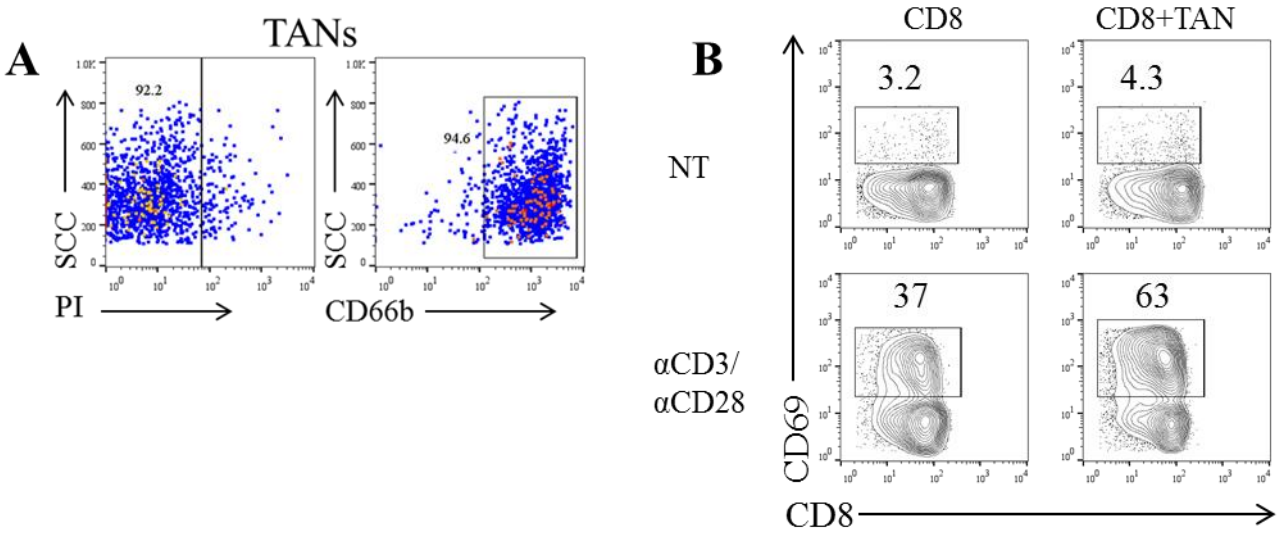
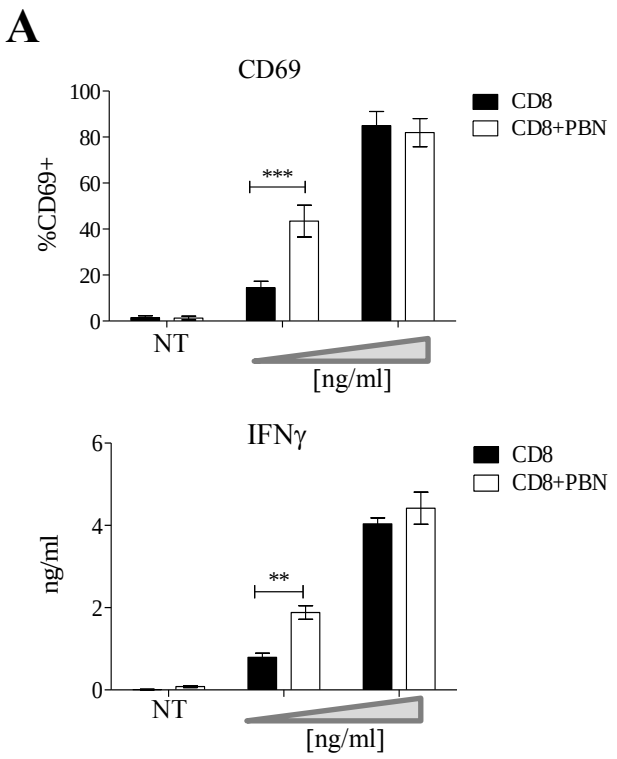

A
CD8
CD66b
B
CD45RO
CD66b
C
TAN
CD69
% CD69+
60
40
20
0
**
CD8
CD8+TAN
n=7
IFNγ
ng/ml
8
6
4
2
0
*
CD8
CD8+TAN
n=3
NT
+αCD3+αCD28
D
CRC PBN
CD69
% CD69+
60
40
20
0
*
CD8
CD8+PBN
n=6
IFNγ
ng/ml
4
3
2
1
0
*
CD8
CD8+PBN
n=5
NT
α CD3/ α CD28
E
HD PBN
CD69
%CD69+
60
40
20
0
***
CD8
CD8+PBN
n=18
IFNγ
ng/ml
2.0
1.5
1.0
0.5
0.0
**
CD8
CD8+PBN
n=5
NT
α CD3/ α CD28
CFSE dilution
%CFSE-
50
40
30
20
10
0
**
CD8
CD8+PBN
n=11
IFNγ
ng/ml
10
8
6
4
2
0
**
CD8
CD8+PBN
n=9
NT
α CD3/ α CD28

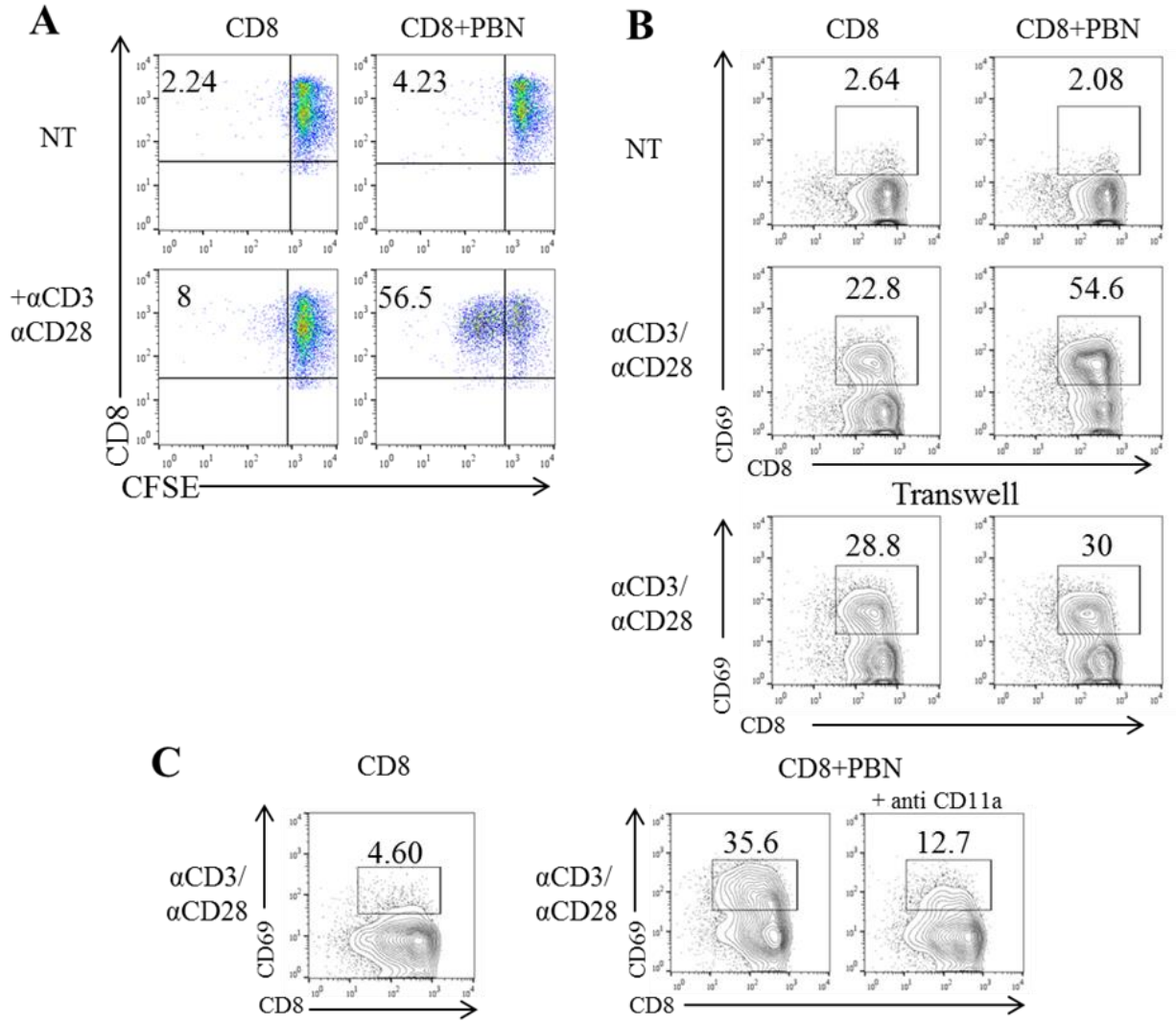
Figure 3. Tumour and peripheral blood-derived neutrophils enhance CD8+ T cell responsiveness. (A-B) Immunofluorescence staining of CD8, CD45RO and CD66b in CRC tissues. Nuclei were stained with DAPI. Pictures are representative of 5 different tissue specimens. Magnification 20x, scale bar: 50 μm. **(C)** Peripheral blood CD8+ T cells from patients with CRC undergoing surgical treatment were co-cultured for 24h with autologous, purified TAN at 1:1 ratio in the presence or absence of suboptimal concentration (1μg/ml) of anti-CD3 (clone UCTH) and anti CD28. CD69 expression was measured by flow-cytometry and IFN-γ release by ELISA. Similar experiments were performed by using PBN from patients with CRC **(D)** and from HD **(E)**. In the latter cases, T cell proliferation and IFN-γ release were also measured upon 72h culture.



Supplementary figure 3. TANs enhance CD8+ T cell responsiveness. (A) Viability showing by PI staining and purity of TANs obtained upon surgical specimens digestion and isolation with anti-CD66b beads. **(B)** Representative dot plots showing CD69 expression in peripheral blood CD8+ cells stimulated by suboptimal in the presence of anti-CD3/ anti-CD28 in presence or absence of autologous TANs.



Supplementary figure 4. TANs enhance CD8+ T cell responsiveness at suboptinmal concentration of anti-CD3. (A) Cumulative flow-cytometry histograms of CD69 expression in CD8+ T cells activated by using different concentrations of surface bound anti-CD3 (TR66, 0.5 and 2µg/ml) and soluble anti CD28 (1µg/ml) upon overnight co-culture in the presence or absence of neutrophils.



Supplementary figure 5. CD8+/PBN cross-talk is contact dependent. (A) Representative plots displaying proliferation assessed by CFSE staining upon 72h culture in peripheral blood CD8+ cells stimulated by suboptimal concentrations of anti-CD3/ anti-CD28 in presence or absence of autologous PBN. **(B)** Representative dot plots showing CD69 expression in peripheral blood CD8+ cells stimulated by suboptimal concentrations of anti-CD3/ anti-CD28 in presence or absence of autologous PBN and in control and transwell conditions, preventing cell-cell contact. **(C)** Representative plots documenting the effects of anti CD11a mAb on CD69 expression in CD8+ T cells activated by a suboptimal concentration of anti-CD3/anti-CD28 in the presence of autologous PBNs.

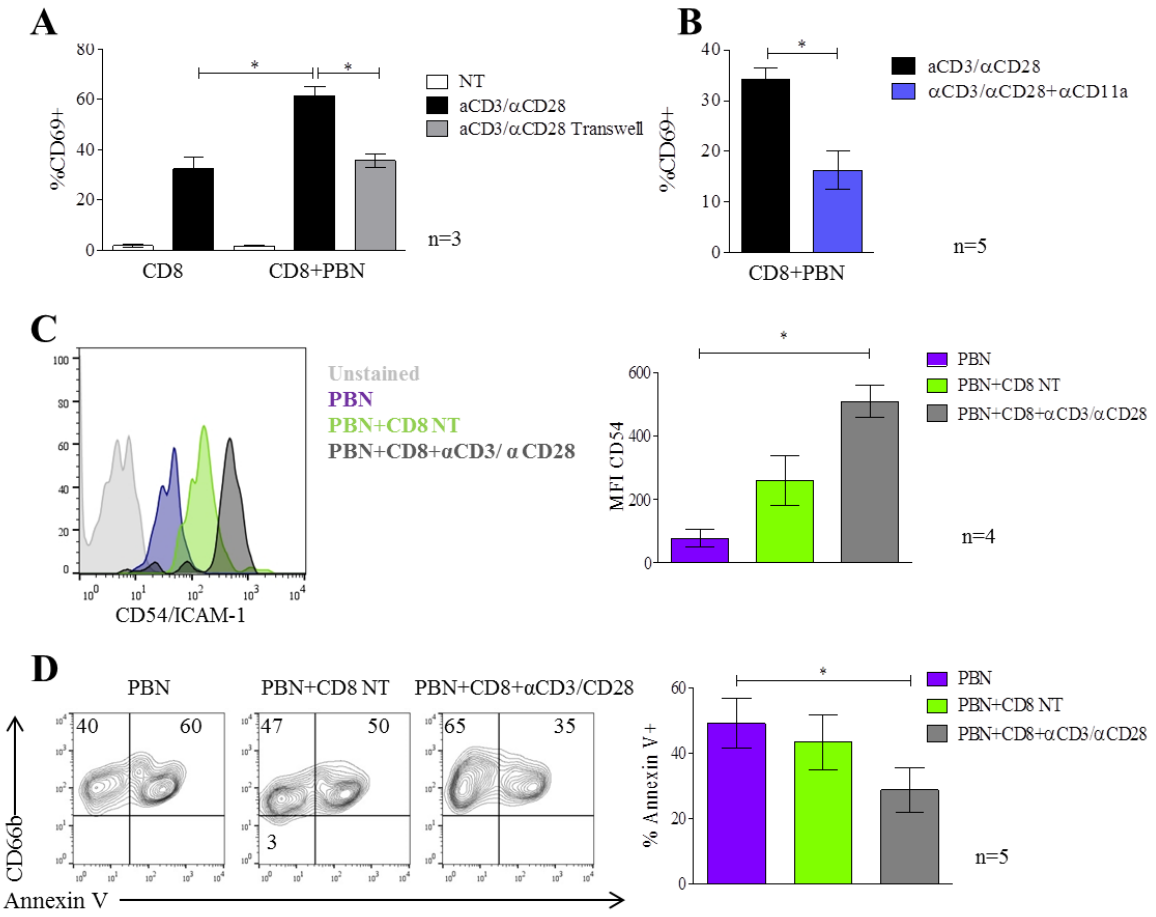


Figure 4. Neutrophil/CD8+ T cell cross talk is mediated through CD11a/CD54 interaction. (A) Peripheral blood CD8+ cells were stimulated by suboptimal concentrations of anti-CD3/ anti-CD28 in presence or absence of autologous PBN and in conditions preventing cell contact (Transwell). **(B)** Cumulative data referring to the effects of anti CD11a mAb on the increase in CD69 expression in CD8+ T cells upon stimulation by suboptimal concentrations of anti-CD3/ anti-CD28 in the presence of autologous PBN. **(C)** CD54/ICAM-1 expression was tested on live PBN, following overnight co-culture in presence or absence of CD8+, in resting state or activated by a suboptimal concentration of anti-CD3 and anti-CD28. The panel reports a representative flow-cytometry histogram and cumulative data from different experiments. **(D)** Viability of PBN following overnight culture in the presence or absence of CD8+ cells in resting state or activated by a suboptimal concentration of anti-CD3 and anti-CD28 was assessed by annexin V/PI staining. The panel reports representative results and cumulative data from independent experiments. *= $P<0.05$.

3.5 Functional features of CD8+ T cells following neutrophil-mediated co-stimulation

Favorable prognosis in CRC has been repeatedly associated with tumor infiltration by "memory" T lymphocytes (18-20). To further characterize neutrophil-mediated co-stimulation of CD8+ T cells, we evaluated phenotypic profiles of lymphocytes activated by optimal anti-CD3 concentrations and CD28 in the presence or absence of granulocytes for six days, e.g. beyond time points usually considered for detection of maximal proliferation (39). Stimulation in the presence of neutrophils induced preferential expansion of CD8+ T central memory cells with a CD45RO+CD62L+ phenotype. These cells were characterized by significantly lower expression of PD-1 exhaustion marker, as compared with those activated in the absence of PBNs or TANs. (Fig. 5A-B)

Repeated TCR-mediated T cell re-stimulation may result in activation induced cell death (AICD) (40). This phenomenon has been extensively characterized in murine models but only to a lesser extent in humans. Recently however, induction of apoptosis following re-stimulation of human CD8+ T cells initially primed in conditions of ineffective co-stimulation has been successfully demonstrated (39). To address these issues in the context of neutrophil-mediated co-stimulation, we investigated apoptosis induction in re-stimulated CD8+ T cells initially primed in the presence or absence of granulocytes. Cells initially activated in the presence of neutrophils underwent apoptosis upon TCR triggered re-stimulation to significantly lower extents, as compared to T cells primed in their absence (Fig. 5C). Taken together, these results indicate that neutrophils enhance activation and promote survival of CD8+ T cells.

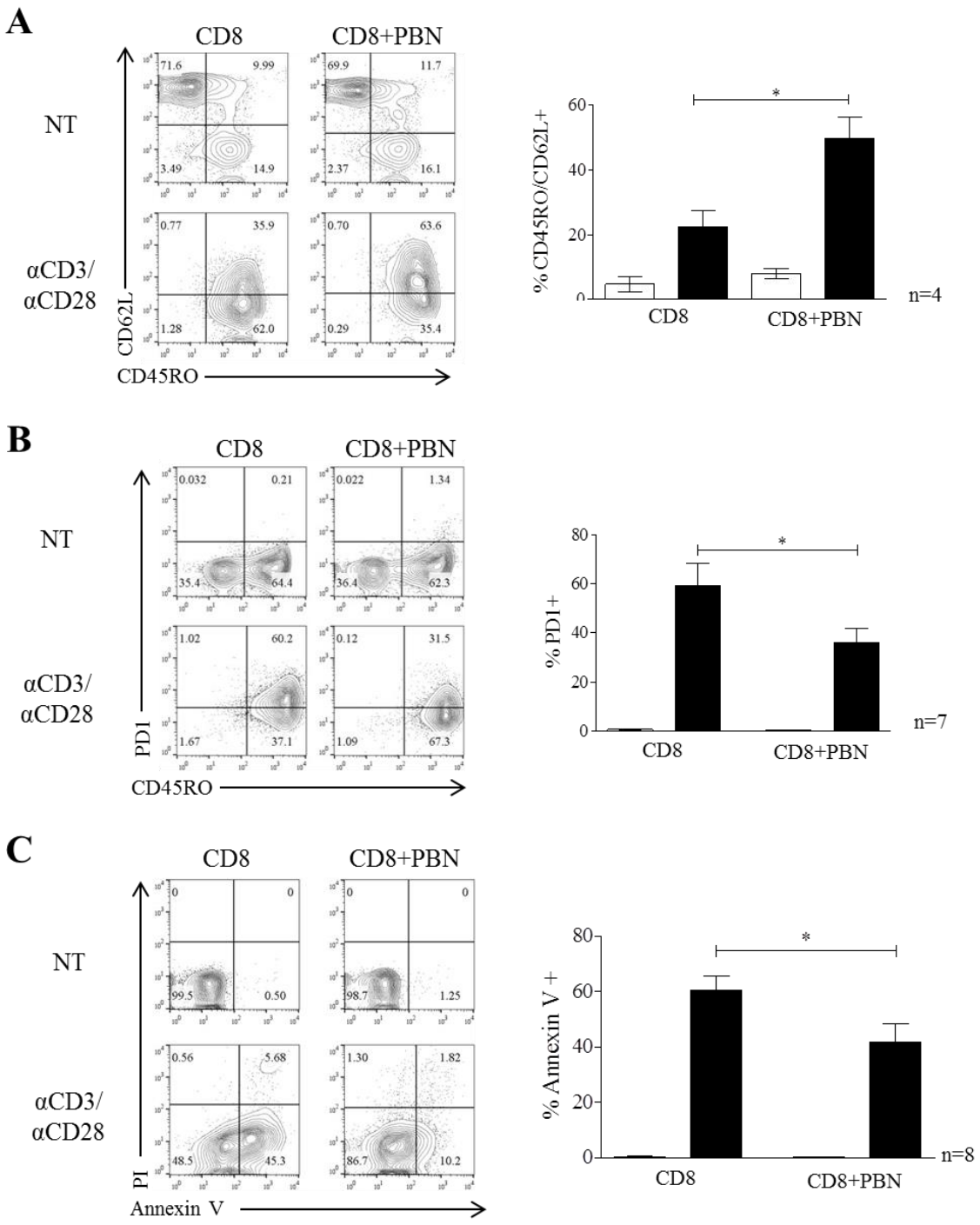


Figure 5. Neutrophils enhance CD8+ central memory differentiation and survival. Peripheral blood CD8+ cells from healthy donors were activated with optimal mitogenic concentrations of anti CD3 mAb (clone TR66, 2μg/ml) and anti CD28 in the presence or absence of autologous PBNs for 6 days. Representative flow-cytometry plots and cumulative data regarding the expression of CD62L central memory marker and PD-1 exhaustion marker in CD45RO+ cells are reported in panels **(A)** and **(B)**, respectively. CD8+ cells untreated or initially activated by anti CD3/anti CD28 in the presence or absence of autologous PBNs were re-stimulated for 24h with anti CD3/anti CD28. Annexin/PI staining was used to evaluate percentages of CD8+ T cells undergoing apoptosis upon re-stimulation. Representative flow-cytometry plots and cumulative data from different experiments are reported in panel **(C)**. *= $P<0.05$.

3.6 Impact of TANs on the prognostic significance of CD8+ T cell infiltration in CRC

"In vitro" mechanistic results urged us to analyze potential prognostic significance of combined CRC infiltration by neutrophils and CD8+ T cells. CRC infiltration by CD66b+ cells was characterized by weak, but significant, correlation with CD8+ T cell infiltration (P<0.001). These findings prompted us to investigate the prognostic significance of combined CRC infiltration by both CD66b+ neutrophils and CD8+ T cells. In our cohort (table 1), 50% of the tumors (325/652) were characterized by poor CD8+ and CD66b+ cell infiltration. While 39% (259/652) and 23% (149/652) of cases showed evidence of high CD66b+ or CD8+ T cell infiltration, respectively, a concomitantly high CD66b+ and CD8+ infiltrate was detectable in 12% of CRC samples (81/652). CRC samples infiltrated by both CD66b+ and CD8+ cells displayed favorable prognosis whereas cancers with low CD66b+ and CD8+ cell infiltration were characterized by poor prognosis (Figure 6A P:<0.0001). Most interestingly, the favorable prognostic significance of CD8+ CRC infiltration was significantly (P: 0.011) enhanced by a concomitant infiltration by CD66b+ neutrophils (Fig. 6). Accordingly, CRC samples with concomitant high infiltration by CD66b+ and CD8+ T cells were more frequently characterized by pN0 stage, e.g. absence of nodal metastases (P=0.03), and a more frequent "pushing" tumor border (P=0.038) (table 2). Taken together, these data indicate that a cross-talk between neutrophils and CD8+ T cells is of clinical relevance in CRC.

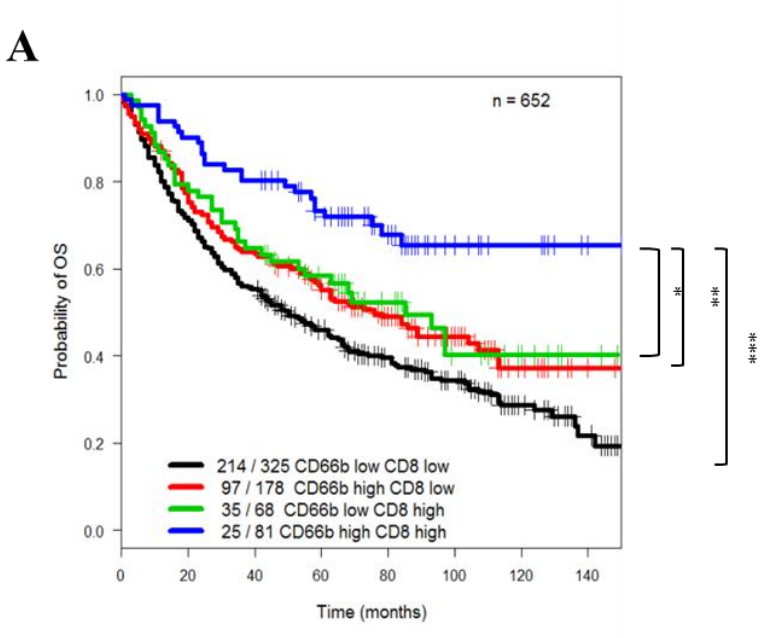


Figure 6. CRC infiltration by CD66b+ enhances the favourable prognostic significance of CD8+ infiltration in CRC. Kaplan–Meier OS curves were designed according to high and low density CD66b+ and CD8+ cell infiltration**.** *= P<0.05; **= P<0.005; ***= P<0.0005

Table 1. Clinical-pathological characteristics of the CRC patient cohort and their association with levels of CD66b+ cell infiltrate.

Characteristics		CD66 low (<10) N = 405	(50 %)	CD66 high (≥10) N = 272	(27 %)	*p*-value*
Age	years (mean)	69.5		69.5		0.887
Gender	Female	202	(50.0)	154	(56.6)	0.126
	Male	203	(50.0)	118	(43.4)	
Tumor location	Left-sided	265	(66.7)	196	(72.1)	0.214
	Right-sided	132	(33.3)	76	(27.9)	
Histologic subtype	Mucinous	25	(5.8)	21	(9.6)	0.299
	Non-mucinous	380	(94.2)	270	(90.4)	
pT stage	pT1-2	63	(15.9)	61	(22.8)	0.027
	pT3-4	332	(84.1)	207	(77.2)	
pN stage	pN0	189	(47.8)	161	(60.1)	0.001
	pN1-2	206	(52.8)	107	(49.9)	
Stage	I	42	(10.8)	51	(19.1)	< 0.0001
	IIA	123	(31.8)	100	(37.6)	
	IIBC	20	(5.2)	8	(3.5)	
	IIIA/B	175	(45.2)	95	(35.7)	
	IIIC	27	(7.0)	11	(4.1)	
Tumor grade	G1-2	367	(93.1)	250	(93.3)	0.945
	G3	27	(6.9)	18	(6.7)	
Vascular invasion	Absent	263	(66.6)	206	(60.1)	0.005
	Present	132	(33.4)	62	(31.9)	
Tumor border	Pushing	106	(26.9)	94	(35.2)	0.028
	Infiltrating	288	(73.1)	173	(74.8)	
PTL inflammation	Absent	315	(79.7)	202	(75.4)	0.216
	Present	80	(20.3)	66	(24.6)	
Microsatellite stability	Deficient	48	(11.8)	35	(12.9)	0.783
	Proficient	357	(88.2)	237	(87.1)	
5-year survival rate	(95%CI)	46.7%	(42.0-51.9)	61.9%	(56.3-68.0)	0.0001

Table 2.Clinical-pathological characteristics of the CRC patient cohort and their association with levels of CD8+ and CD66b+ cell infiltrate.

Characteristics		CD66b low CD8 low N =325 (50 %)		CD66b high CD8 low N =178 (27 %)		CD66b low CD8 high N = 68 (11 %)		CD66b high CD8 high N = 81 (12 %)		*P*-value*	*P*-value**
Age	years (mean)	71		70		70		69		0.480	-
Gender	Female	156	(48.0)	100	(56.2)	38	(55.9)	48	(59.3)	0.145	0.74
	Male	169	(52.0)	78	(43.8)	30	(44.1)	33	(40.7)		
Tumor location	Left-sided	222	(68.5)	132	(74.2)	43	(64.2)	52	(64.2)	0.28	1
	Right-sided	102	(31.5)	46	(25.8)	24	(35.8)	29	(35.8)		
Histologic subtype	Mucinous	19	(5.8)	17	(9.6)	4	(5.9)	2	(2.5)	0.654	0.41
	Non-mucinous	306	(94.2)	161	(90.4)	64	(94.1)	79	(97.5)		
pT stage	pT1-2	47	(14.8)	34	(19.2)	14	(21.5)	21	(26.8)	0.069	0.55
	pT3-4	271	(85.2)	143	(80.1)	51	(78.5)	57	(73.2)		
pN stage	pN0	146	(46.1)	89	(50.9)	40	(60.6)	62	(77.5)	**<0.0001**	**0.03**
	pN1-2	171	(53.9)	86	(49.1)	26	(39.4)	18	(22.5)		
Tumor grade	G1-2	300	(94.6)	170	(96.0)	58	(89.2)	67	(75.9)	**0.008**	0.61
	G3	17	(5.4)	7	(4.0)	7	(10.8)	11	(14.1)		
Vascular invasion	Absent	210	(66.0)	132	(74.6)	45	(69.2)	64	(82.1)	**0.023**	0.07
	Present	108	(34.0)	45	(25.4)	20	(30.8)	14	(17.9)		
Tumor border	Pushing	81	(25.6)	51	(29.0)	19	(29.2)	37	(47.4)	**0.002**	**0.038**
	Infiltrating	236	(74.4)	125	(71.0)	46	(70.8)	41	(52.6)		
PTL inflammation	Absent	258	(81.1)	142	(80.2)	48	(73.8)	51	(65.4)	**0.017**	0.36
	Present	60	(18.9)	35	(19.2)	17	(26.2)	27	(34.6)		
Microsatellite stability	Deficient	37	(15.2)	19	(14.5)	10	(16.5)	16	(32.7)	0.166	0.51
	Proficient	288	(84.8)	159	(85.5)	58	(83.5)	65	(67.3)		

*Clinical-pathological differences between CRC displaying CD66b+ low/CD8+ low density infiltration and the other three subgroups. Jonckheere test if variable numeric (age); otherwise Kruskal Wallis test.

** Clinical-pathological differences between CRC displaying CD66b+ low/CD8+ high density or CD66b+ high/CD8+ high density infiltration. Fisher exact test.

4. Discussion

Analysis of a large clinically annotated TMA including over 600 CRC reveals that CD66b+ cell infiltration is associated with favorable prognosis. While CRC appear to be infiltrated to a larger extent than autologous adjacent healthy tissue, phenotypic profiles of CRC infiltrating neutrophils largely match those detectable in healthy mucosa infiltrating cells. However, in keeping with data regarding early lung cancer infiltrating neutrophils (14;15), TAN appear to express CD54 to higher extents as compared to neutrophils infiltrating autologous adjacent healthy mucosa.

Mechanisms potentially underlying the favorable prognostic significance of CRC infiltration by CD66b+ cells were investigated in detail. Our results indicate that co-culture with autologous neutrophils enhances TCR triggered activation of CD8+ T cells and promotes the expansion of a lymphocyte subset characterized by the expression of "memory" markers and a relative resistance to AICD induced by repeated stimulation. The relevance of these findings to CRC immunobiology is indirectly supported by the co-localization of neutrophils and both CD8+ and CD45RO+ T cells in CRC tissues. Furthermore, most importantly, CRC concomitantly infiltrated by neutrophils and CD8+ T cells are characterized by a significantly more favorable prognosis, as compared to tumors displaying high CD8+ but low CD66b+ cell infiltration. In contrast, high neutrophil infiltration in the absence of concomitant CD8+ lymphocytes only provided a modest prognostic advantage as compared to CRC where neither high CD8+ nor CD66b infiltrate was detectable.

These data suggest that the favorable prognostic significance of infiltration by neutrophils in CRC largely relies on their interaction with CD8+ T cells, possibly based on co-stimulatory mechanisms, as suggested by our "in vitro" results. The potential relevance of these data might obviously extend beyond CRC immunobiology. These interactions might indeed be operational in a wider range of conditions, thus supporting the notion of a highly effective cooperation between innate and adaptive immune responses.

Remarkably, similar results have also emerged from studies conducted in experimental models (41) and in clinical settings, including early stage lung cancer (14;15) and autoimmune and infectious disease. Underlying molecular mechanisms have not been fully clarified. In particular, cell-cell interactions mediated by OX40, CD58, CD59 and their ligands have been proposed (14;39). Alternatively, a role for ROS release has also been suggested (41). Our data suggest that CD11a/CD54 interaction powerfully contributes to the elicitation of the co-

stimulatory effects of neutrophils on CD8+ T cell activation. Nevertheless, further research is warranted to obtain additional insights into underlying molecular mechanisms.

An important limitation in studies on TANs is represented by their short life span, high sensitivity to enzymes necessary for the generation of single cell suspensions from clinical specimens and relatively low numbers, preventing the routine performance of functional studies. However, the consistency of data emerging by using TANs, and PBNs from patients with CRC and healthy donors together with the clinical data, reliably supports the concept of a potentially high clinical relevance of neutrophil-T cell interaction on tumor sites.

Our study also suggests a number of additional important questions. The fact that in a variety of tumors other than CRC, neutrophil infiltration has been suggested to be associated with poor prognosis (42-44) raises the issue of the specificities inherent in neutrophil infiltration in CRC. Our results indicate that the favorable prognostic significance of the latter largely, albeit not exclusively, relies on the interaction between neutrophils and CD8+ T cells, and suggest that lymphocyte infiltration may represent an important pre-requisite for the detection of these effects. Therefore, lack of lymphocyte infiltration or infiltration by "unfavorable" T cells (45) might prevent a full elicitation of anti-tumor effects of neutrophils.

Data regarding neutrophil infiltration in CRC from East Asia patients (29) appear to contradict our results. Genetic background may play an important role in this context. However, additional factors might be involved in determining the prognostic relevance of neutrophil infiltration. CRC oncogenesis is typically characterized by an early increase in bacterial translocation from the gut lumen (45) and gut microbiome composition has recently been shown to decisively impact on chemo- and immunotherapy outcome (46-48). Considering the key role of neutrophils in the response to bacteria, it is tempting to speculate that microbiome composition might decisively affect their ability to actively participate to anti-tumor immune response. Thus, differences in human gut microbiome in different geographic areas (49) might also be of importance in the evaluation of the prognostic significance of neutrophil infiltration in CRC. On the other hand, our data urge studies aimed at the clarification of mechanisms favoring the recruitment of granulocytes within CRC tissues.

In conclusion, our study, providing clear evidence of the prognostic significance of concomitant infiltration by CD8+ cells and neutrophils in CRC, represents one of the first examples (50) of clinically relevant interaction between non-cancerous cells from the tumor microenvironment. Furthermore, it clearly identifies neutrophils as key players in CRC immunobiology.

5. Reference

(1) Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer* 2016;**16**(7):431-46.

(2) Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis* 2012;**33**(5):949-55.

(3) Di CE, Forni G, Lollini P *et al*. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood* 2001;**97**(2):339-45.

(4) Templeton AJ, McNamara MG, Seruga B *et al*. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst* 2014;**106**(6):dju124.

(5) Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;**9**(3):162-74.

(6) Guglietta S, Chiavelli A, Zagato E *et al*. Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. *Nat Commun* 2016;**7**:11037.

(7) Marvel D, Gabrilovich DI. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. *J Clin Invest* 2015;**125**(9):3356-64.

(8) Fridlender ZG, Sun J, Kim S *et al*. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 2009;**16**(3):183-94.

(9) Jablonska J, Leschner S, Westphal K *et al*. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest* 2010;**120**(4):1151-64.

(10) Granot Z, Henke E, Comen EA *et al*. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 2011;**20**(3):300-14.

(11) Finisguerra V, Di CG, Di MM *et al*. MET is required for the recruitment of anti-tumoural neutrophils. *Nature* 2015;**522**(7556):349-53.

(12) Dranoff G, Jaffee E, Lazenby A *et al*. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A* 1993;**90**(8):3539-43.

(13) Stoppacciaro A, Melani C, Parenza M *et al*. Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon gamma. *J Exp Med* 1993;**178**(1):151-61.

(14) Eruslanov EB, Bhojnagarwala PS, Quatromoni JG *et al*. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *J Clin Invest* 2014;**124**(12):5466-80.

(15) Singhal S, Bhojnagarwala PS, O'Brien S *et al*. Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen-Presenting Cell Features in Early-Stage Human Lung Cancer. *Cancer Cell* 2016.

(16) Ferlay J, Shin HR, Bray F *et al*. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;**127**(12):2893-917.

(17) Cunningham D, Atkin W, Lenz HJ *et al*. Colorectal cancer. *Lancet* 2010;**375**(9719):1030-47.

(18) Fridman WH, Pages F, Sautes-Fridman C *et al*. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012;**12**(4):298-306.

(19) Galon J, Costes A, Sanchez-Cabo F *et al*. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;**313**(5795):1960-4.

(20) Pages F, Berger A, Camus M *et al*. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005;**353**(25):2654-66.

(21) Gabitzsch ES, Tsang KY, Palena C *et al*. The generation and analyses of a novel combination of recombinant adenovirus vaccines targeting three tumor antigens as an immunotherapeutic. *Oncotarget* 2015;**6**(31):31344-59.

(22) Mennonna D, Maccalli C, Romano MC *et al*. T cell neoepitope discovery in colorectal cancer by high throughput profiling of somatic mutations in expressed genes. *Gut* 2015.

(23) Zlobec I, Karamitopoulou E, Terracciano L *et al*. TIA-1 cytotoxic granule-associated RNA binding protein improves the prognostic performance of CD8 in mismatch repair-proficient colorectal cancer. *PLoS One* 2010;**5**(12):e14282.

(24) Sconocchia G, Arriga R, Tornillo L *et al*. Melanoma cells inhibit NK cell functions. *Cancer Res* 2012;**72**(20):5428-9.

(25) Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006;**124**(2):263-6.

(26) Nagorsen D, Voigt S, Berg E *et al*. Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. *J Transl Med* 2007;**5**:62.

(27) Sconocchia G, Zlobec I, Lugli A *et al*. Tumor infiltration by FcgammaRIII (CD16)+ myeloid cells is associated with improved survival in patients with colorectal carcinoma. *Int J Cancer* 2010.

(28) Droeser RA, Hirt C, Eppenberger-Castori S *et al*. High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. *PLoS One* 2013;**8**(5):e64814.

(29) Rao HL, Chen JW, Li M *et al*. Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. *PLoS One* 2012;**7**(1):e30806.

(30) Yamamoto T, Kawada K, Itatani Y *et al*. Loss of SMAD4 Promotes Lung Metastasis of Colorectal Cancer by Accumulation of CCR1+ Tumor-associated Neutrophils through CCL15-CCR1 Axis. *Clin Cancer Res* 2016.

(31) Galdiero MR, Bianchi P, Grizzi F *et al*. Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer. *Int J Cancer* 2016;**139**(2):446-56.

(32) Amicarella F, Muraro MG, Hirt C *et al*. Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. *Gut* 2015.

(33) Nebiker CA, Han J, Eppenberger-Castori S *et al*. GM-CSF Production by Tumor Cells Is Associated with Improved Survival in Colorectal Cancer. *Clin Cancer Res* 2014;**20**(12):3094-106.

(34) Trella E, Raafat N, Mengus C *et al*. CD40 ligand-expressing recombinant vaccinia virus promotes the generation of CD8(+) central memory T cells. *Eur J Immunol* 2016;**46**(2):420-31.

(35) Daster S, Eppenberger-Castori S, Hirt C *et al*. Absence of myeloperoxidase and CD8 positive cells in colorectal cancer infiltrates identifies patients with severe prognosis. *Oncoimmunology* 2015;**4**(12):e1050574.

(36) Hirt C, Eppenberger-Castori S, Sconocchia G *et al*. Colorectal carcinoma infiltration by myeloperoxidase-expressing neutrophil granulocytes is associated with favorable prognosis. *Oncoimmunology* 2013;**2**(10):e25990.

(37) Ackermann MF, Lamm KR, Wiegand GW *et al*. Antitumor activity of murine neutrophils demonstrated by cytometric analysis. *Cancer Res* 1989;**49**(3):528-32.

(38) Pelletier M, Micheletti A, Cassatella MA. Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. *J Leukoc Biol* 2010;**88**(6):1163-70.

(39) McKinney EF, Lee JC, Jayne DR *et al*. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature* 2015;**523**(7562):612-6.

(40) Waldmann TA, Dubois S, Tagaya Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity* 2001;**14**(2):105-10.

(41) Schwab L, Goroncy L, Palaniyandi S *et al*. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. *Nat Med* 2014;**20**(6):648-54.

(42) Coffelt SB, Kersten K, Doornebal CW *et al*. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 2015;**522**(7556):345-8.

(43) Fridlender ZG, Albelda SM, Granot Z. Promoting metastasis: neutrophils and T cells join forces. *Cell Res* 2015;**25**(7):765-6.

(44) Liang W, Ferrara N. The Complex Role of Neutrophils in Tumor Angiogenesis and Metastasis. *Cancer Immunol Res* 2016;**4**(2):83-91.

(45) Abreu MT, Peek RM, Jr. Gastrointestinal malignancy and the microbiome. *Gastroenterology* 2014;**146**(6):1534-46.

(46) Iida N, Dzutsev A, Stewart CA *et al*. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science* 2013;**342**(6161):967-70.

(47) Vetizou M, Pitt JM, Daillere R *et al*. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* 2015;**350**(6264):1079-84.

(48) Viaud S, Saccheri F, Mignot G *et al*. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* 2013;**342**(6161):971-6.

(49) Yatsunenko T, Rey FE, Manary MJ *et al*. Human gut microbiome viewed across age and geography. *Nature* 2012;**486**(7402):222-7.

(50) Herrera M, Herrera A, Dominguez G *et al*. Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients. *Cancer Sci* 2013;**104**(4):437-44.

(51) Kostic AD, Chun E, Robertson L *et al*. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013;**14**(2):207-15.

CHAPTER III: “Modulation of neutrophils functions by gut commensal microorganisms”

V Governa[1, 2] V Mele[1], D Calabrese, H Xu[1] E Trella, RA Droeser[1,3], S Däster[1,3], M Bolli[4], L Terracciano[2], G Iezzi[1] and GC Spagnoli[1,6].

[1]Department of Biomedicine, Basel University Hospital and University of Basel, Basel, CH; [2]Molecular Pathology Division, Institute of Pathology, Basel University Hospital and University of Basel, CH; [3]Department of General Surgery, Basel University Hospital and University of Basel, CH; [4]Department of Visceral Surgery, St. Claraspital, Basel, CH; [5]Department of Visceral Surgery, Ospedale Civico, Lugano, CH.qa

(in preparation)

Abstract

Purpose: Human colorectal cancer (CRC) is characterized by a peculiar microenvironment including a large number of microorganisms. Among them, *F.nucleatum* has been associated with CRC progression, triggering tumorigenesis and resulting in severe prognosis. Immune cells also have a fundamental role on CRC development. While immune system, tumor cells and bacteria are key components of the "ménage à trois" of the CRC microenvironment, many aspects of their interactions remain to be investigated. Here, we analyzed the effects of *F.nucleatum* on phenotypes and functional features of CD66b+ neutrophils.

Experimental design: CD66b+ cell infiltration and presence of *F,nucleatum* within CRC tissues (>50 evaluable CRC samples) and corresponding healthy mucosa was assessed by RT-PCR. Phenotypes and cytokine release profiles of peripheral blood CD66b+ cells cultured in the presence of *F.nucleatum* were evaluated by flow cytometry and Luminex assays, respectively. Moreover CD66b+/ CD8+ and CD66b+/Tumor associated stromal cells crosstalk was investigated through in vitro experiments in presence of *F.nucleatum* and *B.fragilis*.

Results: We report that the infiltration by CD66b+ cells in CRC significantly correlates with the presence of *F.nucleatum*. In vitro experiments using CRC lines revealed a high secretion of IL-8 induced by *F.nucleatum*, resulting in an increase of neutrophil recruitment. Functional studies indicate that neutrophils cultured in the presence of *F.nucleatum* are devoid of direct antitumor potential and their viability, phenotype and cytokine release potential is dramatically affected as compared with cultures performed in the presence of control gram- bacteria. Moreover, in presence of *F.nucleatum* CD66b+ cells lose their capability to stimulate CD8+ cells. Importantly, Neutrophils affected by *F.nucleatum* -and not by B.fragilis- induce a high release of IL-6 by tumor associated stromal cells, potentially leading enhanced tumor cell proliferation.

Conclusions: *F.nucleatum* may impair the ability of neutrophils to elicit anti-tumor functions within the CRC microenvironment.

1. Introduction

Neutrophils represent the predominant immune cell type in human peripheral blood and play a major role in the first line immune response against infectious microorganisms. However, their role in anti-tumor responses in humans has not been investigated in detail. In previous studies we have characterized tumor associated neutrophils (TANs) infiltrating human colorectal cancer (CRC) and we have shown that they are able to enhance the responsiveness of autologous CD8+ T cells to TCR triggering. Most importantly, we have also shown that infiltration by neutrophils enhances the prognostic significance of CRC infiltration by CD8+ T cells, thus suggesting that they might effectively promote antitumor immunity (Governa *et al*., in press).

A major peculiarity of CRC microenvironment, is represented by the interplay between gut microbiota and tumor cells as recently reported (1). Indeed tumor progression has been shown to be dependent on both innate and adaptive immune system activation and on quality and quantity of intestinal microflora. Alterations of host-microbiome interaction, frequently referred to as "dysbiosis", have been suggested to significantly contribute to CRC development (2). However, underlying mechanisms are still largely unclear.

Multistep CRC development is accompanied by the expression of Apc, K-Ras and p53 mutations and by morphological alterations, leading to early increase of mucosal permeability and the activation of the mucosal and systemic immune system. Numerous studies have compared microbial community composition in CRC and in normal mucosa and in patients and healthy subjects. In particular, *Fusobacterium nucleatum* has been shown to be most reproducibly associated with CRC (3;4).

Remarkably, *F.nucleatuam* has been shown to modulate immune responses as well. In a mouse model, this bacterium favored CRC progression by releasing short-peptides and short-chain of fatty acids into the tumour microenvironment, serving as chemo-attractants for myeloid-derived suppressors cells (MDSCs). Furthermore, Kostic et al observed increased infiltration of TAMs, TANs, and DCs, in colonic-adenomas of *F.nucleatum* –infected mice, as compared with controls.

High density T cell infiltration has been associated with favorable prognosis in CRC (5;6). Recently, extent of *F.nucleatum* colonization in various CRC patients has been shown to be inversely correlated with CD3+ T cell infiltration (CD8+, CD4+, CD45RO+, FOXP3+ cells) thus suggesting that it might downregulate T cell-mediated antitumour immune responses thereby promoting colorectal tumour progression (7-9).

Based on this background, in this study we explored the potential role *of F.nucleatum* on Neutrophils within the CRC context. To this purpose we have analyzed the correlation between *F.nucleatum* colonization and neutrophil infiltration in CRC microenvironment. Furthermore we have investigated the impact of *F.nucleatum* on neutrophil functions and how this interaction could affect the interplay with other components of the CRC microenvironment, including CD8+ lymphocytes and Tumor associate stromal cells (TASC).

2. Materials and methods

2.1 Tumor cell lines - Clinical specimen collection - Neutrophil and lymphocyte isolation

Please refer to methods described in Chapter II.

2.2 Bacteria

Fusobacterium nucleatum (subsp. *Nucleatum*, ATCC 25586), *Bacteroides vulgatus* (Eggerth and Gagnon, ATCC 8482), and *Bacteroides fragilis* (non enterotoxigenic strain 9343, ATCC 25285), were kindly provided by Dr. Nina Khanna, Department of Biomedicine, University of Basel. They were cultured under anaerobic conditions.

2.3 Real-time reverse transcription PCR assays

Total RNA was extracted from stored CRC tissues or sorted cell populations using Trizol (Invitrogen) and reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen) and Random Primers. Quantitative Real-Time PCR was performed in the ABI prism™ 7700 sequence detection system, using Syber green (Roche) and TaqMan Universal Master Mix and No AmpErase UNG (both from Applied Biosystems).

F.nucleatum primers by Microsyth:

FW- GCCTCACAGCTAGGGACAAC

RW- GAGTAAGGGCCGTGTCTCAG

2.4 CRC/PBN/Bacteria co-cultures

Cells from established cell lines were cultured in the presence or absence of neutrophils (PBN: LS180 = 20:1) and bacteria, at different ratios and tumor cell proliferation was assessed by ^{3}H-Thymidine incorporation (^{3}H-TdR) upon 72h culture.

2.5 Flow cytometry

Cell suspensions from CRCs and tumor-free mucosa, and peripheral blood of healthy donors or patients with CRC, were stained with fluorochrome-conjugated antibodies specific for human

CD66b, CD16, CD11b, (BioLegend) and CD62L, (BD Biosciences). Alternatively, cells were fixed and permeabilized, and intracellular staining was performed by using antibodies specific for MPO (10). Stained cells were analyzed by FACS Calibur flow cytometer (BD Biosciences), using FlowJo software (Tree Star).

2.6 PBN and CD8+ T cell co-cultures

TANs and PBNs, obtained from HD and patients with CRC were co-cultured with autologous peripheral blood CD8+ T lymphocytes isolated by using antibody-coated magnetic beads (Miltenyi Biotec) following gradient centrifugation (11). For co-stimulation experiments, 96-well flat bottom culture plates were coated overnight with anti CD3 mitogenic mAb UCHT-1, (eBiosciences) at sub-optimal concentrations ranging between 0.5 and 5 μg/ml depending from hybridoma and lot. Neutrophils and CD8+ T cells, at a 0,5 10^6/ml concentration each were cultured in RPMI 1640 medium supplemented with GlutaMAX I, HEPES, sodium pyruvate, non-essential amino acids, antibiotics (all from GIBCO) and 5% AB serum (Blood Bank, Kantonsspital Basel), thereafter referred to as complete medium, in the presence of anti-CD28 (1μg/ml, BD Pharmingen). T cell proliferation was measured by assessing carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) dilution in labelled CD8+ T cells following 72h culture by flow cytometry (11). IFN-γ release in culture supernatants was assessed by using commercial ELISA kits (BD Biosciences). Neutrophils were purified as detailed above and re-suspended in complete medium. They were then treated for 1 hour with *Fusobacterium nucleatum* and *Bacteroides Fragilis* heat-killed and opsonized with 10% HS for 15'. Following washing, neutrophils were added to CD8+ cells culture.

2.7 PBN response to bacteria

Phenotypic profiles of bacteria-treated neutrophils (ratio PBN : bacteria = 1:5) were analyzed by flow cytometry at defined time points between 2 and 24 hours, with particular attention to surface markers typically modulated in inflammatory states, such as CD16, CD66b, CD11b and CD62L. In parallel experiments, bacteria-treated neutrophils were co-cultured in complete medium with autologous CD8+ T cells in flat bottom 96 well plates previously sensitized with anti CD3 mAbs at sub-optimal concentrations (see above). Expression of CD69 activation marker, expression of IFN-γ gene and cytokine release and T cell proliferation were evaluated as detailed above. In specific experiments, induction of apoptosis in PBN by Bacteria was tested by Annexin V/PI staining (BioLegend). Supernatant of PBNs cultured with bacteria were

collected upon overnight stimulation and used for Luminex Multiplex assay (Invitrogen, Thermo Fisher Scientific).

2.8 Tumor associated stromal cells (TASC) isolation

Tumor-associated stromal cells were isolated from freshly excised CRC samples, obtained from consenting patients undergoing surgical treatment. The tissue sample was rinsed with PBS (GIBCO) and minced using scissors or scalpels. It was then transferred to digestion medium containing serum-free DMEM (GIBCO), supplemented with Collagenase IV (100x; 20kU/ml; Worthington #CLSS-4), DNAse I (100x; 50 mg/ml; Sigma-Aldrich #D5025), HEPES (10mM; GIBCO #15630-056), Kanamycin (100x; GIBCO #15160-047), Amphotericin B (100x; 250ug/mL; Sigma-Aldrich #A9528), Metronidazol (250x; 200mg/ml, Braun), Cefuroxim (250x; 15mg/ml, Braun).

The suspensions were shaken vigorously for few seconds and then digested for 1 hour at 37°C on continuous smooth rotation. After digestion, the suspensions were filtered through a 100µm (Falcon #352360) and then 70µm (Falcon #352350) nylon mesh Cell Strainer. Cell in suspensions were counted and used for other purposes, while the small chunks remaining on the top of the 100µm filter were picked up with tweezers and transferred into wells of a 12-well plates (about 3 per well). Two milliliters of alpha MEM supplemented with 10% Fetal Bovine serum (GIBCO), Kanamycin (100x; GIBCO #15160-047), GlutaMAX-I (10mM) Sodium Pyruvate (100mM) were added in each well. Cells were cultured at 37°C with 5% CO2. The medium was changed twice per week and the cell outgrowth was monitored by light microscopy.

After an initial isolation period, TASCs were expanded in vitro, characterized by flow cytometry for the purity of the population by using CD90 antibody, and, if necessary, sorted by flow cytometry.

2.9 TASC and CRC cell lines co-culture

LS180 CRC cells line was co-cultured with TASC at different ratios, for 5 days in tumor cell medium. In specific experiments, supernatants collected from PBNs cultured with bacteria overnight, from 4 different HD donors were filtered and added to cultures every 24h for 5 days. Monocultures of TASC or tumor cells were used as controls. At the end of culture periods,

supernatants were collected and IL-6 release was evaluated by ELISA (Biolegend). Additionally, cells were harvested and used for subsequent FACS and gene expression analyses.

2.10 Migration Assay

PBNs suspended in serum-free medium were seeded into upper chambers of transwell plates onto uncoated or matrigel-coated membranes (4 μm pore size, BD Biosciences). Lower chambers contained medium collected upon co-culture of LS180 CRC cell line and *F.nucleatum* (Fn) or *B.fragilis* (Bf). Plates were incubated at 37°C. After 20 hours inserts were removed and the numbers of PBNs that had migrated into the lower chambers were quantified by CyQUANT Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA).

2.11 Measurement of ROS

PBNs from healthy donors are resuspended in HBSS medium and concentrated at a $2x10^6$ /ml concentration. The different concentrations of *F.nucleatum* pre-incubated with HS 5% for 15' RT were directly and rapidly added at the MicroLumat plus (Berchtold Technologies). The indirect amount of produced HOCl (ROS) by oxidant Luminol has been detected in 25 cycles at interval with 5 minutes, for a total of 120 minutes with a constant temperature of 37°. Negative controls were stimuli-free samples.

3. RESULTS

3.1 *F.nucleatum* in CRC patients.

We first addressed the presence of *F.nucleatum* (*Fn*) in tissues from a limited cohort of CRC patients (n=30). Evidence of the presence of *Fn* colonization in a sizeable percentage of cases in our tumor cohort could be reliably obtained by RT-PCR. Importantly in 70% of cases, 16s Fn expression was significantly higher in tumor tissues as compared to autologous healthy mucosa (Figure 1).

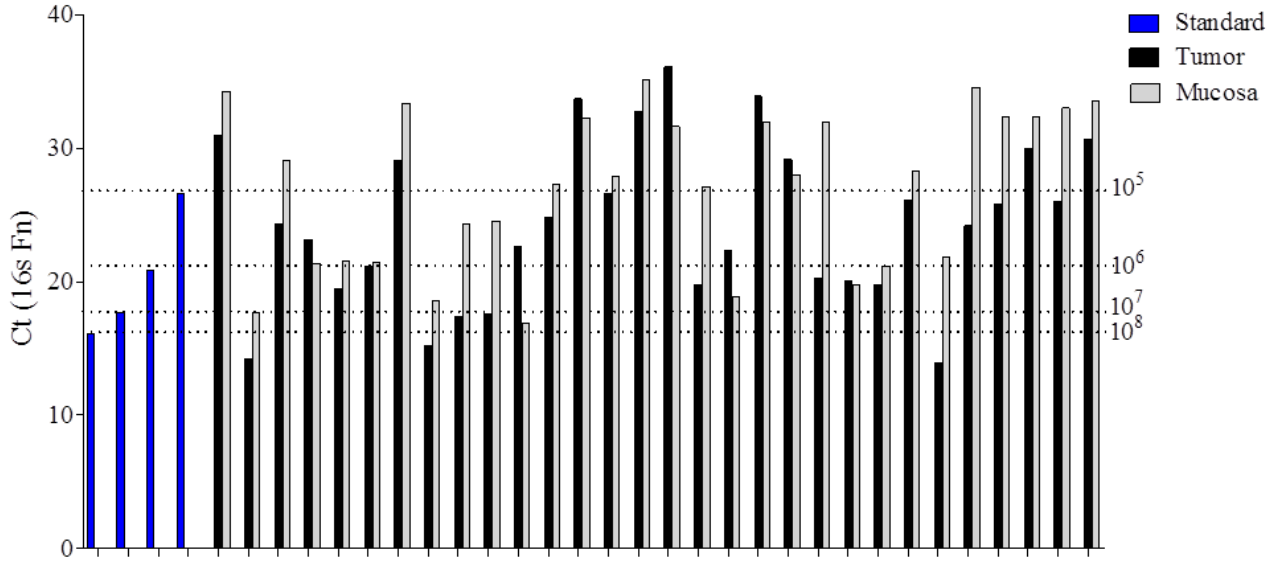
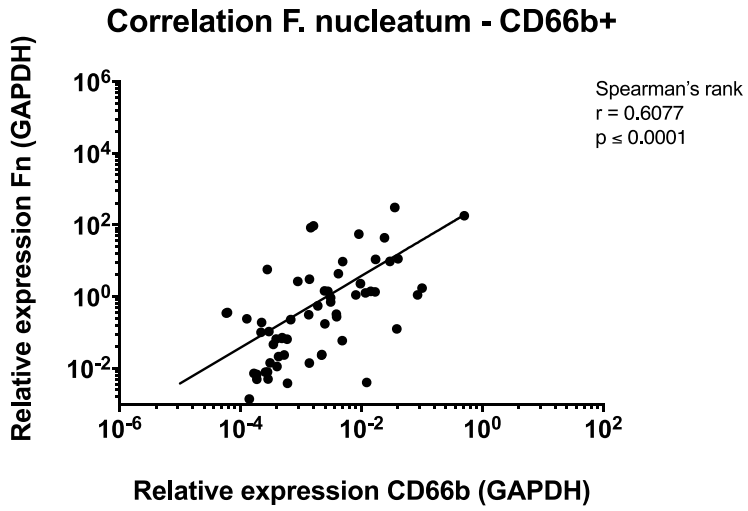


Figure 1. *F.nucleatum* in CRC patients cohort. Colorectal cancer tissue and the corresponding healthy mucosa was collected upon surgery operation and preserved in RNA later. Upon RNA extraction by Trizol the same quantity of RNA was retro-transcripted. 16S of Fn was detected by RT-PCR on 62 samples. Additionally a standard curve was performed using RNA of different quantity of *F.nucleatum*. The amount of *Fn* is here evaluated as Ct.

3.2 Correlation between neutrophil infiltration and *F.nucleatum* colonization in CRC.

In initial studies we comparatively investigated *F.nucleatum* colonization and neutrophil infiltration in healthy mucosa and CRC tissues. We identified 30 CRC samples out of 54 showing evidence of sizeable *F.nucleatum* colonization, as quantified by PCR assays assessing 16S expression. Remarkably, a highly significant correlation (r=0.6077) was observed between extent of *F.nucleatum* colonization and CD66b gene expression tumor (Fig 2A). Although 16S *F.nucleatum* was represented to higher extents in tumor tissues, as compared to corresponding healthy mucosa, a positive correlation was also observed in normal tissues (Fig 2B).

A



B

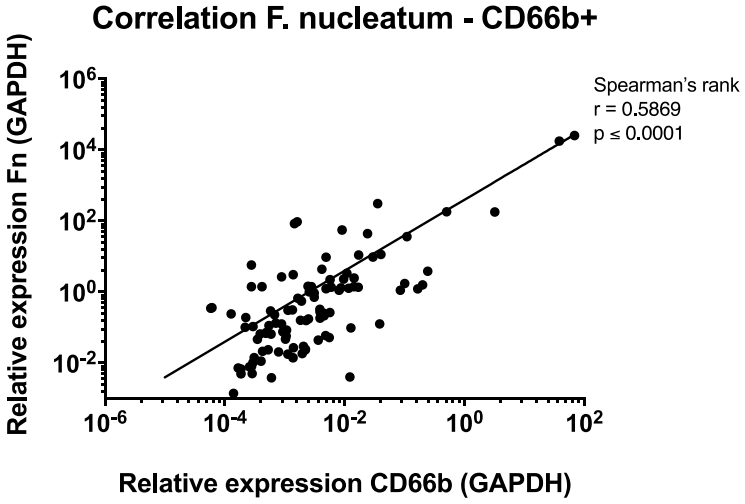


Figure 2. *F.nucleatum*/ CD66b correlation *ex vivo*. Colorectal cancer tissue and the corresponding healthy mucosa was collected upon surgery operation and preserved in RNA later. Upon RNA extraction by Trizol the

same quantity of RNA was retro-transcripted. 16S of *Fn* was detected by RT-PCR on 62 samples. Additionally a standard curve was performed using RNA of different quantity of *F.nucleatum.* After quantification of Fn detectable on the different CRC samples and corresponding mucosa, the samples with quantifiable amount of 16S *Fn* were normalized on GAPDH and correlated to the gene expression of CD66b+ cells displayed into corresponding samples.

3.3 *F.nucleatum* impacts neutrophils phenotype and functionality

It is now well recognized that the CRC microenvironment critically impacts on clinical outcome. My previous results identified tumor infiltration granulocytes as positive prognostic marker in CRC and neutrophils as active partners of adaptive T cell responses. Additionally, we found that CRC-infiltrating neutrophils are a heterogeneous population of cells differentially expressing CD66b, CD16, CD62L, CD54 and MPO. Thus, we hypothesized that heterogeneity of TANs in CRC, as compared to PBNs, and their differential density as compared to autologous healthy mucosa, may depend on the composition and the inherent complexity of the tumour microenvironment.

Based on this background, we investigated how components of CRC-microenvironment, such as cytokines released by other CRC-infiltrating immune cells and specific microbiota strains could contribute to TAN phenotypic heterogeneity and density within tumor tissue.

First, we observed that IFNγ and GM-CSF, whose expression in CRC tissues is associated with favorable prognosis enhance PBN survival “in vitro” and induce upregulation of important adhesion molecules such as CD11b and CD54/ICAM-1 on PBN surface (Figure 3A-B).

Interestingly, as mentioned before, very recent observations have documented that gut microbiome may be profoundly altered in CRC patients. In particular, the Gram-negative strain *F.nucleatum* is known to preferentially colonize human CRC (3) and to induce gut tumorigenesis and Granulocyte recruitment *in vivo* (12).

PBNs stimulated with heat-inactivated *F. nucleatum* (Fn) showed a rapid decrease in CD16 expression and reduced viability (Figure 4 A-C-D), accompanied by an increased expression of CD66b, CD11b, CD54 and a down-regulation of CD62L and intracellular MPO expression (Figure 4E). On the other hand, PBNs stimulation with a different gram–negative strain, such as *Bacteroides fragilis* (Bf) that also colonize CRC, did not appear to induce a similar phenotypic profile.

Based on the differential phenotype displayed by PPBNs in presence of *F.nucleatum* we then hypothesized that *F.nucleatum* could also affect PBNs functions. Therefore we collected supernatants of PBNs stimulated with Fn and Bf upon overnight incubation and we assessed cytokine and chemokine release in these conditions (Figure 5A).

PBNs stimulated by FN show an increased IL8 release as compared to controls and Bf stimulated PBN. Interestingly *F.nucleatum* had the capability to trigger CCL2 and IL1Ra release by PBNs. These factors are known to be endowed with pro-inflammatory and immune suppressive potential and may induce tumourigenesis (13;14). Our results show that *F.nucleatum* is able to confer to PBNs a distinct functional profile that may impair the functions of other cells of the tumor microenvironment.

Taken together these results suggest that cytokines associated to prolonged CRC survival, namely IFN-γ and/or GM-CSF, could account for the accumulation of live CD16+/MPO+ in CRC tissues. However we have observed that pre-incubation of purified neutrophils with *F.nucleatum* modulates their phenotypes in a manner closely reminding phenotypic profiles frequently observed in CRC or mucosa infiltrating neutrophils. Moreover PBNs stimulated by *F.nucleatum* are characterized by a pro-tumoural factor release profile.
Thus, local cytokine production and bacterial colonization may modulate phenotype and functions of TANs thereby differentially impacting on tumor microenvironment.

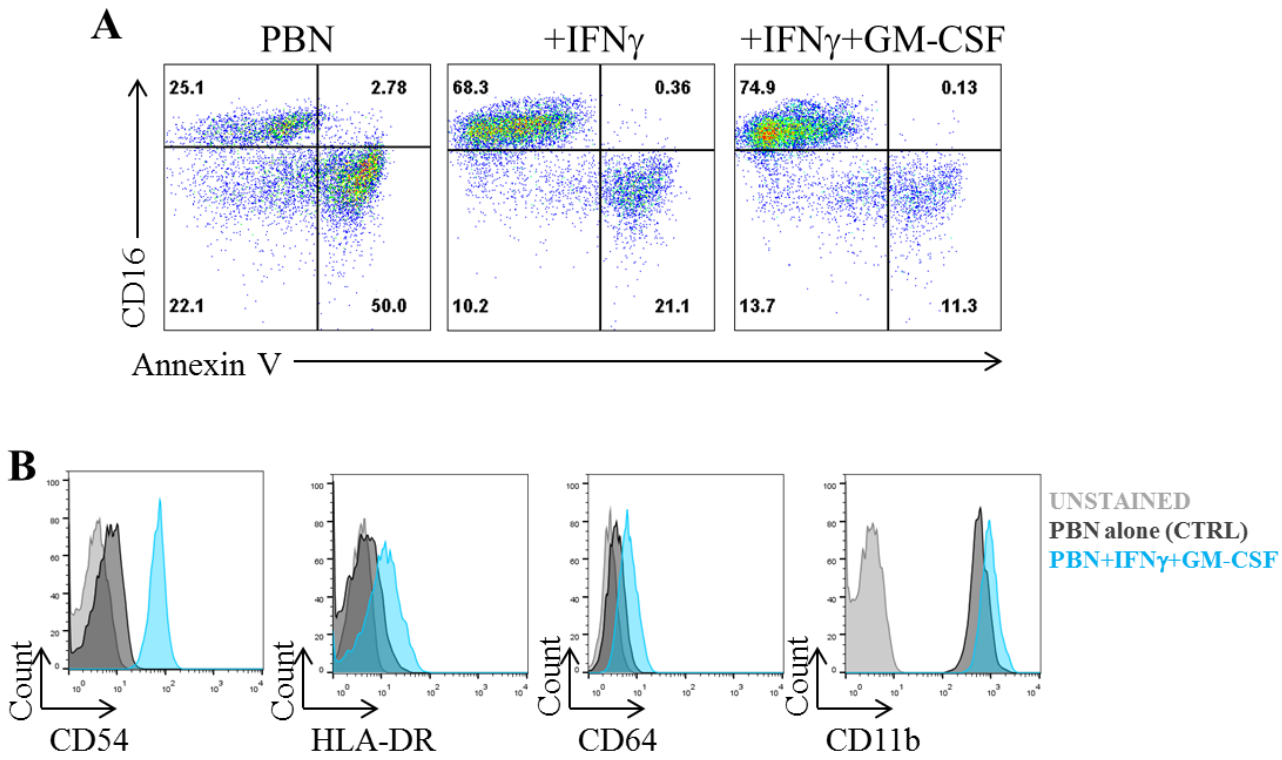


Figure 3. Cytokine-activated PBN. (A) PBNs were cultured in presence of IFNγ (1ng/ml) and GM-CSF (1ng/ml) and upon 24h culture PBNs phenotype was assessed by FACS analysis. Representative plot shows that IFNγ promotes PBN survival, as evaluated by Annexin V staining. Furthermore CD16 expression is maintained and increased CD54, CD11b, CD64 and HLA-DR expression is also observed **(B).**

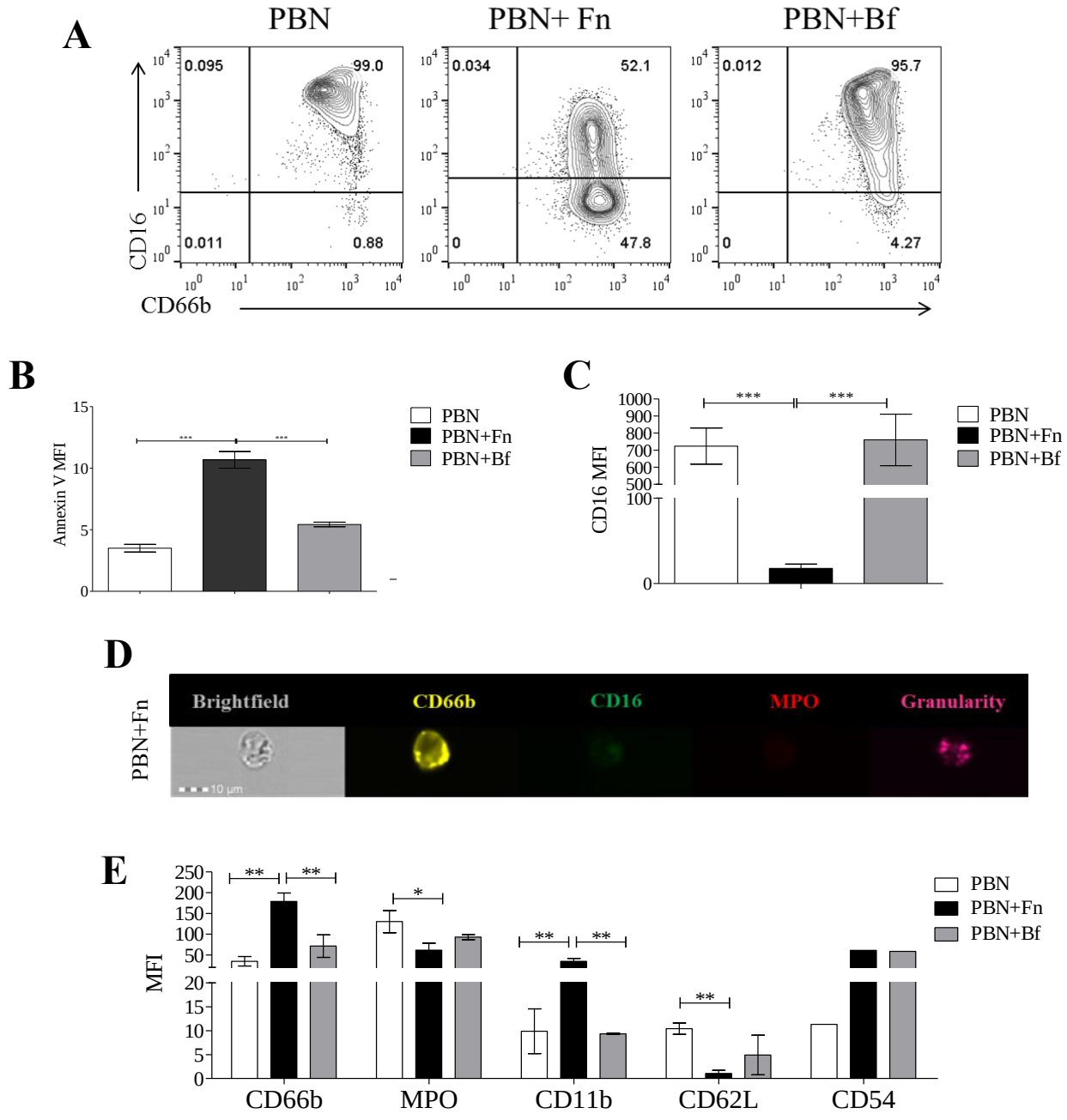


Figure 4. Modulation of markers' expression in PBNs by bacterial stimuli. (A-C) PBNs from HD were cultured in the presence or absence of bacteria (ratio 1:5) and viability and phenotypic profiles were assessed after 2h. **(**Representative plot displays a significant reduction of CD16 expression in presence of Fn but not of Bf. **(B)** PBNs viability was impaired as well upon stimulation with Fn. **(D)** Representative pictures from ImageStream analysis of CD66b, CD16 and MPO expression in PBNs in presence of Fn. **(E)** CD54 and CD11b and CD66b upregulation is observed upon stimulation with Fn and, to a lower extent, in presence of Bf. In contrast CD62L and intracellular MPO expression are reduced upon co-culture with Fn.

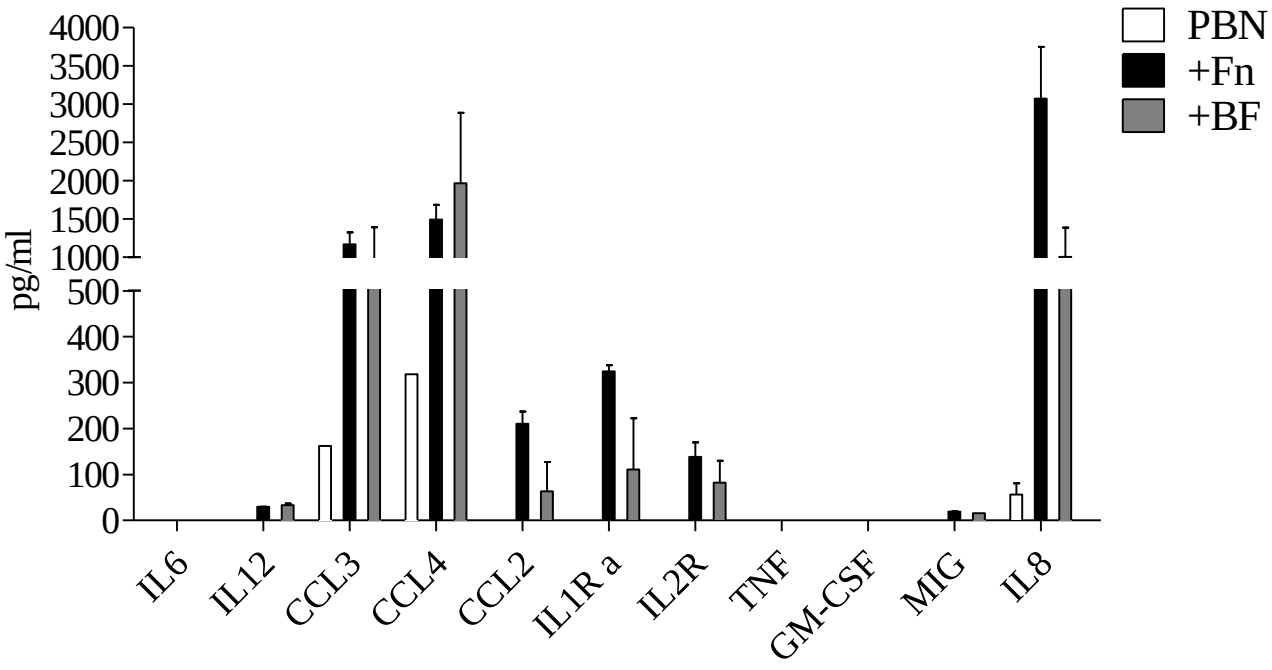


Figure 5. Cytokine release profile of PBNs upon stimulation with bacteria. Peripheral blood neutrophils from four different healthy donors were cultured for 2 hours in the presence or absence of the indicated bacterial stimuli. Supernatants were then collected and the release of the indicated factors was assessed by Luminex technology.

3.4 Direct effects of *F.nucleatum* on CRC cell lines proliferation and PBNs migration.

Colorectal colonization by the Gram-negative microbiota *F.nucleatum* has been associated with CRC outgrowth *in vivo* and CRC cell proliferation *in vitro*. In our hands, we could not observe any increase of CRC cell lines proliferation in presence of different concentrations of *F.nucleatum* (Figure 6A). However, we observed an enhancement of IL8 production by LS180 cell line in presence of *F.nucleatum* resulting in increased PBNs migration as compared to control and Bf stimulated tumor cells (Figure 6 B-C).

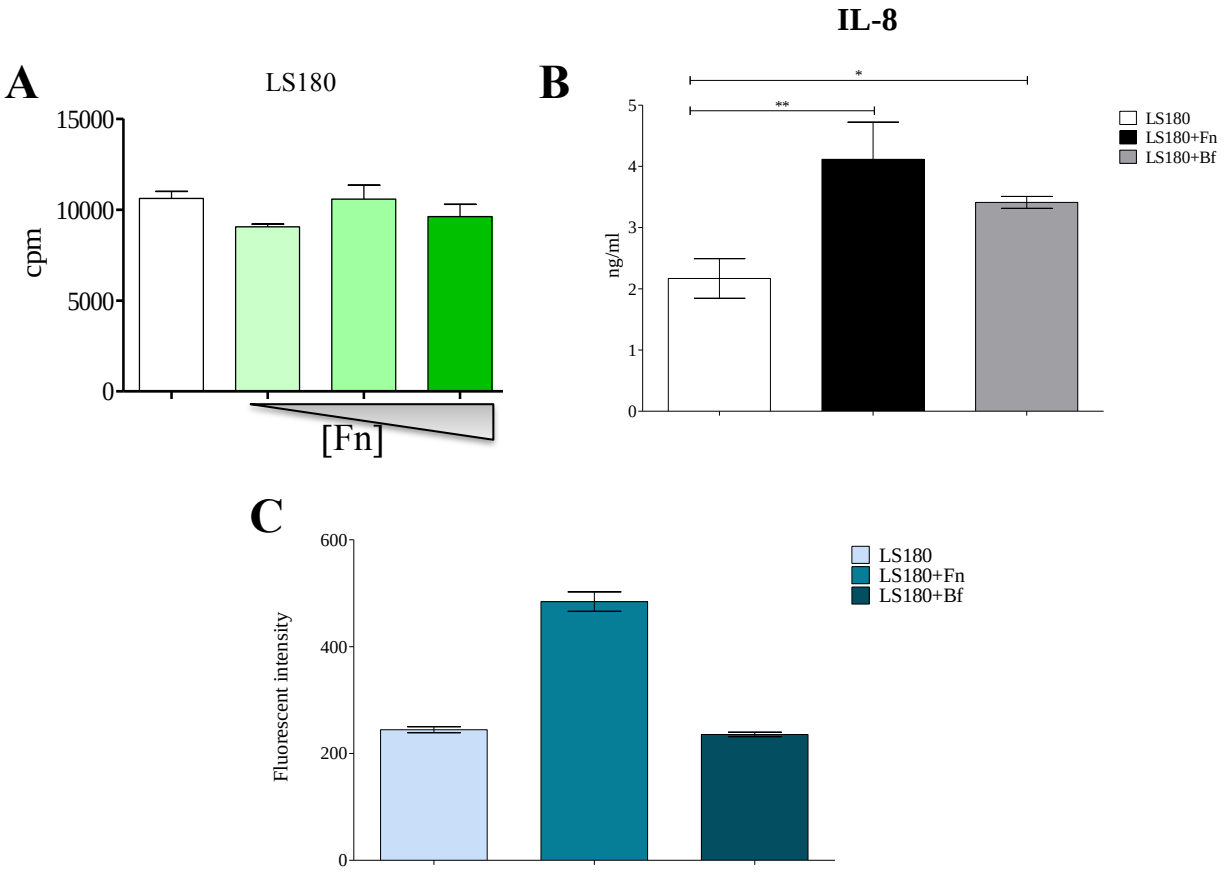


Figure 6. Direct effect of *F.nucleatum* on CRC cell lines. (A) Proliferative responses of LS180 cells cultured in the presence or absence of *F. nucleatum* (*Fn*) at different ratios (1:1000,1:500,1:250) as assessed by ^{3}H-Thymidine incorporation after 72h. **(B)** Supernatant of LS180 treated with *Fn* or *B.fragilis* (Bf) was collected following overnight incubation and IL-8 release was measured by ELISA assay. **(C)** Migration assay using transwell plates was performed to investigate neutrophil migration in basal condition or following stimulation by different bacterial strains.

3.5 Neutrophils and *F.nucleatum cross-talk* does not influence CRC cell growth

We were intrigued by the negative effect of *F.nucleatum* on PBN survival. We hypothesized that PBNs could act as "scavengers" of CRC-associated bacteria and therefore directly impact on tumor cells killing by release of cytotoxic compounds. Indeed PBNs in presence of *F.nucleatum* display a major production of ROS, MPO, and TRAIL, (Figure 7A-B-C). Therefore we investigated whether cytotoxic effects of PBNs on CRC tumor growth could be detectable upon *F.nucleatum* stimulation.

However, although stimulated PBNs release high amounts of MPO/ROS, they fail to display cytotoxic activity towards CRC cells (Figure. 7D). Indeed our results shown that PBNs are unable to kill tumor cells upon stimulation with *F.nucleatum* and other microrganisms (data not shown).

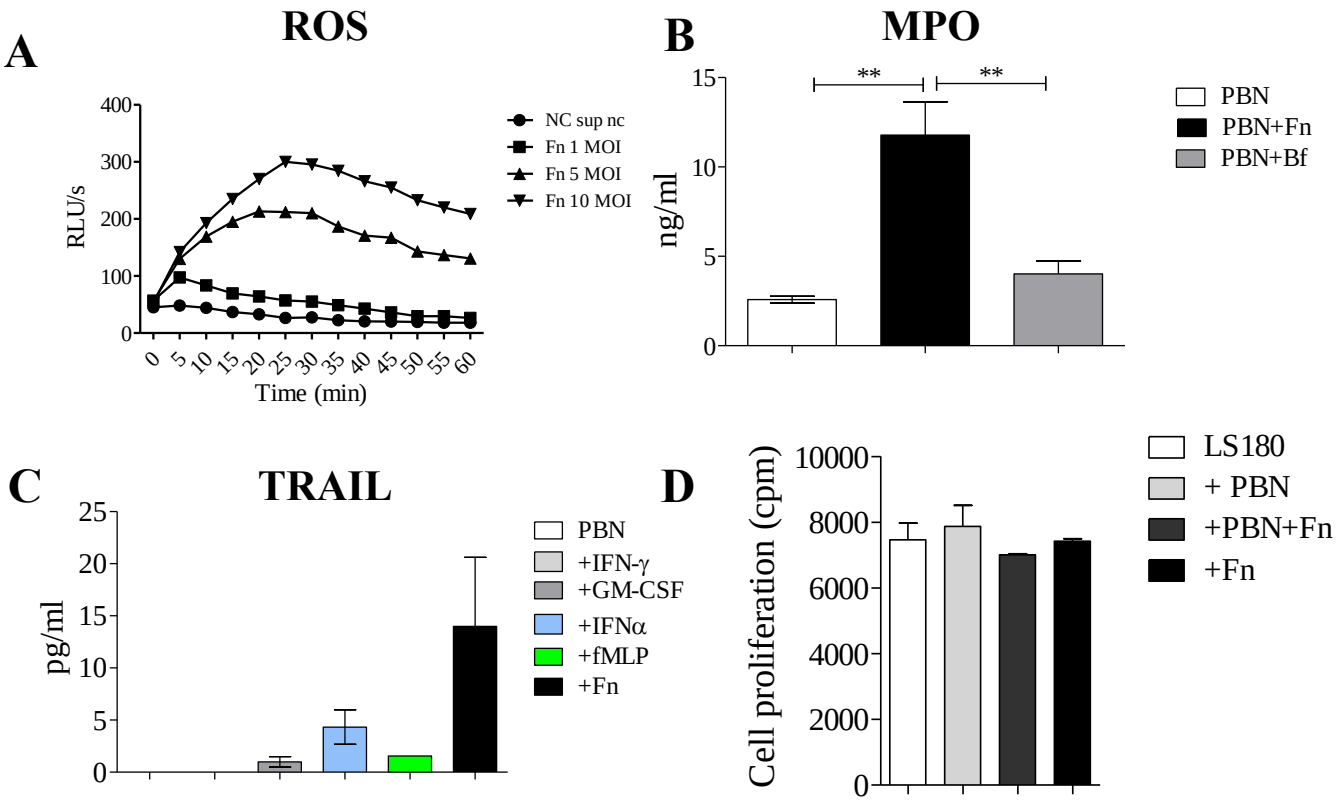


Figure 7. Release of cytotoxic effector factors induced by Fn on PBNs does not impair CRC lines proliferation. (A) ROS production by Neutrophils in the presence or absence of *F. nucleatum.* **(B-C)** Supernatant from LS180 cells cultured overnight in the presence or absence of Fn and Bf was collected and MPO and TRAIL release was measured by ELISA assays. **(D)** Proliferative responses of LS180 cells cultured in the absence or presence of neutrophils (PBN: LS180 ratio = 1:5) and *F.nucleatum*, as assessed by ^{3}H-Thymidine incorporation

after 72h culture. Neutrophils activated by *F. nucleatum* do not impact on CRC cells proliferation (unpaired t-test; $p \geq 0.05$).

3.6 *F.nucleatum* abolishes the PBNs stimulatory effects on CD8

Our previous experiments (see chapter II) clearly indicate that granulocytes mediate co-stimulation of adaptive T cell responses independently of their putative antigen presentation potential.

To further characterize neutrophil-mediated co-stimulation of CD8+ T cells, we investigated whether the priming of PBNs with the different, previously described, bacteria may lead to divergent immunomodulation. Our data indicate that pre-incubation with Fn, but not with Bf, fully abolishes their co-stimulatory potential, as measured by CD69+ expression (data not shown), proliferation (Figure 8A-B) and IFN-γ release.

The response of CD8+ cells to PBN might be abolished primarily due to the low viability of PBNs cultured in the presence of *F.nucleatum.* However, a role of the production of immunosuppressive factors, including IL1Ra, induced by *F.nucleatum* on PBNs, should not be disregarded.

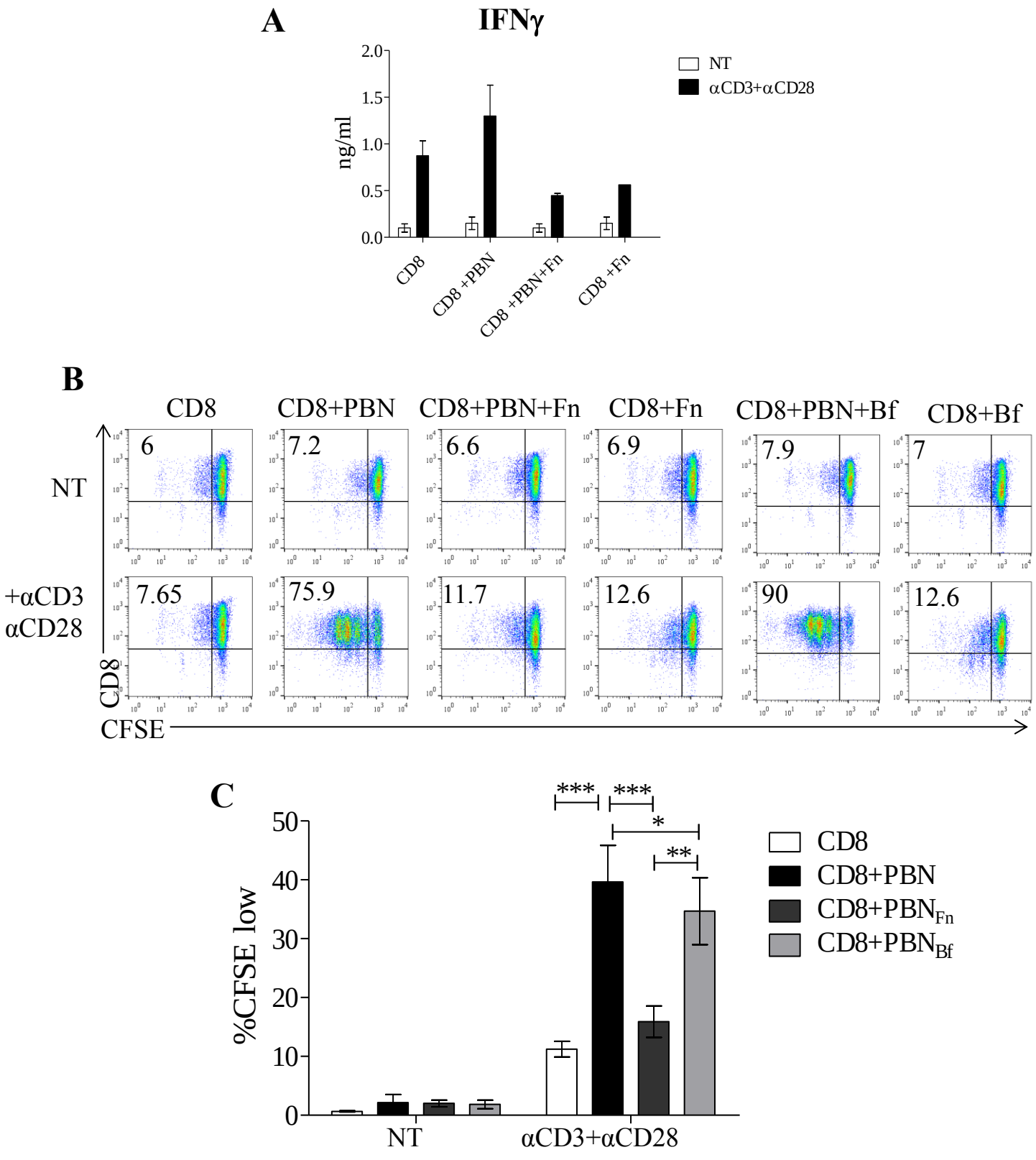


Figure 8. (A-B) Peripheral blood CD8+ T cells were co-cultured for 72h with purified PBNs at 1:1 ratio in the presence or absence of suboptimal concentration (1µg/ml) of anti-CD3 (clone UCTH) and anti CD28. PBNs were previously treated for 1 hour with heat-killed *F.nucleatum* (*Fn*) or *Bacteroides Fragilis* (Bf). Following washing PBNs were then added to CD8+ cells cultures. The supportive role of neutrophils on CD8+ T cell proliferation induced by anti CD3/CD28 was abolished following treatment with *F.nucleatum* as showed by CFSE assays. Representative flow-cytometry plots and cumulative data from different experiments are reported.

3.7 Indirect effects of PBN on tumor growth.

We then considered the possibility that PBNs activated by *F.nucleatum* could modulate CRC growth by conditioning the functions of tumor associated stromal cell (TASC), which have been shown to play crucial roles on CRC progression (15). Our data suggest that co-culture of CRC and TASC in the presence of supernatants of PBN activated by *F. nucleatum* (sFn) results in enhanced tumour cell proliferation and IL-6 release likely by TASC (Figure 9A-D). A similar experiment performed in presence of supernatants of PBN activated by *B.fragilis* (sBf) did not provide any evidence of pro-tumorigenic effects (Figure 9B).

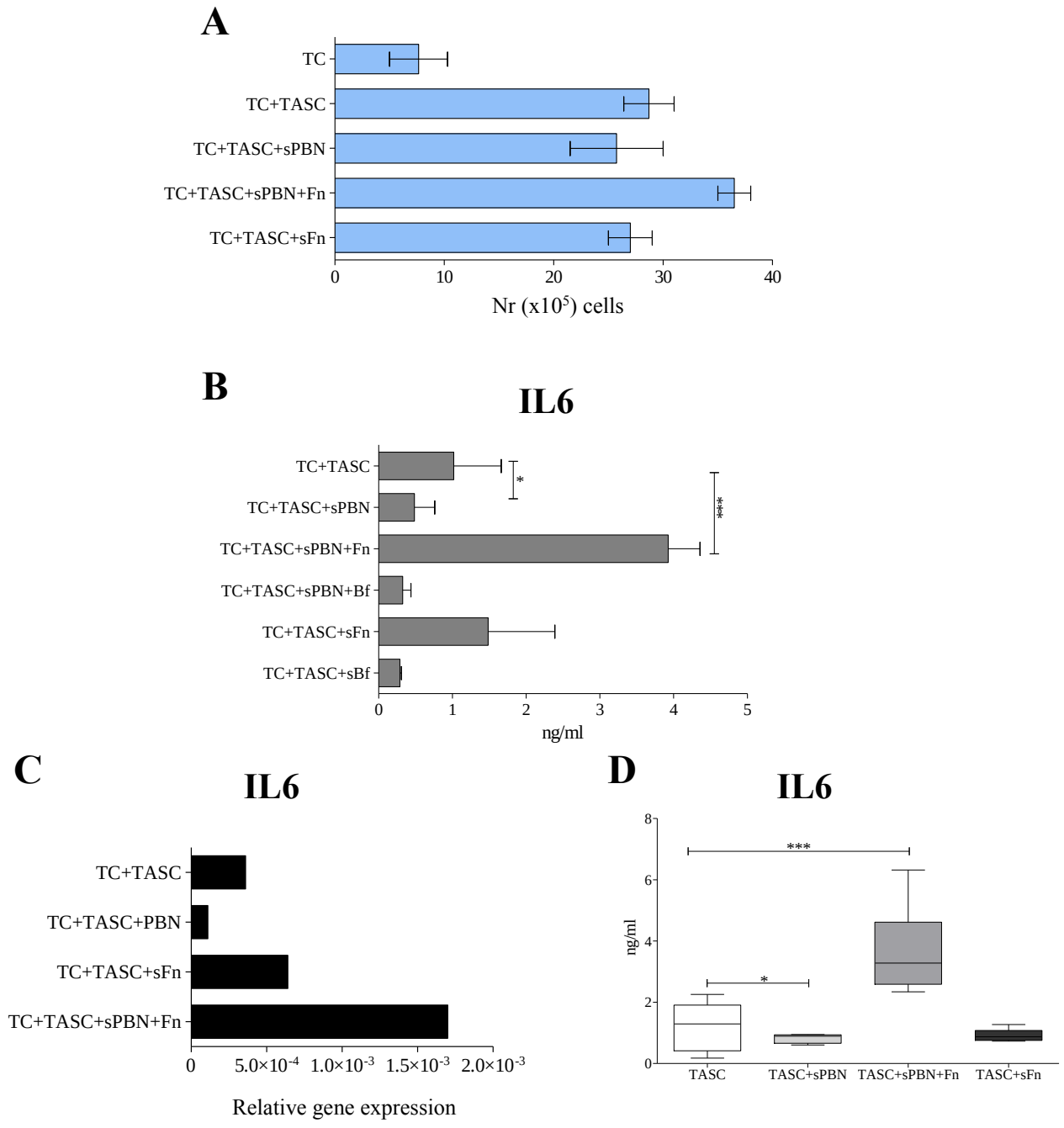


Figure 9. sPBNs enhance IL6 release by TASC upon culture with *F.nucleatum.* LS180 CRC cell line, was cultured alone (TC) or with TACS (TC+TASC) at ratio of 1:1 for 5 days and with supernatants previously collected from PBN alone (sPBN) or cultured with *F.nucleatum* (sPBN+Fn) or *B.fragilis* (sPBN Bf). The different supernatants were collected and filtered were added every 24h. The co-cultured cells and the controls were then harvested to assess cell numbers and FACS analysis to normalize EpCAM and CD90 expression. **(A)** TASC induce an increased proliferation of CRC cells. **(B)** Supernatants of the different conditions were tested for IL6 by ELISA assay. Data (mean ± SD) from three independent experiments are shown. **(C)** Expression levels of IL-6 genes were evaluated by quantitative PCR, using GAPDH as reference gene. **(D)** IL6 release by TASC without TC was also tested.

4. Discussion

Our work demonstrates that bacteria present in CRC microenvironment and potentially associated to tumor progression may impact on phenotype and functions of Neutrophils *in vitro*. Considering the important findings by other groups on the relevance of *F.nucleatum* colonization in CRC, we focused on this bacterial strain.

Our results show that *F.nucleatum* colonization of CRC or healthy mucosa is significantly correlated with density of infiltrating neutrophils. However, our "in vitro" data indicate that PBN rapidly undergo apoptosis in the presence of *F.nucleatum*, whereas these effects are delayed, in the presence of other gram–negative bacteria, such as *B.fragilis*. Importantly, PBNs cultured in presence of *F.nucleatum* appear to express CD66b and CD11b to higher extents than controls and PBNs stimulated by other gram–negative bacteria. In contrast, expression of CD16 and MPO is rapidly down regulated in PBNs cultured with *F.nucleatum*.

On the other hand, interestingly, in the presence of *F.nucleatum* CRC cell lines produce high amounts of IL-8, potentially able to recruit neutrophils to tumor tissues. PBNs released high amounts of MPO, ROS CCL2, CCL4, IL1Ra and IL8 upon stimulation by *F.nucleatum*. However, these activities did not promote the cytotoxic potential of neutrophils against tumor cells, but were rather associated with immunosuppressive effects since PBNs cultured in the presence of *F.nucleatum* failed to co-stimulate CD8+ cells activated by TCR triggering.

Taken together, these data lead us to hypothesize a scenario where tumor cells in presence of *F.nucleatum* recruit neutrophils into tumor niche as a "cell trap" where these cells are induced to undergo apoptosis and the elicitation of their CD8+ T cell co-stimulation potential is prevented, as also suggested by mouse models. High *F.nucleatum* loads may then impact on the functionality of a variety of immune cell players. Previous work, for instance, has shown that *F.nucleatum* high colonization is inversely associated with the density of CRC infiltrating CD3+ cells, although not significantly associated with CD8+ lymphocyte density.

Interestingly, we also observed that supernatants from *F.nucleatum* stimulated PBN enhance the release of IL6 by tumor associated stromal cells (TASC), which might, in turn, promote tumor proliferation. Still unclear however, is the nature of the neutrophil-derived soluble factors involved in the elicitation of these effects.

Additional studies are warranted to investigate the effects induced on neutrophils by other components of the gut microbiome. Nevertheless, our data suggest, that bacteria may

differentially attract neutrophils to the tumor niche, and modulate their phenotype and immune functions.

Taken together, our results contribute to the characterization of complex interactions taking place in CRC microenvironment elucidating the relationship occurring between microbiota and neutrophils, key components of innate immune system. Our findings further underline the potential relevance of the interaction between microbiota and immune cells as target for CRC prevention and treatment.

CHAPTER IV: discussion and outlook

1. Discussion

Neutrophils play an indispensable and established role in defense against microorganisms infection, however new appreciation for their significance in cancer immunobiology has started to emerge (16).

During the last decade important studies has shown the profound influence of these dynamic cells throughout each step of carcinogenesis. In fact in mice models it has been demonstrated that neutrophils are able to acquire distinct phenotypes and even opposing functions by polarizing into N1 and N2 cells in analogy with the well described M1 and M2 polarized macrophage states. The molecular mechanisms underlying neutrophil polarization are not clearly defined. Cytokines present within the tumor microenvironment could drive this polarization process although N2 cells appear to display immature neutrophils characteristics. Therefore whether N2 are activated or immature neutrophils remain to be elucidated. Furthermore in most of the experimental models studied, neutrophils appear to elicit pro-tumorigenic and even immunosuppressive functions.

The role of neutrophils in humans concerns still needs to be investigated. Neutrophil infiltration in cancers is frequently considered as a negative prognostic factor but the biological mechanisms underlying their clinical relevance are unclear.

Colorectal cancer microenvironment critically affects disease progression and is characterized by peculiar features. In particular, tumor infiltration by memory CD8+ T cells has repeatedly been reported to be associated with favorable prognosis. Furthermore, defined commensal bacterial strains, preferentially found to be associated with CRC, have been suggested to favor tumor progression (17). Taken together these data indicate that the interaction between cancerous cells, immune system and commensal microorganisms may play a key role in the control of tumor progression and impact CRC clinical course.

Original reports from our group and data presented in my thesis do indicate that, at difference with cancers from different histological and anatomical origin, CRC infiltration by myeloid cells is also associated with favorable prognosis. During the first phase of our study we have explored “in vitro” mechanisms potentially underlying the “in vivo” favorable prognosis. First, we hypothesized a direct tumor killing by neutrophils. Despite activation by a variety of stimuli, in our hands neutrophils were unable to exert cytotoxic or cytostatic effects on established CRC cell lines. Indeed high MPO/ROS production induced by neutrophil activation did not suffice to induce tumor cells death or to arrest their proliferation.

Most interestingly, we have observed that granulocyte infiltration appears to significantly enhance the favorable prognostic significance of CD8+ T cell infiltration in CRC. These findings pose the question of the analysis of the granulocyte/CD8+ T cell cross-talk potentially taking place in CRC tissues and of the ability of different bacterial strains to condition it.

While activated T cells are known to release cytokines promoting neutrophils survival and activation, the ability of neutrophils to support TCR triggered T cell activation is debated. Our data do indicate that, in the presence of neutrophils, anti-CD3 stimulated CD8+ T cells are characterized by higher expression of activation markers, and higher proliferation and IFN-γ release. This co-stimulatory effect induced by PBN on CD8+ cells is cell-to-cell contact dependent. CD11a (LFA-1) on CD8+ and CD54 (ICAM1) on Neutrophils, are the molecules involved in this interaction, although a role of other co-stimulatory molecules such OX40L, or 4-BBL cannot be excluded. These findings might shed some light on the favorable prognostic significance of CRC infiltration by both CD8+ T cells and granulocytes. Importantly, these effects are only visible upon sub-optimal T cell activation, but become undetectable when optimal concentrations of mitogenic anti-CD3 mAbs are used. In the latter conditions however, whereas neutrophils do not enhance CD8+ T cell proliferation, they induce a preferential expansion of CD8+ central memory T cells, showing a decreased expression of PD-1 "exhaustion" marker. CRC infiltration by cells displaying this phenotypic profile is indeed known to be associated with favorable prognosis.

A major peculiarity of CRC microenvironment is represented by its close interaction with large numbers of microorganisms. Therefore, we considered the hypothesis that defined bacterial species may influence neutrophil functions and, as a consequence, their interplay with tumor cells and CD8+ cells.

Within this frame, we have shown that *F.nucleatum* specifically impacts on PBN phenotype and viability. Indeed, the expression of CD66b and CD16, main phenotypic markers of neutrophils, are modulated by *F.nucleatum*. Moreover *F.nucleatum* dramatically reduces PBN life span. *F.nucleatum* was able to induce in neutrophils a major production of ROS and MPO and the release of CCL2, CCL4, IL1Ra and IL8. These activities did not promote the cytotoxic potential of neutrophils against tumor cells, but could rather be of immunosuppressive significance.

Additionally we have observed that PBN stimulated supernatants collected by with *F.nucleatum* enhance the release of IL6 by tumor associated stromal cells (TASC) that may trigger tumor proliferation.

I am aware of the fact that my work suffers from a number of limitations inherent in the short life span of granulocytes and difficulties associated with assays with tissue-derived cells, usually available in small numbers, and barely amenable to standard assessments. Furthermore, experimental models frequently fail to provide reliable support to these studies due to major differences between human and murine granulocyte compartments.

Nevertheless, my experimental findings, especially if considered in combination with clinical data, strongly suggest that the interaction between neutrophils and CD8+ T cells may be endowed with major anti-tumour significance. Although we did not observe any direct killing of tumor cells induced by neutrophils we could speculate that they might indirectly, but effectively, promote anti-tumour CD8+ cell functions. Furthermore, I could document the inhibitory effects of *F.nucleatum* on neutrophil co-stimulatory functions and survival, possibly resulting in the enhancement of tumor cell proliferation mediated by TASC activation.

Taken together, my results urge an integrated analysis of human tumor microenvironment taking into account its different components and the specificities inherent in anatomic location and histological origin of different cancer types. Moreover, they further underline the critical relevance of the interactions between non-transformed cells and commensal flora, potentially impacting clinical course of malignant diseases and patient survival.

2. Outlook

Our data do suggest a number of additional studies of clinical, translational and basic immunology relevance.

First, they help defining immune infiltrate features directly associated with disease prognosis. Thus, the inclusion of neutrophil infiltration in the analysis of CRC immune contexture could be of relevance in clinical decision making, recommending the use of adjuvant treatments in patients bearing poorly infiltrated CRC following “curative” surgery or less aggressive surgical approaches in cancers where biopsies could provide evidence of high immune cell infiltration. It is tempting to speculate that these measures might have a major impact on quality of life and, ultimately, on patient survival.

Second, our initial data show that microbiome composition might condition the composition of the immune cell infiltrate in CRC. Treatments aimed at modifying gut microbiome, promoting colonization with strains associated with CRC infiltration by prognostically significant immune cells while decreasing colonization by “unfavorable” strains could be envisaged. Our findings may provide an interesting experimental set up for a preliminary analysis of potentially relevant features of defined microorganisms.

Third, the effects of Fn on neutrophil phenotypes, viability and functions urges a detailed analysis of underlying molecular mechanisms. Their significance might extend beyond the CRC immunobiology and apply to other areas, including infectious and autoimmune diseases and IBD.

Last but not least, our results contribute to the overall clarification of the co-stimulatory significance of granulocyte interaction with T cells. These studies suffer from the poor viability of human neutrophils “in vitro” and by a relative scarcity of experimental models, considering the poor matching of human and murine neutrophil features “in vivo”. However, this research area appears to be highly promising and might contribute to the definition of a better integrated vision of the interaction between innate and adaptive immune systems.

3. Reference List

(1) Rubinstein MR, Wang X, Liu W *et al*. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. *Cell Host Microbe* 2013;14(2):195-206.

(2) Arthur JC, Gharaibeh RZ, Muhlbauer M *et al*. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. *Nat Commun* 2014;5:4724.

(3) Castellarin M, Warren RL, Freeman JD *et al*. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012;22(2):299-306.

(4) Kostic AD, Gevers D, Pedamallu CS *et al*. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* 2012;22(2):292-8.

(5) Galon J, Costes A, Sanchez-Cabo F *et al*. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313(5795):1960-4.

(6) Pages F, Berger A, Camus M *et al*. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005;353(25):2654-66.

(7) Mima K, Cao Y, Chan AT *et al*. Fusobacterium nucleatum in Colorectal Carcinoma Tissue According to Tumor Location. *Clin Transl Gastroenterol* 2016;7(11):e200.

(8) Mima K, Nishihara R, Qian ZR *et al*. Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis. *Gut* 2016;65(12):1973-80.

(9) Mima K, Sukawa Y, Nishihara R *et al*. Fusobacterium nucleatum and T Cells in Colorectal Carcinoma. *JAMA Oncol* 2015;1(5):653-61.

(10) Droeser RA, Hirt C, Eppenberger-Castori S *et al*. High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. *PLoS One* 2013;8(5):e64814.

(11) Trella E, Raafat N, Mengus C *et al*. CD40 ligand-expressing recombinant vaccinia virus promotes the generation of CD8(+) central memory T cells. *Eur J Immunol* 2016;46(2):420-31.

(12) Kostic AD, Gevers D, Pedamallu CS *et al*. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* 2012;22(2):292-8.

(13) Pantschenko AG, Pushkar I, Anderson KH *et al*. The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression. *Int J Oncol* 2003;23(2):269-84.

(14) Pantschenko AG, Pushkar I, Miller LJ *et al*. In vitro demonstration of breast cancer tumor cell sub-populations based on interleukin-1/tumor necrosis factor induction of interleukin-8 expression. *Oncol Rep* 2003;10(4):1011-7.

(15) Mele V, Muraro MG, Calabrese D *et al*. Mesenchymal stromal cells induce epithelial-to-mesenchymal transition in human colorectal cancer cells through the expression of surface-bound TGF-beta. *Int J Cancer* 2014;134(11):2583-94.

(16) Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Annu Rev Pathol* 2014;9:181-218.

(17) Kostic AD, Chun E, Robertson L *et al*. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013;14(2):207-15.

Appendix

The study by Doester et al. identifies myeloperoxidase (MPO+) as an independent positive prognostic marker in CRC patients. We also proved by flow cytometry and immunofluorescence that not all MPO+ cells are CD66b positive. This implies that MPO+ cells include other immune population as macrophages, thus indicating that MPO cannot be considered as a reliable marker to identify neutrophils. Based on that, we investigated whether the CD66b marker -uniquely express on neutrophils-, could be associated with a differential prognostic significance as compared to MPO, in particular when analyzed in combination with CD8+ cell infiltration.

In my work presented in chapter II (Governa et al., under revision), I observed that CD66b+ cell infiltration represents a positive prognostic factor in CRC similarly to MPO+ cell infiltration, with a higher p-value (0.0001 versus 0.038). Most importantly, we could identified a synergistic effect on the overall survival rate based on CRC infiltration by both CD8+ and CD66b+ cells that, in contrast, was not detectable with MPO+ and CD8+ cells.

My contribution to this study consisted in the identification of the MPO+ CD66b- subset. I subsequently proposed and developed the idea to further investigate CD66b+ cells infiltration, which later became the main aim of my PhD project.

BRIEF REPORT

OncoImmunology 4:12, e1050574; December 2015; © 2015 Taylor & Francis Group, LLC

Absence of myeloperoxidase and CD8 positive cells in colorectal cancer infiltrates identifies patients with severe prognosis

Silvio Däster[1,2], Serenella Eppenberger-Castori[3], Christian Hirt[1,2], Savas D Soysal[1,2], Tarik Delko[1], Christian A Nebiker[1,2], Benjamin Weixler[1], Francesca Amicarella[2], Giandomenica Iezzi[2], Valeria Governa[2], Elisabetta Padovan[2], Valentina Mele[2], Giuseppe Sconocchia[4], Michael Heberer[2], Luigi Terracciano[3], Christoph Kettelhack[1], Daniel Oertli[1], Giulio C Spagnoli[2], Urs von Holzen[1,5], Luigi Tornillo[3,†], and Raoul A Droeser[1,2,†,*]

[1]Department of Surgery; University Hospital Basel; Basel, Switzerland; [2]Institute of Surgical Research and Hospital Management; Department of Biomedicine; University of Basel; Basel, Switzerland; [3]Institute of Pathology; University Hospital Basel; Basel, Switzerland; [4]Institute of Translational Pharmacology; Department of Biomedicine; National Research Council; Rome, Italy; [5]Indiana University School of Medicine South Bend; Indiana University Health Goshen Center for Cancer Care; Goshen, IN USA

[†]These authors contributed equally to this work.

Keywords: CD8+, human colorectal cancer, myeloperoxidase, prognostic markers, tissue microarray

Abbreviations: CRC, colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair; MPO, myeloperoxidase; NK, natural killer; PTL, peritumoral lymphocytes; TMA, tissue microarray.

Colorectal cancer (CRC) infiltration by cells expressing myeloperoxidase (MPO) or CD8 positive T lymphocytes has been shown to be independently associated with favorable prognosis. We explored the relationship occurring between CD8+ and MPO+ cell CRC infiltration, its impact on clinical-pathological features and its prognostic significance in a tissue microarray (TMA) including 1,162 CRC. We observed that CRC showing high MPO+ cell infiltration are characterized by a prognosis as favorable as that of cancers with high CD8+ T cell infiltration. However, MPO+ and CD8+ CRC infiltrating cells did not synergize in determining a more favorable outcome, as compared with cancers showing $MPO^{high}/CD8^{low}$ or $MPO^{low}/CD8^{high}$ infiltrates. Most importantly, we identified a subgroup of CRC with $MPO^{low}/CD8^{low}$ tumor infiltration characterized by a particularly severe prognosis. Intriguingly, although MPO+ and CD8+ cells did not co-localize in CRC infiltrates, an increased expression of TIA-1 and granzyme-B was detectable in T cells infiltrating CRC with high MPO+ cell density.

Introduction

Despite advanced multimodal treatment regimens, CRC is still the fourth most common cause of cancer-related death worldwide in both genders.[1] Besides specific mutations[2,3] and genomic and epigenomic instability, [4,5] the interaction of malignant cells with the tumor microenvironment has been demonstrated to play critical roles in cancer development and progression. [6,7]

Infiltration by immunocompetent cells and *cytokine* and *chemokine* gene expression significantly influence CRC outcome.[8-11] Indeed, the analysis of CRC "immune contexture"[12] has been suggested to outperform the prognostic significance of tumor node metastasis (TNM) staging and might contribute to personalized treatment decisions.[13,14] In particular, CRC infiltration by CD8+ T cells has repeatedly been shown to be associated with favorable clinical outcome.[8,10,15,16]

On the other hand, the role of innate immune system has not been studied in comparable detail. Controversial data have been reported regarding CRC infiltration by natural killer (NK) cells[17-19] and macrophages.[20-23] Recently, we have observed that CRC infiltration by cells expressing MPO, an enzyme typically released by activated granulocytes, represents an independent favorable prognostic factor, as emerging from the analysis of a large cohort of patients.[24]

Experimental models have suggested that antitumor effects of granulocytes are largely dependent on the interaction with T cells.[25-27] Furthermore, most recently, early stage lung cancer-associated neutrophils have been shown to be able to stimulate T cell responses in humans.[28]

Prompted by these reports, in this study we have explored the relationship occurring between CD8+ and MPO+ cell infiltration in CRC, its impact on clinical-pathological features and its prognostic significance.

*Correspondence to: Raoul A Droeser; Email: raoul.droeser@usb.ch
Submitted: 01/09/2015; Revised: 05/07/2015; Accepted: 05/07/2015
http://dx.doi.org/10.1080/2162402X.2015.1050574

The study published by Amicarella et al. shows that Th17 cell infiltration in CRC is correlated with MPO+ and CD16+ cell infiltration. Moreover, in vitro data have demonstrated that in addition to IL-17, Th17+ cells release a spectrum of chemokines and cytokines, including IL-8, capable to promote the recruitment of neutrophils. Th17+ cells did not significantly impact on CRC prognosis when detected in the stroma. However, we could show that intratumoral Th17+ cell infiltration is associated with a favorable prognosis. Considering that CRC infiltration by Th17+ cells was correlated with CD8+ and MPO+ cell cell infiltration, we could speculate that the positive prognostic significance of intratumoral Th17 + cells is might be due to their role in the recruitment of other cell subsets associated with positive prognosis, including CD8+, MPO+ and CD16+ cells.

This has prompted me to investigate the functional role of neutrophil infiltration into CRC.

OPEN ACCESS

ORIGINAL ARTICLE

Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer

F Amicarella,[1] M G Muraro,[1] C Hirt,[1] E Cremonesi,[1] E Padovan,[1] V Mele,[1] V Governa,[1,2] J Han,[1,3] X Huber,[1,4] R A Droeser,[4] M Zuber,[5] M Adamina,[6] M Bolli,[7] R Rosso,[8] A Lugli,[9] I Zlobec,[9] L Terracciano,[2] L Tornillo,[2] P Zajac,[1] S Eppenberger-Castori,[2] F Trapani,[2] D Oertli,[4] G Iezzi[1]

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2015-310016)

[1]Department of Biomedicine, Institute of Surgical Research, University of Basel, Basel, Switzerland
[2]Institute of Pathology, University of Basel, Basel, Switzerland
[3]Department of General Surgery, Shanghai East Hospital, Tongji University, Shanghai, China
[4]Department of General Surgery, University Hospital Basel, Basel, Switzerland
[5]Department of Visceral Surgery, Kantonsspital Olten, Olten, Switzerland
[6]Department of Visceral Surgery, Kantonsspital St Gallen, St. Gallen, Switzerland
[7]Department of Visceral Surgery, St Claraspital, Basel, Switzerland
[8]Department of Visceral Surgery, Ospedale Civico Lugano, Lugano, Switzerland
[9]Institute of Pathology, University of Bern, Bern, Switzerland

Correspondence to
Dr Giandomenica Iezzi, Department of Biomedicine, Institute of Surgical Research (ICFS), Basel University Hospital, Hebelstrasse 20, Basel 4031, Switzerland; giandomenica.iezzi@usb.ch

Received 19 May 2015
Revised 11 November 2015
Accepted 30 November 2015
Published Online First 30 December 2015

To cite: Amicarella F, Muraro MG, Hirt C, *et al. Gut* 2017;**66**:692–704.

ABSTRACT

Background The immune contexture predicts prognosis in human colorectal cancer (CRC). Whereas tumour-infiltrating CD8+ T cells and myeloid CD16+ myeloperoxidase (MPO)+ cells are associated with favourable clinical outcome, interleukin (IL)-17-producing cells have been reported to correlate with severe prognosis. However, their phenotypes and functions continue to be debated.

Objective To investigate clinical relevance, phenotypes and functional features of CRC-infiltrating, IL-17-producing cells.

Methods IL-17 staining was performed by immunohistochemistry on a tissue microarray including 1148 CRCs. Phenotypes of IL-17-producing cells were evaluated by flow cytometry on cell suspensions obtained by enzymatic digestion of clinical specimens. Functions of CRC-isolated, IL-17-producing cells were assessed by in vitro and in vivo experiments.

Results IL-17+ infiltrates were not themselves predictive of an unfavourable clinical outcome, but correlated with infiltration by CD8+ T cells and CD16+ MPO+ neutrophils. Ex vivo analysis showed that tumour-infiltrating IL-17+ cells mostly consist of CD4+ T helper 17 (Th17) cells with multifaceted properties. Indeed, owing to IL-17 secretion, CRC-derived Th17 triggered the release of protumorigenic factors by tumour and tumour-associated stroma. However, on the other hand, they favoured recruitment of beneficial neutrophils through IL-8 secretion and, most importantly, they drove highly cytotoxic CCR5+CCR6+CD8+ T cells into tumour tissue, through CCL5 and CCL20 release. Consistent with these findings, the presence of intraepithelial, but not of stromal Th17 cells, positively correlated with improved survival.

Conclusions Our study shows the dual role played by tumour-infiltrating Th17 in CRC, thus advising caution when developing new IL-17/Th17 targeted treatments.

Significance of this study

What is already known on this subject?
- Infiltration of colorectal cancers (CRCs) by defined populations of immune cells predicts clinical outcome irrespective of tumour stage.
- CRC-infiltrating CD8+ T cells and CD16+ myeloperoxidase (MPO)+ neutrophils have been found to be associated with prolonged survival, whereas infiltration by interleukin (IL)-17-producing cells, as evaluated in a limited number of cases, has been suggested to correlate with more severe prognosis.
- IL-17 is a proinflammatory cytokine mediating protumorigenic and proangiogenic effects.
- Monoclonal antibodies targeting IL-17/IL-17-receptor or impairing expansion of IL-17-producing cells may represent a new therapeutic option in CRC.

What are the new findings?
- Analysis of a large cohort of CRCs shows that tumour-infiltrating IL-17-producing cells are not themselves predictive of poor clinical outcome.
- Intraepithelial localisation of CRC-infiltrating IL-17+ cells is associated with improved survival.
- CRC infiltration by IL-17+ cells correlates with the presence of beneficial CD8+ T cells and CD16+ MPO+ neutrophils.
- CRC-infiltrating IL-17+ cells, mostly consisting of polyfunctional T helper 17 cells (Th17), can recruit highly cytotoxic CD8+ T cells into tumour nests through CCL5 and CCL20 release.

How might it impact on clinical practice in the foreseeable future?
- By disclosing the dual role played by CRC-Th17, our findings question therapeutic approaches aimed at inhibiting Th17 development or expansion, possibly resulting in impaired tumour infiltration by beneficial effector cells. The positive contribution of Th17 to anti-tumour immune responses should not be disregarded when developing new IL-17/Th17 targeted treatments in CRC.

INTRODUCTION

The tumour immune contexture—that is, type, location, density and functional orientation of tumour-infiltrating immune cells,[1] predicts clinical outcome in human colorectal cancer (CRC). In particular, CD45RO+ memory T lymphocytes, cytotoxic CD8+ T cells (CTLs) and interferon (IFN)-γ-producing T helper 1 cells (Th1) have been found to be associated with prolonged survival, irrespective of tumour stage (5–7). Unexpectedly, Foxp3+ regulatory T cells (Tregs),[2 3] CD16+ and myeloperoxidase (MPO)+ myeloid cells,[4–6] also

 DOI: 10.1002/eji.201545554 Eur. J. Immunol. 2016. 46: 420–431

European Journal of Immunology

CD40 ligand-expressing recombinant vaccinia virus promotes the generation of CD8$^+$ central memory T cells

Emanuele Trella[1], Nermin Raafat[1,5], Chantal Mengus[1], Emmanuel Traunecker[2], Valeria Governa[1], Swantje Heidtmann[3], Michael Heberer[1], Daniel Oertli[4], Giulio C. Spagnoli[1] and Paul Zajac[1]

[1] Institute of Surgical Research, Department of Biomedicine, University of Basel, Basel, Switzerland
[2] Department of Biomedicine, University of Basel, Basel, Switzerland
[3] Department of Visceral Surgery, Kantonspital, Aarau, Switzerland
[4] Department of Surgery, University Hospital, Basel, Switzerland
[5] Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Central memory CD8$^+$ T cells (T_{CM}) play key roles in the protective immunity against infectious agents, cancer immunotherapy, and adoptive treatments of malignant and viral diseases. CD8$^+$ T_{CM} cells are characterized by specific phenotypes, homing, and proliferative capacities. However, CD8$^+$ T_{CM}-cell generation is challenging, and usually requires CD4$^+$ CD40L$^+$ T-cell "help" during the priming of naïve CD8$^+$ T cells. We have generated a replication incompetent CD40 ligand-expressing recombinant vaccinia virus (rVV40L) to promote the differentiation of human naïve CD8$^+$ T cells into T_{CM} specific for viral and tumor-associated antigens. Soluble CD40 ligand recombinant protein (sCD40L), and vaccinia virus wild-type (VV WT), alone or in combination, were used as controls. Here, we show that, in the absence of CD4$^+$ T cells, a single "in vitro" stimulation of naïve CD8$^+$ T cells by rVV40L-infected nonprofessional CD14$^+$ antigen presenting cells promotes the rapid generation of viral or tumor associated antigen-specific CD8$^+$ T cells displaying T_{CM} phenotypic and functional properties. These observations demonstrate the high ability of rVV40L to fine tune CD8$^+$ mediated immune responses, and strongly support the use of similar reagents for clinical immunization and adoptive immunotherapy purposes.

Keywords: Antigen presenting cells · Central memory T cells · CD40-CD40L · CD4$^+$ T cells · CD8$^+$ T cells · Immunological memory · Vaccinia virus

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Generation of immunological memory represents a key tenet of the adaptive immune system. Memory T cells display the exquisite ability to respond to previously experienced antigens with clonal expansion and with the rapid elicitation of multiple effector function [1]. However, the origin of memory CD8$^+$ T cells is still debated, particularly concerning the relationship between effector and memory cells [2].

Current understanding is consistent with the "one cell, multiple fates" model postulating that memory and effector T cells

Correspondence: Dr. Paul Zajac
e-mail: paul.zajac@usb.ch

OX40 expression enhances the prognostic significance of CD8 positive lymphocyte infiltration in colorectal cancer

Benjamin Weixler[1], Eleonora Cremonesi[2], Roberto Sorge[3], Manuele Giuseppe Muraro[2], Tarik Delko[1], Christian A. Nebiker[1], Silvio Däster[1], Valeria Governa[2], Francesca Amicarella[2], Savas D. Soysal[1,2], Christoph Kettelhack[1], Urs W. von Holzen[1,4], Serenella Eppenberger-Castori[5], Giulio C. Spagnoli[2], Daniel Oertli[1], Giandomenica Iezzi[2], Luigi Terracciano[5], Luigi Tornillo[5], Giuseppe Sconocchia[6,*] and Raoul A. Droeser[1,2,*]

[1] Department of Surgery, University Hospital Basel, Basel, Switzerland

[2] Institute of Surgical Research and Hospital Management (ICFS) and Department of Biomedicine, University Hospital Basel, Basel, Switzerland

[3] Department of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy

[4] IU Health Goshen Center for Cancer Care, Goshen, IN, USA

[5] Institute of Pathology, University Hospital Basel, Basel, Switzerland

[6] Institute of Translational Pharmacology, National Research Council, Rome, Italy

[*] These authors have contributed equally to this work

Correspondence to: *Raoul A. Droeser,* ***email:*** *Raoul.Droeser@usb.ch*

Keywords: OX40, CD8, colorectal cancer, prognosis, microenvironment

Received: *August 15, 2015* ***Accepted:*** *September 18, 2015* ***Published:*** *September 29, 2015*

ABSTRACT

Background: OX40 is a TNF receptor family member expressed by activated T cells. Its triggering by OX40 ligand promotes lymphocyte survival and memory generation. Anti-OX40 agonistic monoclonal antibodies (mAb) are currently being tested in cancer immunotherapy. We explored the prognostic significance of tumor infiltration by OX40+ cells in a large colorectal cancer (CRC) collective.

Methods: OX40 gene expression was analyzed in 50 freshly excised CRC and corresponding healthy mucosa by qRT-PCR. A tissue microarray including 657 clinically annotated CRC specimens was stained with anti-OX40, -CD8 and -FOXP3 mAbs by standard immunohistochemistry. The CRC cohort was randomly split into training and validation sets. Correlations between CRC infiltration by OX40+ cells alone, or in combination with CD8+ or FOXP3+ cells, and clinical-pathological data and overall survival were comparatively evaluated.

Results: OX40 gene expression in CRC significantly correlated with FOXP3 and CD8 gene expression. High CRC infiltration by OX40+ cells was significantly associated with favorable prognosis in training and validation sets in univariate, but not multivariate, Cox regression analysis. CRC with $OX40^{high}/CD8^{high}$ infiltration were characterized by significantly prolonged overall survival, as compared to tumors with $OX40^{low}/CD8^{high}$, $OX40^{high}/CD8^{low}$ or $OX40^{low}/CD8^{low}$ infiltration in both uni- and multivariate analysis. In contrast, prognostic significance of OX40+ and FOXP3+ cell infiltration was not enhanced by a combined evaluation. Irrespective of TNM stage, CRC with $OX40^{high}/CD8^{high}$ density infiltrates showed an overall survival similar to that of all stage I CRC included in the study.

Conclusions: $OX40^{high}/CD8^{high}$ density tumor infiltration represents an independent, favorable, prognostic marker in CRC with an overall survival similar to stage I cancers.

Gut Online First, published on February 6, 2018 as 10.1136/gutjnl-2016-313498

ORIGINAL ARTICLE

Gut microbiota modulate T cell trafficking into human colorectal cancer

Eleonora Cremonesi,[1] Valeria Governa,[2] Jesus Francisco Glaus Garzon,[3] Valentina Mele,[1] Francesca Amicarella,[1] Manuele Giuseppe Muraro,[2] Emanuele Trella,[2] Virginie Galati-Fournier,[4] Daniel Oertli,[5] Silvio Raffael Däster,[5] Raoul A Droeser,[5] Benjamin Weixler,[5] Martin Bolli,[6] Raffaele Rosso,[7] Ulrich Nitsche,[8] Nina Khanna,[9] Adrian Egli,[10] Simone Keck,[4] Julia Slotta-Huspenina,[11] Luigi M Terracciano,[12] Paul Zajac,[2] Giulio Cesare Spagnoli,[2] Serenella Eppenberger-Castori,[12] Klaus-Peter Janssen,[8] Lubor Borsig,[3] Giandomenica Iezzi[1]

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2016-313498).

For numbered affiliations see end of article.

Correspondence to
Dr Giandomenica Iezzi, Cancer Immunotherapy, Department of Biomedicine, University of Basel, Hebelstrasse 20, Basel 4031, Switzerland; giandomenica.iezzi@usb.ch

Received 30 November 2016
Revised 22 August 2017
Accepted 9 September 2017

ABSTRACT

Objective Tumour-infiltrating lymphocytes (TILs) favour survival in human colorectal cancer (CRC). Chemotactic factors underlying their recruitment remain undefined. We investigated chemokines attracting T cells into human CRCs, their cellular sources and microenvironmental triggers.

Design Expression of genes encoding immune cell markers, chemokines and bacterial 16S ribosomal RNA (16SrRNA) was assessed by quantitative reverse transcription-PCR in fresh CRC samples and corresponding tumour-free tissues. Chemokine receptor expression on TILs was evaluated by flow cytometry on cell suspensions from digested tissues. Chemokine production by CRC cells was evaluated in vitro and in vivo, on generation of intraperitoneal or intracecal tumour xenografts in immune-deficient mice. T cell trafficking was assessed on adoptive transfer of human TILs into tumour-bearing mice. Gut flora composition was analysed by 16SrRNA sequencing.

Results CRC infiltration by distinct T cell subsets was associated with defined chemokine gene signatures, including CCL5, CXCL9 and CXCL10 for cytotoxic T lymphocytes and T-helper (Th)1 cells; CCL17, CCL22 and CXCL12 for Th1 and regulatory T cells; CXCL13 for follicular Th cells; and CCL20 and CCL17 for interleukin (IL)-17-producing Th cells. These chemokines were expressed by tumour cells on exposure to gut bacteria in vitro and in vivo. Their expression was significantly higher in intracecal than in intraperitoneal xenografts and was dramatically reduced by antibiotic treatment of tumour-bearing mice. In clinical samples, abundance of defined bacteria correlated with high chemokine expression, enhanced T cell infiltration and improved survival.

Conclusions Gut microbiota stimulate chemokine production by CRC cells, thus favouring recruitment of beneficial T cells into tumour tissues.

To cite: Cremonesi E, Governa V, Garzon JFG, *et al*. *Gut* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/gutjnl-2016-313498

INTRODUCTION

Infiltration by immune cells heavily impacts on clinical outcome in human colorectal cancer (CRC).[1] High densities of cytotoxic CD8+ T cells,[2 3] IFN-g expressing T-helper 1 cells (Th1),[4] CXCR5+ follicular T-helper cells (Tfh)[5] and, surprisingly, Foxp3+ regulatory T cells (Tregs)[6 7] are associated with prolonged patients' survival. Consistent with positive role of T-helper cells, expression of HLA class II antigens was also reportedly associated with favourable clinical course.[8] In contrast, prognostic significance of interleukin (IL)-17-producing T-helper cells (Th17) is controversial: their presence within CRC tissues was reported to correlate either with poor[4] or improved prognosis.[9] In addition, innate immune cells were also shown to predict clinical outcome. Tumour infiltration by CD16+ myeloperoxidase + myeloid cells, mostly consisting of activated neutrophils, is an independent predictor of favourable prognosis.[10–12] Notably, infiltration by neutrophils or natural killer (NK) cells was also found to increase favourable prognostic significance of cytotoxic CD8+ T cells.[12 13]

Chemotactic factors driving these cell populations into CRC tissues remain largely undefined. Expression of defined chemokines, including CXCL9[14] CXCL10[14] CXCL16[15] and CX3CL1[5] was reported to correlate with high densities of tumour-infiltrating lymphocytes (TILs) and to predict favourable clinical outcome. However, putative responding cell subsets within immune infiltrating cells have not been carefully characterised. Furthermore, chemokine sources and microenvironmental stimuli leading to chemokine production within CRC tissues were not addressed so far.

On intestinal tumourigenesis, gut commensal bacteria translocate across altered epithelia and stimulate immune cells infiltrating lamina propria to release proinflammatory cytokines.[16] However, whether gut flora-derived microbial stimuli also promote production of chemotactic factors was not evaluated yet. Here we investigated chemokine–chemokine receptor network underlying T cell infiltration into CRCs.

MATERIALS AND METHODS

Clinical specimen collection and processing

Clinical specimens were collected from consenting patients undergoing surgical treatment at Basel University Hospital, St. Claraspital in Basel and Ospedale Civico di Lugano, Switzerland (Swiss

www.oncotarget.com Oncotarget, 2018, Vol. 9, (No. 36), pp: 24443-24456

Research Paper

Hemidesmus indicus induces immunogenic death in human colorectal cancer cells

Eleonora Turrini[1,*], Elena Catanzaro[1,*], Manuele G. Muraro[2,*], Valeria Governa[3], Emanuele Trella[2], Valentina Mele[3], Cinzia Calcabrini[1], Fabiana Morroni[4], Giulia Sita[4], Patrizia Hrelia[4], Massimo Tacchini[5] and Carmela Fimognari[1]

[1]Department for Life Quality Studies, Alma Mater Studiorum–University of Bologna, Rimini, Italy

[2]Oncology Surgery, Department of Biomedicine, University Hospital of Basel and University of Basel, ZLF, Basel-Switzerland

[3]Cancer Immunotherapy, Department of Biomedicine, University Hospital of Basel and University of Basel, ZLF, Basel-Switzerland

[4]Department of Pharmacy and Biotechnology, Alma Mater Studiorum–University of Bologna, Bologna, Italy

[5]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

[*]These authors contributed equally to this work

Correspondence to: *Carmela Fimognari,* ***email:*** *carmela.fimognari@unibo.it*

Keywords: *Hemidesmus indicus; immunogenic cell death; adjuvant activity; botanical drugs; colorectal cancer*

Received: *July 29, 2017* ***Accepted:*** *April 16, 2018* ***Published:*** *May 11, 2018*

ABSTRACT

The ability of anticancer treatments to promote the activation of tumor-reactive adaptive immune responses is emerging as a critical requirement underlying their clinical effectiveness. We investigated the ability of *Hemidesmus indicus*, a promising anticancer botanical drug, to stimulate immunogenic cell death in a human colorectal cancer cell line (DLD1). Here we show that *Hemidesmus* treatment induces tumor cell cytotoxicity characterized by surface expression of calreticulin, increased HSP70 expression and release of ATP and HMGB1. Remarkably, the exposure to released ICD-inducer factors from *Hemidesmus*-treated DLD1 cells caused a modest induction of CD14-derived dendritic cells maturation, as demonstrated by the increased expression of CD83. Moreover, at sub-toxic concentrations, H.i. treatment of monocytes and dendritic cells induced their mild activation, suggesting its additional direct immunostimulatory activity. These data indicate that *Hemidesmus indicus* induces immunogenic cell death in human tumor cells and suggest its potential relevance in innovative cancer immunotherapy protocols.

INTRODUCTION

According to the FDA guidelines, a botanical drug is set up from a botanical drug substance and is proposed for use as a drug. In this record, FDA, without precedent for its history, proposes to endorse botanical drugs in extract as a new class of drugs. A standard FDA-approved drug is constituted by a well-characterized active principle. Conversely, a botanical drug, by definition, is made out of multiple compounds [1]. Such complex composition may give advantages, particularly in managing complex diseases with polymorphic nature that cannot respond to the standard single drugs.

Some natural products have been found to stimulate antitumor immune response [2]. For instance, an extract from the Japanese traditional medicine *Juzen-taho-to* composed of 10 medicinal plants prompted a CD8 T-cell-immunity-based anticancer response in a murine melanoma model [3]. A gallotannin-rich standardized fraction from *Caesalpinia spinosa* is endowed with immune system dependent-anticancer activity. Indeed, it induces immunogenic cell death (ICD), dendritic cells (DCs) activation, and increased generation of melanoma associated antigen-specific T cells [4].

A number of studies indicate that responsiveness to specific anticancer drugs is critically dependent

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

Biology of Human Tumors

Clinical Cancer Research

The Interplay Between Neutrophils and CD8$^+$ T Cells Improves Survival in Human Colorectal Cancer

Valeria Governa[1,2], Emanuele Trella[1], Valentina Mele[1], Luigi Tornillo[2], Francesca Amicarella[1], Eleonora Cremonesi[1], Manuele Giuseppe Muraro[1], Hui Xu[1], Raoul Droeser[1,3], Silvio R. Däster[1,3], Martin Bolli[4], Raffaele Rosso[5], Daniel Oertli[3], Serenella Eppenberger-Castori[2], Luigi M. Terracciano[2], Giandomenica Iezzi[1], and Giulio C. Spagnoli[1]

Abstract

Purpose: Tumor infiltration by different T lymphocyte subsets is known to be associated with favorable prognosis in colorectal cancer. Still debated is the role of innate immune system. We investigated clinical relevance, phenotypes, and functional features of colorectal cancer–infiltrating CD66b$^+$ neutrophils and their crosstalk with CD8$^+$ T cells.

Experimental Design: CD66b$^+$ and CD8$^+$ cell infiltration was analyzed by IHC on a tissue microarray including >650 evaluable colorectal cancer samples. Phenotypic profiles of tissue-infiltrating and peripheral blood CD66b$^+$ cells were evaluated by flow cytometry. CD66b$^+$/CD8$^+$ cells crosstalk was investigated by *in vitro* experiments.

Results: CD66b$^+$ cell infiltration in colorectal cancer is significantly associated with increased survival. Interestingly, neutrophils frequently colocalize with CD8$^+$ T cells in colorectal cancer. Functional studies indicate that although neutrophils are devoid of direct antitumor potential, coculture with peripheral blood or tumor-associated neutrophils (TAN) enhances CD8$^+$ T-cell activation, proliferation, and cytokine release induced by suboptimal concentrations of anti-CD3 mAb. Moreover, under optimal activation conditions, CD8$^+$ cell stimulation in the presence of CD66b$^+$ cells results in increasing numbers of cells expressing CD45RO/CD62L "central memory" phenotype. Importantly, combined tumor infiltration by CD66b$^+$ and CD8$^+$ T lymphocytes is associated with significantly better prognosis, as compared with CD8$^+$ T-cell infiltration alone.

Conclusions: Neutrophils enhance the responsiveness of CD8$^+$ T cells to T-cell receptor triggering. Accordingly, infiltration by neutrophils enhances the prognostic significance of colorectal cancer infiltration by CD8$^+$ T cells, suggesting that they might effectively promote antitumor immunity. *Clin Cancer Res; 23(14); 3847–58. ©2017 AACR.*

Introduction

Granulocytes account for 50%–70% of leukocytes in humans. They represent a first-line defense against bacterial and fungal infections (1, 2). However, clinical and prognostic relevance of granulocyte infiltration in human cancers is debated (1–3). A number of studies suggest that high granulocyte/lymphocyte ratios in peripheral blood are associated with poor prognosis in different malignancies (4). Furthermore, myeloid cells of the granulocytic lineage at different maturation stages were shown to represent sizeable subsets of myeloid-derived suppressor cells (MDSC), promoting tumor growth and inhibiting cancer-specific adaptive responses (5).

More recently, the possibility that neutrophils might promote antitumor immune responses of clinical relevance has started to be explored (2). In particular, the ability of neutrophils to polarize into N1 and N2 functional profiles, similarly to macrophages, has been documented in experimental models (6, 7). Furthermore, tumor "educated" neutrophils were shown to elicit antimetastatic effects (8), and interaction of hepatocyte growth factor (HGF) with its receptor MET was suggested to play a key role in the recruitment of neutrophils mediating antitumor activities (9). Earlier studies indicated that production by tumor cells of G-CSF or granulocyte-macrophage colony-stimulating factor (GM-CSF), promoting neutrophil survival and activation, could induce adaptive antitumor immune responses and regression of established tumors based on neutrophil–T-cell interaction (10, 11). Furthermore, the ability of early-stage lung cancer–infiltrating neutrophils to support T-cell proliferation and antitumor responses has been demonstrated (12, 13). However, their prognostic significance was not addressed.

Colorectal cancer represents the third cause of cancer-related mortality worldwide. Tumor–node–metastasis (TNM) staging,

[1]Department of Biomedicine, Basel University Hospital and University of Basel, Basel, Switzerland. [2]Molecular Pathology Division, Institute of Pathology, Basel University Hospital and University of Basel, Switzerland. [3]Department of General Surgery, Basel University Hospital and University of Basel, Switzerland. [4]Department of Visceral Surgery, St. Claraspital, Basel, Switzerland. [5]Department of Visceral Surgery, Ospedale Civico, Lugano, Switzerland.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

E. Trella and V. Mele contributed equally to this article.

Corresponding Author: Giulio C. Spagnoli, Surgical Oncology Lab, University Hospital and University of Basel, Department of Biomedicine 20 Hebelstrasse, Basel 4031, Switzerland. Phone: 416-1265-2378; Fax: 416-1265-3990; E-mail: giulio.spagnoli@usb.ch

doi: 10.1158/1078-0432.CCR-16-2047

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

Translational Relevance

Composition of tumor microenvironment impacts on cancer progression and clinical course. Regarding colorectal cancer, infiltration by $CD8^+$ T lymphocytes is associated with improved survival, but the role of myeloid cells is unclear. We have observed that colorectal cancer infiltration by $CD66b^+$ neutrophils is associated with favorable prognosis. We hypothesized that their prognostic significance may be related to their ability to support $CD8^+$ T-cell responses. Indeed, we found that colorectal cancer–derived tumor associated and peripheral blood neutrophils from patients with colorectal cancer and healthy donors enhance $CD8^+$ lymphocyte responsiveness to T-cell receptor (TCR) complex triggering. Most importantly, the prognostic significance of $CD8^+$ T-cell infiltration in colorectal cancer is significantly improved by a concomitant neutrophil infiltration. These data powerfully support the use of a refined analysis of colorectal cancer immune contexture for clinical decision making and identify neutrophils as important players in antitumor immune responses in colorectal cancer.

routinely used to identify patients eligible for different treatments is frequently ineffective in predicting colorectal cancer clinical course (14).

Clinical relevance of the composition of tumor infiltrate in colorectal cancer has been extensively investigated. Colorectal cancer infiltration by $CD8^+$ and memory T cells has been consistently associated with favorable prognosis (15, 16). The specificity of these cells is largely unclear. Recognition of differentiation antigens or neoantigens (17) expressed by tumor cells was reported. Alternatively, bystander effects related to T-cell responses against antigens from microbial commensal could also be hypothesized. Interestingly, expression of activation markers by colorectal cancer–infiltrating lymphocytes correlates with prolonged survival (18).

The role of the innate immune system is unclear. NK-cell infiltration is modest and apparently devoid of prognostic significance (19). Although tumor infiltration by myeloid cells is associated with poor prognosis in a variety of cancers (20), macrophage infiltration has been suggested to correlate with favorable prognosis in colorectal cancer (21).

The role of neutrophils has not been comparably explored. We previously observed that colorectal cancer infiltration by $CD16^+$ myeloid cells correlates with favorable outcome (22). Similarly to neutrophils, these cells are HLA-class II and largely myeloperoxidase (MPO)+ (23). Data from other groups suggest that high tumor infiltration by $CD66b^+$ neutrophils may correlate with either benign or poor prognosis in patients with colorectal cancer. In a cohort of East Asian patients ($n = 229$), neutrophil infiltration was associated with severe prognosis (24). Moreover, neutrophil infiltration in colorectal cancer–derived lung metastases has been suggested to be associated with severe prognosis following surgical excision (25). However, neutrophil infiltration in colorectal cancer was reported to be associated with responsiveness to 5-fluorouracil (5-FU) treatment (26). Thus, clinical significance of tumor-associated neutrophils (TAN)-infiltrating colorectal cancer is still unclear and underlying functional mechanisms remain to be elucidated.

We have analyzed the prognostic significance of colorectal cancer–infiltrating $CD66b^+$ neutrophils by using a clinically annotated tissue microarray (TMA) including >650 cases. In addition, we have comparatively evaluated the phenotypes of neutrophils from healthy and cancerous colon tissues and peripheral blood from patients and healthy donors (HD). Their ability to support adaptive immune responses was specifically addressed. Finally, the prognostic relevance of the association of neutrophils with $CD8^+$ T cells infiltrating colorectal cancer microenvironment was explored.

Materials and Methods

TMA construction

The TMA used in this work was constructed using >650 nonconsecutive, formalin-fixed, and paraffin-embedded primary colorectal cancer samples, from the tissue BioBank of the Institute of Pathology of the University Hospital Basel (Switzerland; refs. 18, 22). A semiautomated tissue arrayer was used to transfer punches of a 0.6-mm diameter from tissue blocks onto glass slides. Punches derived from tumor centers and consisted of at least 50% tumor cells. Clinical–pathologic data for patients included in the TMA are summarized in Supplementary Tables S1 and S2. Use of clinical information was approved by the local ethical authorities.

IHC

TMA slides were incubated with primary antibodies specific for CD8, CD16, MPO (18, 22, 23), and CD66b (clone G10F5, Biolegend). Secondary staining and negative controls were performed as described previously (18, 22, 23). Colorectal cancer infiltration by cells expressing defined markers was scored by experienced pathologists.

Tumor cell lines

Established human colorectal cancer cell lines (DLD1, HCT116, SW480, HT29, and SW620) were purchased from European Collection of Authenticated Cell Cultures (ECACC). DLD1 and HCT116 were cultured in RPMI1640 supplemented with 10% FBS, GlutaMAX-I, nonessential amino acids (NEAA), 100 mmol/L sodium pyruvate, 10 mmol/L HEPES (all from Gibco). HT29 cells were cultured in McCoy 5A medium (Sigma-Aldrich), supplemented with 10% FBS, GlutaMAX-I, and kanamycin (Gibco). SW480 and SW620 cells were cultured in L15 medium (Sigma-Aldrich), supplemented with 10% FBS, GlutaMAX-I, and kanamycin (Gibco). Absence of mycoplasma contamination was verified by PCR, prior to experimental procedures.

Clinical specimen collection and processing

Clinical specimens from consenting patients undergoing surgical treatment at Basel University Hospital St. Claraspital, Basel, Switzerland, and Ospedale Civico (Lugano, Switzerland), were obtained according to procedures approved by local ethical commissions. Tumor tissues and corresponding tumor-free mucosa fragments were embedded in optimal cutting temperature compound for further histologic evaluation or enzymatically digested by using an enzyme cocktail including 200 U/mL collagenase IV (Worthington Biochemical Corporation) and 0.2 mg/mL DNAse I (Sigma-Aldrich for 1 hour at 37°C) to obtain single-cell suspensions, as described previously (27).

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

Neutrophil and lymphocyte isolation

TANs were isolated from tumor cell suspensions by positive selection of CD66b$^+$ cells with antibody-coated microbeads according to the manufacturer's instructions (Miltenyi Biotec, code: 130-104-913). Heparinized peripheral blood was collected from patients with colorectal cancer prior to surgery or from HDs, and density-gradient centrifugation was performed. Sedimented fractions containing high-density neutrophils were washed and treated with dextran 4% (T500, Pharmacia) in saline solution and residual erythrocytes in supernatants were lysed by using lysis buffer (Miltenyi Biotec). Peripheral blood neutrophils (PBN) were further enriched by positively removing contaminating cells, using the EasySep Human Neutrophil Enrichment Kit (StemCell Technologies). Purity of isolated PBN and TAN was evaluated by flow cytometry upon staining for the neutrophils/myeloid markers CD66b, CD16, myeloperoxidase (MPO), and CD11b and exceeded 98% and 80%, respectively, in cells used in functional assays. Average percentages of apoptotic cells in PBN and TAN suspensions used in functional assays, as measured by Annexin V/PI staining (BioLegend), did not exceed 5% and 20%, respectively. Peripheral blood and tumor-infiltrating CD8$^+$ lymphocytes (TIL) were isolated from peripheral blood mononuclear cells (PBMC) obtained by gradient centrifugation or digested tumor specimens, respectively, by using anti CD8-coated magnetic beads (Miltenyi Biotec), as described previously (28).

Colorectal cancer/neutrophil cocultures

Colorectal cancer cells from established cell lines were cultured in the presence or absence of neutrophils untreated or previously treated for 1 hour with IFNγ or fMLP (Sigma) at different ratios and tumor cell proliferation was assessed by ^{3}H-Thymidine incorporation (^{3}H-TdR). In specific experiments, induction of apoptosis in tumor cells was tested by Annexin V/PI staining.

Flow cytometry

Cell suspensions from colorectal cancers and tumor-free mucosa, and peripheral blood of HDs or patients with colorectal cancer, were stained with fluorochrome-conjugated antibodies specific for human CD66b, CD16, CD11b (BioLegend) and CD54, CXCR1, CXCR2 (BD Biosciences). Alternatively, cells were fixed and intracellular staining was performed with antibodies specific for MPO (23). Stained cells were analyzed by FACSCalibur flow cytometer (BD Biosciences), using FlowJo software (Tree Star).

Imagestream

Following CD66b, CD16, and intracellular MPO-specific staining, cells were washed and resuspended in PBS supplemented with 0.5% FBS and 5 mmol/L EDTA, prior to processing through ImageStream, Mark II Imaging Flow Cytometer (Amnis, EMD Millipore). Analysis was performed using IDEAS software (Amnis, EMD Millipore) and neutrophils from colorectal cancer tissue, healthy mucosa, and peripheral blood were identified on the basis of brightfield morphology, granularity, and CD66b expression.

Neutrophil and CD8$^+$ T-cell cocultures

PBNs and TANs, obtained from HD and colorectal cancer patients, were cocultured with CD8$^+$ T cells from autologous peripheral blood or tumor specimens. For costimulation experiments, 96-well flat-bottom culture plates were coated overnight with anti-CD3 mitogenic mAb (TR66, a gift of Dr. Lanzavecchia, Bellinzona, Switzerland), or UCHT-1 (eBiosciences), at suboptimal concentrations ranging between 0.5 and 5 μg/mL depending from hybridoma and lot. Neutrophils and CD8$^+$ T cells at a 0.5 10^6/mL concentration each were cultured in RPMI1640 medium supplemented with GlutaMAX I, HEPES, sodium pyruvate, nonessential amino acids, antibiotics (all from Gibco), and 5% AB serum (Blood Bank, Kantonsspital, Basel, Switzerland), thereafter referred to as complete medium, in the presence of anti-CD28 (1 μg/mL, BD Pharmingen). After 24-hour incubation, expression of CD69 early T-cell activation marker was evaluated by flow cytometry. T-cell proliferation was measured by assessing carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) dilution in labeled CD8$^+$ T cells following 72-hour culture by flow cytometry (28). IFNγ release in culture supernatants was assessed by using commercial ELISA Kits (BD Biosciences). When indicated, coculture experiments were performed by using Transwell plates (Corning), or in the presence of anti-CD11a (BioLegend) or control reagents.

Immunofluorescence

Colorectal cancer sections were fixed with formalin 4% for 15 minutes at room temperature and blocked with 2% goat serum diluted in PBS containing 0.3% Triton X-100 for 1 hour at room temperature. They were then incubated with rabbit polyclonal anti human CD8 (Abcam) or rabbit polyclonal anti human CD45RO (BiorByt) and mouse monoclonal anti human CD66b (Biolegend) for 1 hour at 37°C. Slides were washed with PBS and incubated for 1 hour at room temperature with goat anti-mouse Alexa Fluor 488 and anti-rabbit 546–conjugated antibodies (Invitrogen). Nuclei were counterstained with 4,6-diamidino-2-phenyldole (DAPI, Invitrogen). Sections were examined using Olympus BX61 fluorescence microscope (Olympus) and images were captured with 10× and 20× magnification using an F-View II camera (Olympus) and AnalySiS software (Soft Imaging System GmbH).

Statistical analysis

Statistical significance of differential expression of activation markers, cytokine release, and cell proliferation was analyzed by Student t and Wilcoxon/Mann–Whitney tests, as appropriate.

Associations with survival were explored using the Cox proportional hazards regression model. Cut-off values used to classify colorectal cancer with low or high immune cell infiltration were obtained by regression tree analysis (rpart package). On the basis of this calculation and on the test evaluability, threshold value for CD66b$^+$ infiltration was set at 10 cells per punch. After dichotomization, Kaplan–Meier curves were plotted, and compared by log-rank test.

Kruskal–Wallis and Jonckheere–Terpstra tests were used to determine the association of CD66b$^+$ and CD8$^+$ cell infiltration and clinical–pathologic features depending on continuous or discrete nature of the variable. Any missing clinical–pathologic information was assumed to be missing at random. Subsequently, CD66b$^+$ and CD8$^+$ cell infiltration data were entered into multivariate Cox regression analysis with clinical–pathologic variables and HRs and 95% confidence intervals (CI) were used to determine prognostic effects on survival time.

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

Data were analyzed using the Statistical Package Software R (Version 3.2.4, www.r-project.org). *P* values <0.05 were considered statistically significant.

Ethical commission approval

Ethikkommission Nordwest- und Zentralschweiz (EKNZ), no. 2014-388.

Results

Prognostic significance of CD66b$^+$ cell infiltration in colorectal cancer

In previous studies, we had observed that colorectal cancer infiltration by MPO$^+$ cells is associated with favorable prognosis (23). However, this enzyme is produced by different cells of the myeloid lineage. Therefore, to precisely identify TANs in colorectal cancer, we stained a TMA including >650 colorectal cancer with a mAb recognizing CD66b, a classical neutrophil marker (26). Colorectal cancer–infiltrating CD66b$^+$ cells could be efficiently detected in punches from fixed, paraffin-embedded tissues (Fig. 1A and B). In this cohort of patients, high colorectal cancer infiltration by CD66b$^+$ cells, as dichotomized by using a cut-off value ($n = 10$) obtained by regression tree analysis (see "Materials and Methods"), was associated with increased overall survival (OS; Fig. 1C, $P = 0.0001$). Similar results were observed when CD66b$^+$ cell infiltration was analyzed by dichotomizing data using median ($n = 5$) or mean ($n = 16.5$) values as cutoff ($P = 0.0003$ and 0.001, respectively) or by using nondichotomized continuous log 10–transformed CD66b$^+$ cell infiltration values ($P < 0.0001$). Interestingly, high-density CD66b$^+$ cell infiltration was significantly associated with pT1–2 stage ($P = 0.027$), pN0 stage ($P = 0.001$), clinical stage ($P < 0.0001$), absence of vascular invasion ($P = 0.005$), and "pushing" (18) tumor borders ($P = 0.028$; Supplementary Table S1).

Phenotypic characterization of tissue-infiltrating and peripheral blood CD66b$^+$ cells in patients with colorectal cancer

Prompted by data supporting their prognostic significance, we investigated phenotypic profiles of neutrophils infiltrating colorectal cancer, adjacent healthy mucosa, and autologous peripheral blood.

Colorectal cancer were characterized by a significantly ($P = 0.0009$) higher infiltration by CD66b$^+$ cells, as compared with autologous healthy mucosa (Fig. 2A), although wide variations were observed from patient to patient. Imagestream analysis indicated that TAN and mucosa-associated neutrophils included

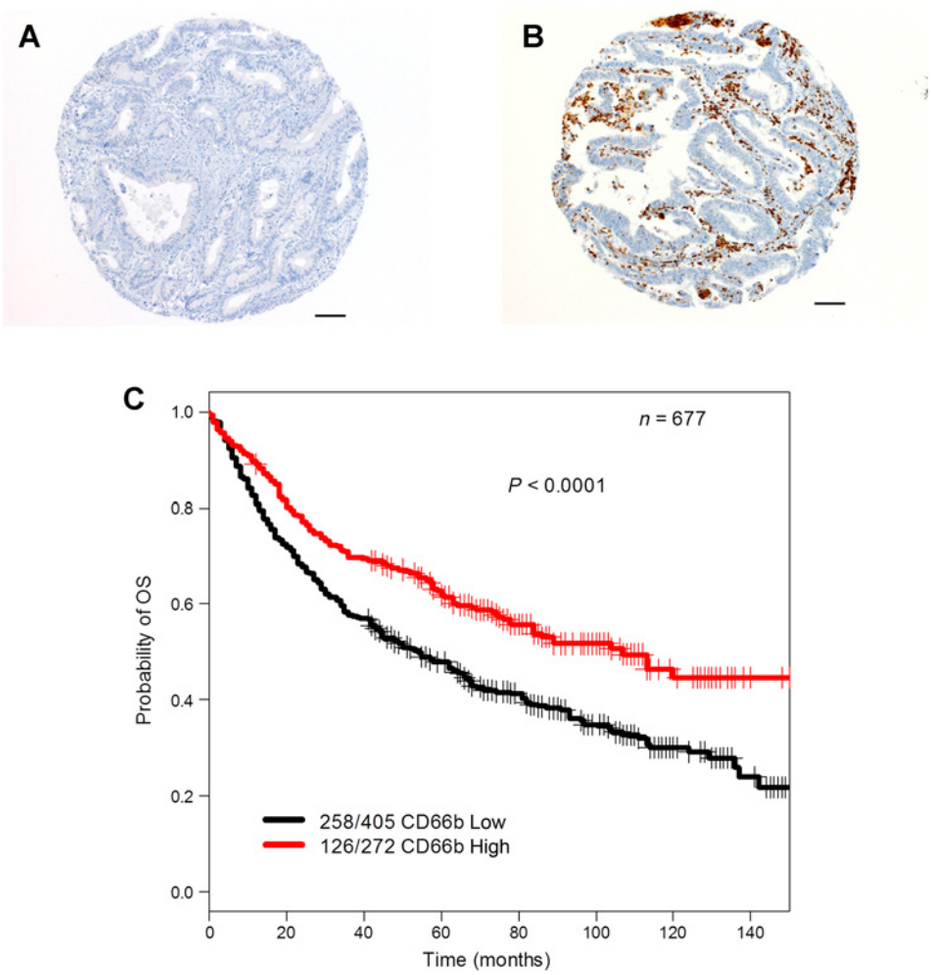


Figure 1.
CD66b$^+$ cell infiltration in colorectal cancer is associated with favorable prognosis. Colorectal cancer samples were stained with a CD66b specific mAb. Tumor punches are representative of low (**A**) and high (**B**) density of colorectal cancer infiltration by CD66b$^+$ cells. Magnification: 10×, scale bar, 100 μm. **C,** Kaplan–Meier curves illustrating overall survival (OS) probability according to CD66b$^+$ cell density. Numbers of deaths/total cases within each category are indicated.

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

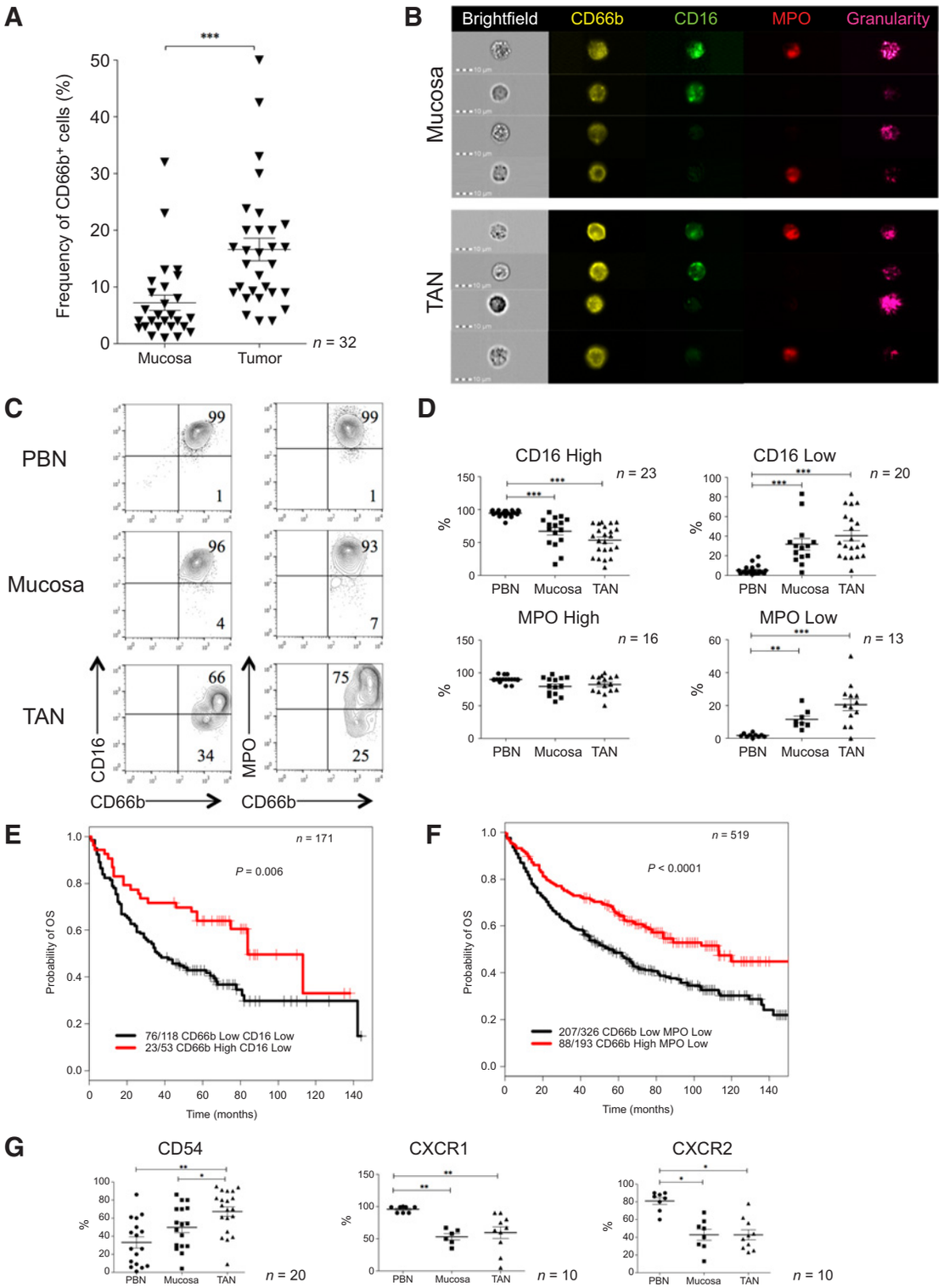


Figure 2.

TANs phenotype. **A,** Percentages of CD66b$^+$ cells in colorectal cancer tissues and autologous healthy mucosa were determined by flow cytometry within cell suspensions following enzymatic tissue digestion. **B,** Representative imagestream pictures of CD66b, CD16, and MPO expression on tumor and autologous healthy mucosa-derived neutrophils. **C,** Representative flow cytometry plots of CD16, CD66b, and MPO-specific staining of autologous PBNs, healthy mucosa–derived neutrophils and TAN from a colorectal cancer patient. **D,** Cumulative analysis of percentages of CD16 low/high and MPO low/high cells within gated CD66b$^+$ cells from PB, healthy mucosa, and tumors. **E** and **F,** Kaplan–Meier OS curves designed according to CD66b$^+$ high/low and CD16$^+$ low (**E**) or CD66b$^+$ high/low and MPO$^+$ low (**F**) cell infiltration in colorectal cancer. **G,** Cumulative results showing expression of CD54 and chemokine receptors on PBNs, healthy mucosa-derived neutrophils and TANs, as gated on CD66b$^+$ cells. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

$CD66b^+$ cells with variable expression of MPO and CD16 (Fig. 2B).

To obtain accurate quantitative data, phenotypic profiles of tissue-infiltrating neutrophil were analyzed by flow cytometry in comparison to PBNs. Representative examples are shown in Fig. 2C. In keeping with previous reports (22, 23), we observed that sizeable percentages of tissue-infiltrating $CD66b^+$ neutrophils express MPO and CD16 to lower extents as compared with autologous PBN (Fig. 2C and D). On the basis of this background, and considering that MPO and CD16 are also expressed by cells other than neutrophils, we explored the relative prognostic significance of the expression of these markers in the TMA under investigation. We observed that colorectal cancer infiltration by $CD66b^+$ cells is associated with improved OS also in the absence of a concomitantly high $CD16^+$ or MPO^+ cell infiltration (Fig. 2E and F). Furthermore, in the presence of a high $CD66b^+$ cell infiltration, presence or absence of concomitant $CD16^+$ or MPO^+ high-density infiltration did not significantly modify survival curves ($P = 0.75$ and 0.79, respectively).

Expression of other markers was also investigated. CD66b and CD11b are expressed in similarly high percentages of TANs and PBNs (data not shown), whereas CXCR1 and CXCR2 are expressed to lower extents in TANs, as compared with autologous PBNs (Fig. 2G). This phenotypic profile is shared by neutrophils infiltrating adjacent autologous healthy mucosa (Fig. 2D and G). However, higher percentages of TANs express CD54, as compared with autologous PBNs and mucosa-infiltrating neutrophils (Fig. 2G).

Notably, phenotypic profiles of PBNs from patients with colorectal cancer and HD are similar (Supplementary Fig. S1).

Neutrophils do not directly inhibit colorectal cancer cell proliferation

Data from TMA analysis consistent with an antitumor potential of colorectal cancer infiltration by neutrophils prompted us to explore possible mechanisms of action. Direct effects on colorectal cancer cells were first considered (29). However, short life span, and relatively low numbers of cells recovered from clinical specimens hampered routine use of TANs in these functional assays. Therefore, these experiments were performed by using PBNs from patients with colorectal cancer and HDs. Coculture in the presence of granulocytes did not decrease proliferation (Supplementary Fig. S2A and S2B) nor induced apoptosis (data not shown) in a panel of colorectal cancer cell lines. Prior treatment of neutrophils with IFNγ or fMLP also failed to impact on viability and proliferation potential of cocultured colorectal cancer cells (Supplementary Fig. S2C).

Neutrophil/$CD8^+$ lymphocyte cross-talk: effects on TCR–triggered T-cell activation

Alternative mechanisms of action underlying favorable prognostic significance of TANs in colorectal cancer might be related to their ability to exert indirect antitumor effects, mediated by other cell subsets. Colorectal cancer infiltration by $CD8^+$ T cells has widely been reported to associate with favorable prognosis (15), although their antigen specificity is largely unclear. Cytokines released by activated T cells, including GM-CSF and IFNγ, are able to activate neutrophils and to prolong their survival (30). More recently, neutrophils infiltrating early-stage lung cancers, but not their peripheral blood counterpart, were shown to promote T-cell response to anti-CD3 triggering (12, 13).

Initial studies suggested that $CD66b^+$ granulocytes frequently colocalize with $CD8^+$ and $CD45RO^+$ T lymphocytes within tumor tissues (Fig. 3A and B). On the basis of these findings, we tested the ability of TANs derived from enzyme-digested colorectal cancer specimens to modulate responses of autologous peripheral blood $CD8^+$ T cells to anti-CD3 triggering. Upon addition of TANs to $CD8^+$ lymphocyte cultures, a significantly ($P = 0.006$) increased expression of CD69 early activation marker induced by suboptimal concentrations of anti-CD3 mAb in the presence of anti CD28 mAb was observed. Furthermore, importantly, IFNγ release in these cultures was also significantly enhanced ($P = 0.01$; Fig. 3C). Consistent with data from experiments with TANs, we observed that interaction with PBNs from patients (Fig. 3D) and HD (Fig. 3E) resulted in significant increases in CD69 expression and IFNγ release by autologous $CD8^+$ lymphocytes upon stimulation with suboptimal concentrations of anti-CD3 mAb and anti-CD28 mAb. Representative flow cytometry plots are reported in Supplementary Fig. S3B and cumulative data are reported in Fig. 3D and E. T-cell proliferation, as assessed by CFSE dilution at 72 hours, was also significantly enhanced. Representative flow cytometry profiles are shown in Supplementary Fig. S3D, whereas cumulative data are reported in Fig. 3E. In contrast, these costimulatory effects were undetectable in T cells activated with optimal mitogenic concentrations of anti-CD3 mAb (Supplementary Fig. S3A).

Neutrophil-mediated costimulation critically required cell-to-cell contact as it was not observed in experiments performed in Transwell plates (Fig. 4A; Supplementary Fig. S3B). Furthermore, blocking of CD11a on $CD8^+$ T cells, preventing binding to CD54/ICAM-1 expressed by neutrophils, significantly ($P = 0.015$) inhibited elicitation of costimulatory functions (Fig. 4B; Supplementary Fig. S3C). Notably, CD54/ICAM-1 expression appeared to be upregulated in neutrophils upon culture in the presence of resting and activated $CD8^+$ T cells (Fig. 4C). Furthermore, coculture with activated $CD8^+$ T cells improved neutrophil viability (Fig. 4D).

These data indicate that untreated TAN and PBN are able to costimulate $CD8^+$ T cells, and that these effects are detectable in suboptimal activation conditions.

Neutrophil-mediated costimulation results in increased memory $CD8^+$ T-cell numbers

Favorable prognosis in colorectal cancer has been repeatedly associated with tumor infiltration by "memory" T lymphocytes (15, 16). To further characterize neutrophil-mediated costimulation of $CD8^+$ T cells, we evaluated phenotypic profiles of lymphocytes activated by optimal anti-CD3 concentrations and CD28 in the presence or absence of granulocytes for 5 days, thus, beyond time points usually considered for detection of maximal proliferation (31). Remarkably, peripheral blood $CD8^+$ T-cell stimulation in the presence of PBN resulted in significantly increased percentages of "central" memory cells expressing a $CD45RO^+/CD62L^+$ phenotype. Representative examples and cumulative data are reported in Fig. 5A. However, in the presence of TAN, these effects were only detectable in four of seven experiments performed with cells from different patients (data not shown).

Similar experiments were also performed by using TAN and autologous tumor-derived $CD8^+$ TIL. These cells are characterized

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

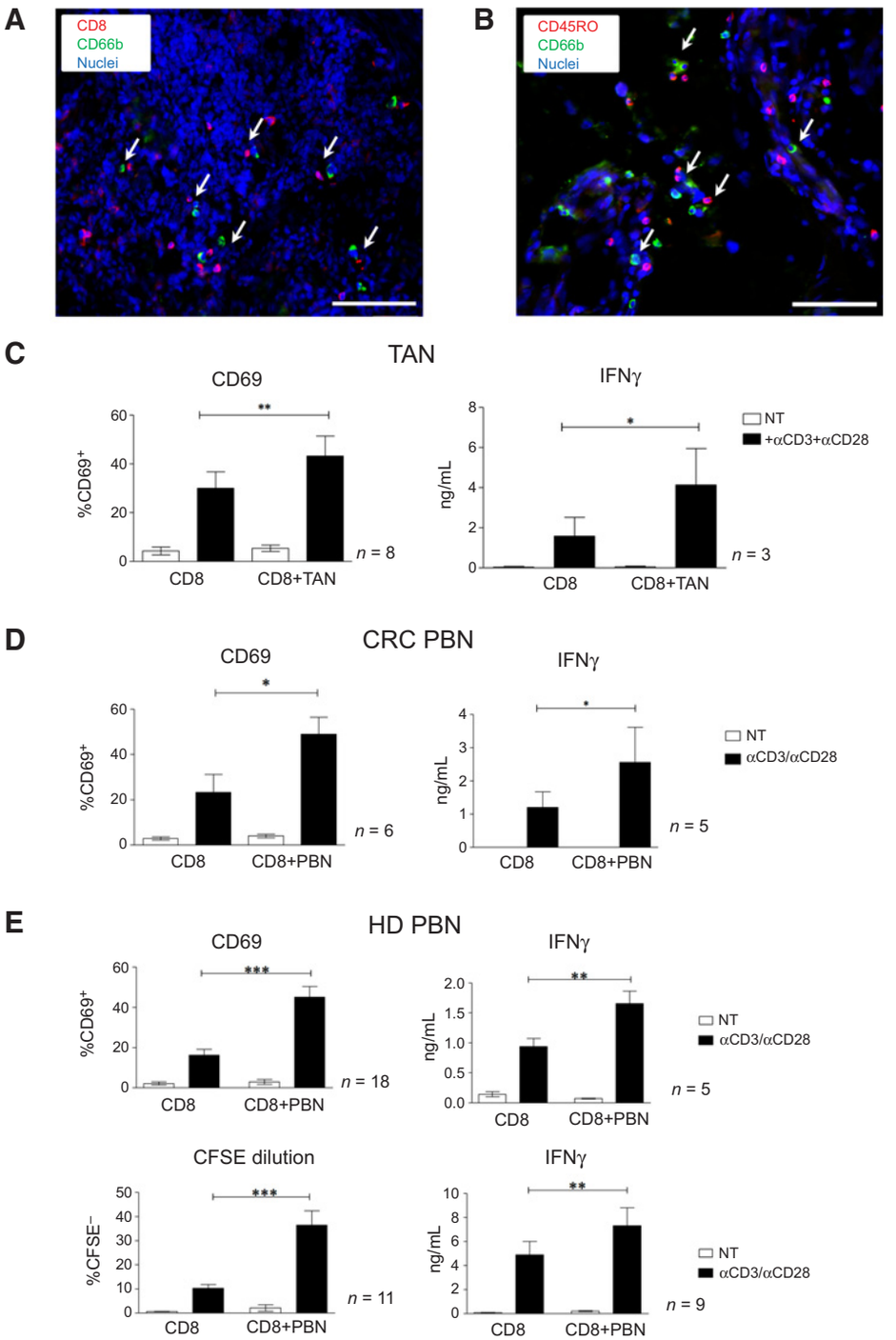


Figure 3.
Tumor and peripheral blood–derived neutrophils enhance CD8$^+$ T-cell responsiveness. **A** and **B,** Immunofluorescence staining of CD8, CD45RO, and CD66b in colorectal cancer tissues. Nuclei were stained with DAPI. Pictures are representative of five different tissue specimens. Magnification 20×; scale bar, 50 μm. **C,** Peripheral blood CD8$^+$ T cells from patients with colorectal cancer undergoing surgical treatment were cocultured for 24 hours with autologous, purified TAN at 1:1 ratio in the presence or absence of suboptimal concentration (1 μg/mL) of anti-CD3 (clone UCTH) and anti-CD28. CD69 expression was measured by flow cytometry and IFNγ release by ELISA. Similar experiments were performed by using PBN from patients with colorectal cancer (**D**) and from HD (**E**). In the latter cases, T-cell proliferation and IFNγ release were also measured upon 72-hour culture.

by phenotypic profiles different from autologous peripheral blood CD8$^+$ T cells. In particular, significantly higher percentages of CD8$^+$ TIL, as compared with peripheral blood CD8$^+$ T cells, do express CD69 (71.9% ± 19.1% vs. 1.6% ± 0.6%, $n = 4$, $P = 0.008$) or CD45RO (64.5% ± 25.7% vs. 22.5 ± 10%, $n = 4$, $P = 0.02$), consistent with a locally "activated" state. However, we observed that the percentage of CD62L$^+$ cells was markedly increased in anti-CD3/CD28–stimulated cultures performed in the presence of autologous TAN, as compared with cultures performed in their absence, in CD8$^+$ TILs derived from three out of four tumors tested, whereas it was identical in a fourth. Accordingly, IFNγ release upon anti CD3/CD28 stimulation in cultures of CD8$^+$ TIL from two out of three different tumor specimens tested was higher in the presence than in the absence of autologous TAN. Representative data and cumulative results are shown in Fig. 5B and C.

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

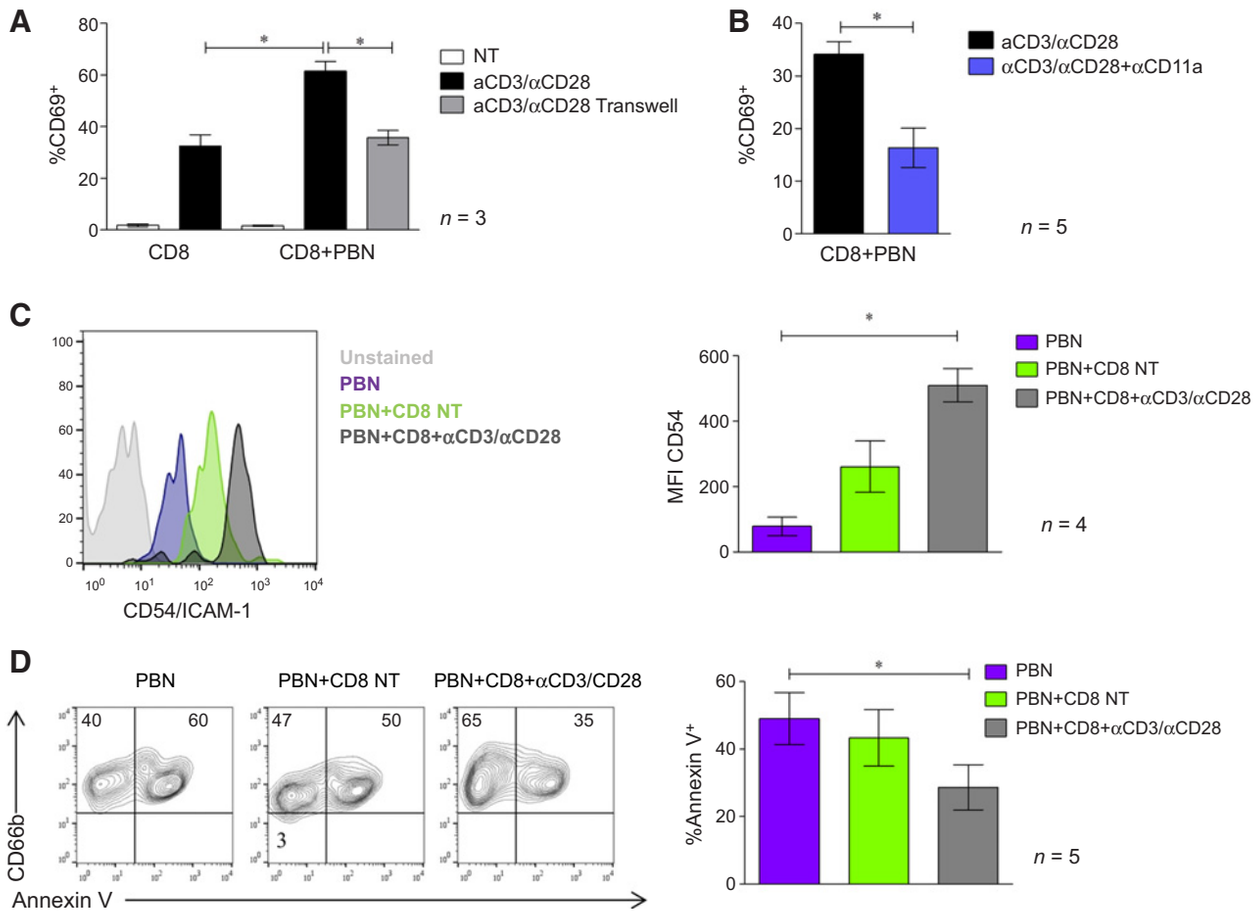


Figure 4.

Neutrophil/CD8$^+$ T-cell cross talk is mediated through CD11a/CD54 interaction. **A,** Peripheral blood CD8$^+$ cells were stimulated by suboptimal concentrations of anti-CD3/anti-CD28 in presence or absence of autologous PBN and in conditions preventing cell contact (Transwell). **B,** Cumulative data referring to the effects of anti-CD11a mAb on the increase in CD69 expression in CD8$^+$ T cells upon stimulation by suboptimal concentrations of anti-CD3/anti-CD28 in the presence of autologous PBN. **C,** CD54/ICAM-1 expression was tested on live PBN, following overnight coculture in presence or absence of CD8$^+$, in resting state or activated by a suboptimal concentration of anti-CD3 and anti-CD28. The panel reports a representative flow cytometry histogram and cumulative data from different experiments. **D,** Viability of PBN following overnight culture in the presence or absence of CD8$^+$ cells in resting state (NT) or activated by a suboptimal concentration of anti-CD3 and anti-CD28 was assessed by Annexin V/PI staining. The panel reports representative results and cumulative data from independent experiments. *, $P < 0.05$.

Impact of TANs on the prognostic significance of CD8$^+$ T-cell infiltration in colorectal cancer

"*In vitro*" mechanistic results urged us to analyze potential prognostic significance of combined colorectal cancer infiltration by neutrophils and CD8$^+$ T cells. Colorectal cancer infiltration by CD66b$^+$ cells was characterized by weak, but significant, correlation with CD8$^+$ T-cell infiltration ($P < 0.001$). These findings prompted us to investigate the prognostic significance of combined colorectal cancer infiltration by both CD66b$^+$ neutrophils and CD8$^+$ T cells. In our cohort (Supplementary Table S2), 50% of the tumors (325/652) were characterized by poor CD8$^+$ and CD66b$^+$ cell infiltration. Although 39% (259/652) and 23% (149/652) of cases showed evidence of high CD66b$^+$ or CD8$^+$ T-cell infiltration, respectively, a concomitantly high CD66b$^+$ and CD8$^+$ infiltrate was detectable in 12% of colorectal cancer samples (81/652). Colorectal cancer samples infiltrated by both CD66b$^+$ and CD8$^+$ cells displayed favorable prognosis, whereas cancers with low CD66b$^+$ and CD8$^+$ cell infiltration were characterized by poor prognosis (Fig. 6A, $P < 0.0001$). Most interestingly, the favorable prognostic significance of CD8$^+$ colorectal cancer infiltration was significantly ($P = 0.011$) enhanced by a concomitant infiltration by CD66b$^+$ neutrophils (Fig. 6). Accordingly, colorectal cancer samples with concomitant high infiltration by CD66b$^+$ and CD8$^+$ T cells were more frequently characterized by pN0 stage (absence of nodal metastases $P = 0.03$), and a more frequent "pushing" tumor border ($P = 0.038$; Supplementary Table S2).

Several models with additive inclusion of single clinical–pathologic data were also tested. Age and gender of the patients and pT or pN stages of the tumors did not affect the significant prognostic impact of CD66b$^+$ and CD8$^+$ cell infiltration. However, when vascular invasion or invasive margins were added to the model, colorectal cancer infiltration by CD66b$^+$ and CD8$^+$ cells lost its independent prognostic value (data not shown).

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

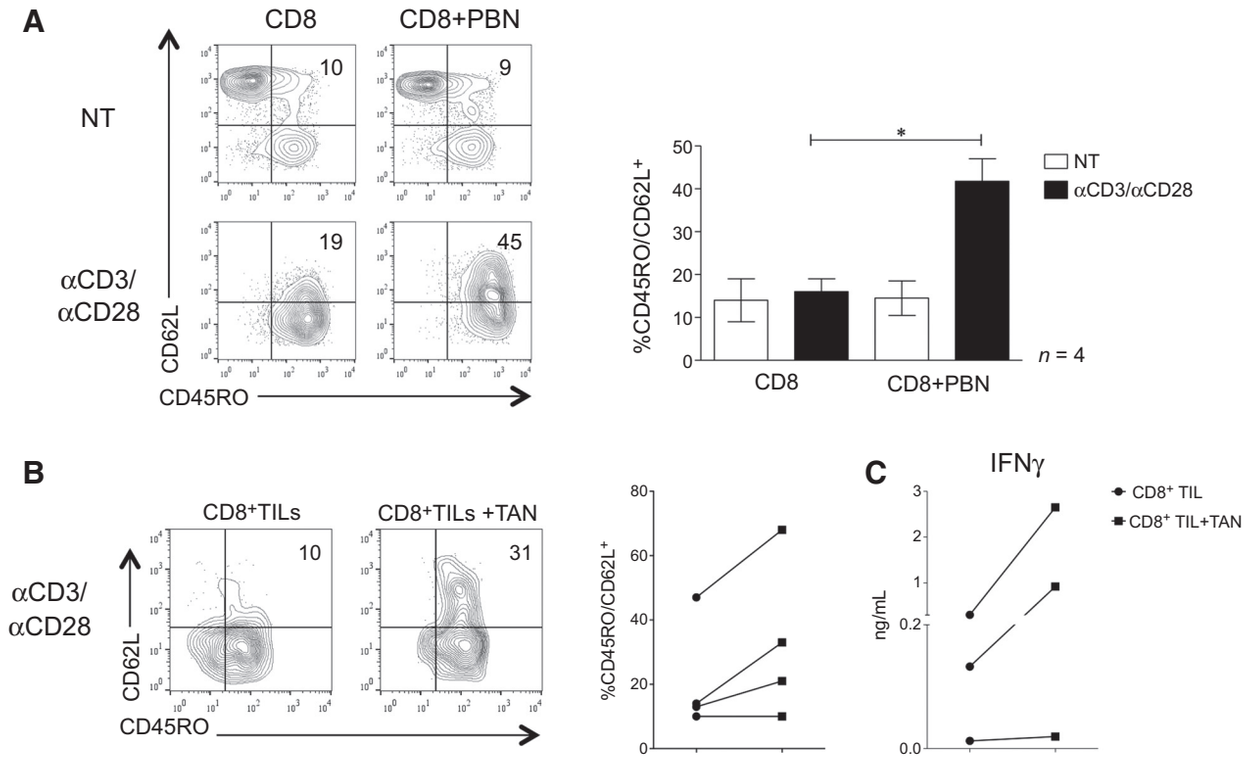


Figure 5.

Neutrophils enhance CD8$^+$ central memory differentiation and survival. Peripheral blood CD8$^+$ cells from HDs were activated with optimal mitogenic concentrations of anti-CD3 mAb (clone TR66, 2 μg/mL) and anti-CD28 in the presence or absence of autologous PBNs for 5 days. Representative flow cytometry plots and cumulative data regarding the expression of CD62L central memory marker in CD45RO$^+$ cells are reported in **A**. Similar experiments were also performed by using freshly derived CD8$^+$ TIL and autologous TANs from colorectal cancer specimens. **B,** Representative flow cytometry plots and cumulative data regarding the expression of CD62L central memory marker in CD8$^+$ TILs stimulated in the presence or absence of autologous TAN. In unstimulated cultures, CD62L was expressed in <4% of CD8$^+$ TIL. Cumulative data regarding IFNγ release are also reported (**C**).

Discussion

The role of neutrophils in tumor immunobiology and the prognostic significance of neutrophil infiltration in cancer tissues are controversial (1, 2). Early studies have documented a direct cytotoxic potential of neutrophils against defined tumor cell lines (29). However, granulocytes have also been shown to actively contribute to the generation of microenvironmental conditions favoring tumor growth, particularly in cancers associated with chronic inflammation. Their ability to degrade extracellular matrix and to promote angiogenesis has been shown to play critical roles in tumor progression (32). More recently, neutrophils were shown to accumulate in premetastatic niches with pro- or antitumor functions in different experimental models (33, 34). Importantly, the ability of neutrophils at different maturation stages to suppress immune responses has been clearly demonstrated in experimental model (35, 36). However, the phenotypic and functional characterization of human MDSC of the granulocytic lineage has not been elucidated in comparable detail (37). Recent studies suggest that local microenvironmental conditions might result in the polarization of neutrophils toward pro- or antitumor functional states (38), possibly characterized by different physical and functional profiles (36). Remarkably, depending on anatomical locations and histologic origins, human cancers may be characterized by highly diverse microenvironmental conditions, potentially impacting on the clinical significance of granulocyte infiltration.

In this study, we report that the analysis of a large clinically annotated TMA including over 600 colorectal cancer reveals that CD66b$^+$ cell infiltration is associated with favorable prognosis. CD66b is expressed by neutrophils and eosinophils. However, in keeping with previously published data (39), we observed that >90% of CD66b$^+$ colorectal cancer–infiltrating cells were neutrophils. Although colorectal cancer appears to be infiltrated to a larger extent than autologous adjacent healthy tissue, phenotypic profiles of colorectal cancer–infiltrating neutrophils largely match those detectable in healthy mucosa–infiltrating cells. However, in agreement with data regarding early lung cancer–infiltrating neutrophils (12, 13), TANs appear to express CD54 to higher extents as compared with neutrophils infiltrating autologous adjacent healthy mucosa, consistent with an "activated" phenotypic profile.

Mechanisms potentially underlying the favorable prognostic significance of colorectal cancer infiltration by CD66b$^+$ cells were investigated in detail. Our results indicate that coculture with autologous neutrophils enhances T-cell receptor–triggered activation of CD8$^+$ T cells and may promote the expansion of a lymphocyte subset characterized by the expression of "memory" markers. The relevance of these findings to colorectal cancer immunobiology is indirectly supported by the colocalization of

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

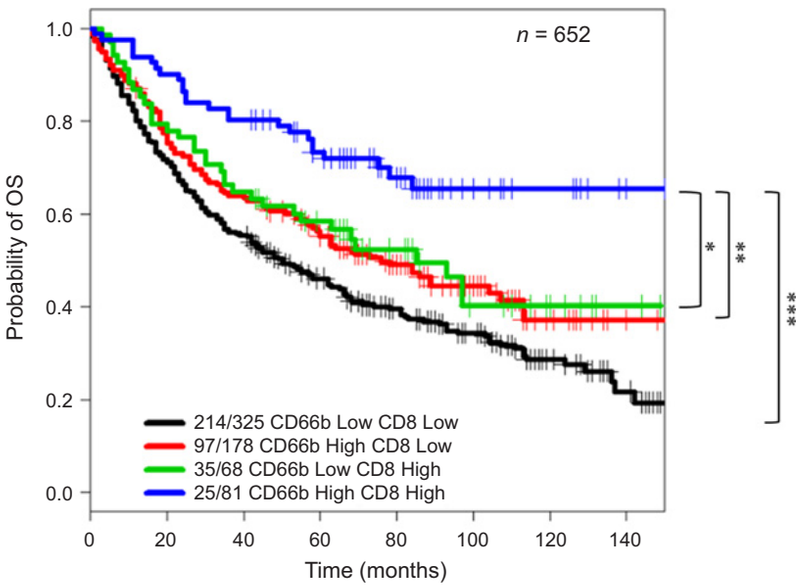


Figure 6.
Colorectal cancer infiltration by CD66b$^+$ enhances the favorable prognostic significance of CD8$^+$ infiltration in colorectal cancer. Kaplan–Meier OS curves were designed according to high- and low-density CD66b$^+$ and CD8$^+$ cell infiltration. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

neutrophils and both CD8$^+$ and CD45RO$^+$ T cells in colorectal cancer tissues. Furthermore, most importantly, colorectal cancer concomitantly infiltrated by neutrophils and CD8$^+$ T cells are characterized by a significantly more favorable prognosis, as compared with tumors displaying high CD8$^+$ but low CD66b$^+$ cell infiltration. High neutrophil infiltration "per se," in the absence of concomitant CD8$^+$ lymphocytes, provided a more modest, but still highly significant, prognostic advantage as compared with colorectal cancer where neither high-density CD8$^+$ nor CD66b$^+$ infiltrates were detectable. Therefore, absence of lymphocyte infiltration or infiltration by "unfavorable" T cells might prevent a full elicitation of antitumor effects of neutrophils.

These data may suggest that the favorable prognostic significance of infiltration by neutrophils in colorectal cancer might, at least in part rely on their interaction with CD8$^+$ T cells, possibly based on costimulatory mechanisms, as suggested by our "*in vitro*" results. Admittedly, literature reports based on "*in vitro*" studies with human cells in this area are controversial (30, 40, 41). However, in our experience, different techniques, and, in particular, the use of human, as opposed to calf serum, might largely account for the observed discrepancies.

The potential relevance of these results might obviously extend beyond colorectal cancer immunobiology. Neutrophil–CD8$^+$ T-cell interactions might indeed be operational in a wider range of conditions, thus supporting the notion of a highly effective cooperation between innate and adaptive immune responses.

Remarkably, similar results have also emerged from studies conducted in experimental models (42) and in clinical settings, including early-stage lung cancer (12, 13) and autoimmune and infectious disease (31). Underlying molecular mechanisms have not been fully clarified. In particular, cell–cell interactions mediated by OX40, CD58, CD59, and their ligands have been proposed (12, 31). Alternatively, a role for ROS release has also been suggested (42). Our data suggest that CD11a/CD54 interaction powerfully contributes to the elicitation of the costimulatory effects of neutrophils on CD8+ T-cell activation. Nevertheless, further research is warranted to obtain additional mechanistic insights.

Overall, an important limitation in studies on TANs is represented by their short life span, high sensitivity to enzymes necessary for the generation of single-cell suspensions from clinical specimens and relatively low numbers, preventing the routine performance of functional studies. However, the consistency of "*in vitro*" results data emerging by using TANs, and PBNs from patients with colorectal cancer and HDs together with the clinical data, appears to support the notion of a potentially high relevance of neutrophil–T-cell interaction on tumor sites.

Our study suffers from a number of additional limitations largely inherent in research requiring the use of clinical materials. Reported clinical data stem from a retrospective study. However, performance of prospective studies, currently being planned, is delayed by overall survival rates in patients with colorectal cancer frequently exceeding 50% at 5 years following surgery. Furthermore, repeated biopsies of metastatic sites are usually not included in routine clinical procedures. Therefore, the performance of longitudinal studies addressing the role of neutrophils in different stages of tumor progression is problematic. Finally, numbers of neutrophils and CD8$^+$ T cells which may be obtained from freshly excised colorectal cancer are usually modest and barely amenable to standard cellular immunology assays, particularly in autologous settings. For instance, direct tumor cytotoxicity studies could only performed with resting or activated peripheral blood neutrophils from patients and HDs and we were unable to separate sufficient numbers of high/normal and low-density granulocytes (36) from tumor suspensions.

Remarkably, our results also appear to suggest functional discrepancies between PBN and autologous TAN as regarding, for instance, the capacity of expanding "central memory" cells. These data urge further research aimed at clarifying whether these discrepancies are due to functional impairments of TAN in specific tumor microenvironments (7) or to tumor infiltration by CD66$^+$ cell subpopulations of different functional significance (36, 37).

Our study also poses a number of additional important questions. The fact that in a variety of tumors other than colorectal cancer, neutrophil infiltration has been suggested to be associated with poor prognosis (35, 43, 44) raises the issue of the specificities inherent in neutrophil infiltration in colorectal cancer. The

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

microenvironment of these tumors presents a variety of peculiar characteristics. Similar to other cancers (45), colorectal cancer infiltration by "memory" CD8$^+$ T cells has been shown to be associated with favorable prognosis. However, tumor infiltration by cells expressing FOXP3, a classical regulatory T-cell marker, also appears to correlate with a less severe clinical course (46). Furthermore, at difference with tumors of different histologic origin (20) colorectal cancer infiltration by macrophages is also associated with favorable prognosis (21). Most remarkably, colorectal cancer cells have previously been shown (47) to produce GM-CSF, possibly enhancing viability and functions of granulocytes eventually recruited within tumor microenvironment.

Data regarding neutrophil infiltration in colorectal cancer from East Asia patients (24) appear to contradict our results. Genetic background may play an important role in this context. However, additional factors might be involved in determining the prognostic relevance of neutrophil infiltration. Colorectal cancer oncogenesis is typically characterized by an early increase in bacterial translocation from the gut lumen (48) and gut microbiome composition has recently been shown to decisively impact on chemo- and immunotherapy outcome (49). Considering the key role of neutrophils in the response to bacteria, it is tempting to speculate that microbiome composition might decisively affect their ability to actively participate to antitumor immune response. Thus, differences in human gut microbiome in different geographic areas (50) might also be of importance in the evaluation of the prognostic significance of neutrophil infiltration in colorectal cancer. Within this context, our data also urge studies aimed at the clarification of mechanisms favoring the recruitment of granulocytes within colorectal cancer tissues.

In conclusion, our study, providing clear evidence of the prognostic significance of concomitant infiltration by CD8$^+$ cells and neutrophils in colorectal cancer, represents an additional example of clinically relevant interaction between noncancerous cells from the tumor microenvironment. Furthermore, it clearly identifies neutrophils as key players in colorectal cancer immunobiology.

Disclosure of Potential Conflicts of Interest

L. Tornillo reports receiving speakers bureau honoraria from AMGEN Switzerland AG. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: V. Governa, V. Mele, L. Tornillo, R. Rosso, G. Iezzi, G.C. Spagnoli

Development of methodology: V. Governa, G.C. Spagnoli

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Governa, E. Trella, L. Tornillo, F. Amicarella, E. Cremonesi, M.G. Muraro, H. Xu, R Droeser, S.R. Däster, M. Bolli, R. Rosso, L. Terracciano

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Governa, E. Trella, V. Mele, L. Tornillo, E. Cremonesi, M.G. Muraro, S. Eppenberger-Castori, L. Terracciano, G. Iezzi, G.C. Spagnoli

Writing, review, and/or revision of the manuscript: V. Governa, V. Mele, F. Amicarella, R. Droeser, S.R. Däster, D. Oertli, S. Eppenberger-Castori, G. Iezzi, G.C. Spagnoli

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Governa, R. Droeser, S.R. Däster, G.C. Spagnoli

Study supervision: D. Oertli, G.C. Spagnoli

Acknowledgments

We thank Marco Cassatella for advice on neutrophils purification and activation techniques, Elisabetta Padovan, Luca Quagliata, Jean Pieters, Alfred Zippelius, Nina Khanna, Pascal Forrer, Marco Lepore, Christian Hirt, and Paul Zajac for critical discussions.

Grant Support

This work was partially funded by an SNF grant (to G.C. Spagnoli), an SNF Professorship grant (to G. Iezzi), and a grant from Swiss Cancer League to (R.A. Droeser). V. Governa was funded by a grant from the Swiss Centre for Applied Human Toxicology (SCAHT) and Sophienstiftung zur Förderung der klinischen Krebsforschung, Zürich, Switzerland, and Novartis Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 17, 2016; revised December 14, 2016; accepted December 21, 2016; published OnlineFirst January 20, 2017.

References

1. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. Nat Rev Cancer 2016;16:431–46.
2. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? Carcinogenesis 2012;33:949–55.
3. Di Carlo E, Forni G, Lollini P, Colombo MP, Modesti A, Musiani P. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 2001;97:339–45.
4. Templeton AJ, McNamara MG, Seruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. J Natl Cancer Inst 2014;106:dju124.
5. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162–74.
6. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 2009;16:183–94.
7. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. J Clin Invest 2010;120:1151–64.
8. Granot Z, Henke E, Comen EA, King TA, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell 2011;20:300–14.
9. Finisguerra V, Di CG, Di MM, Serneels J, Costa S, Thompson AA, et al. MET is required for the recruitment of anti-tumoural neutrophils. Nature 2015;522:349–53.
10. Dranoff G.GM-CSF-based cancer vaccines. Immunol Rev 2002;188:147–54.
11. Stoppacciaro A, Melani C, Parenza M, Mastracchi A, Bassi C, Baroni C, et al. Regression of an established tumor genetically modified to release granulocyte colony-stimulating factor requires granulocyte-T cell cooperation and T cell-produced interferon gamma. J Exp Med 1993;178:151–61.
12. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. J Clin Invest 2014;124:5466–80.
13. Singhal S, Bhojnagarwala PS, O'Brien S, Moon EK, Garfall AL, Rao AS, et al. Origin and role of a subset of tumor-associated neutrophils with antigen-presenting cell features in early-stage human lung cancer. Cancer Cell 2016;30:120–35.
14. Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, et al. Colorectal cancer. Lancet 2010;375:1030–47.
15. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

16. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005;353:2654–66.
17. Mennonna D, Maccalli C, Romano MC, Garavaglia C, Capocefalo F, Bordoni R, et al. T cell neoepitope discovery in colorectal cancer by high throughput profiling of somatic mutations in expressed genes. Gut 2015 Dec 17. [Epub ahead of print].
18. Zlobec I, Karamitopoulou E, Terracciano L, Piscuoglio S, Iezzi G, Muraro MG, et al. TIA-1 cytotoxic granule-associated RNA binding protein improves the prognostic performance of CD8 in mismatch repair-proficient colorectal cancer. PLoS One 2010;5:e14282.
19. Sconocchia G, Arriga R, Tornillo L, Terracciano L, Ferrone S, Spagnoli GC. Melanoma cells inhibit NK cell functions. Cancer Res 2012;72:5428–9.
20. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 2006;124:263–6.
21. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C. Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. J Transl Med 2007;5:62.
22. Sconocchia G, Zlobec I, Lugli A, Calabrese D, Iezzi G, Karamitopoulou E, et al. Tumor infiltration by FcgammaRIII (CD16)+ myeloid cells is associated with improved survival in patients with colorectal carcinoma. Int J Cancer 2011;128:2633–72.
23. Droeser RA, Hirt C, Eppenberger-Castori S, Zlobec I, Viehl CT, Frey DM, et al. High myeloperoxidase positive cell infiltration in colorectal cancer is an independent favorable prognostic factor. PLoS One 2013;8:e64814.
24. Rao HL, Chen JW, Li M, Xiao YB, Fu J, Zeng YX, et al. Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. PLoS One 2012;7:e30806.
25. Yamamoto T, Kawada K, Itatani Y, Inamoto S, Okamura R, Iwamoto M, et al. Loss of SMAD4 promotes lung metastasis of colorectal cancer by accumulation of CCR1+ tumor-associated neutrophils through CCL15-CCR1 axis. Clin Cancer Res 2016 Aug 4. [Epub ahead of print].
26. Galdiero MR, Bianchi P, Grizzi F, Di Caro G, Basso G, Ponzetta A, et al. Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer. Int J Cancer 2016;139:446–56.
27. Amicarella F, Muraro MG, Hirt C, Cremonesi E, Padovan E, Mele V, et al. Dual role of tumour-infiltrating T helper 17 cells in human colorectal cancer. Gut 2015 Dec 30. [Epub ahead of print].
28. Trella E, Raafat N, Mengus C, Traunecker E, Governa V, Heidtmann S, et al. CD40 ligand-expressing recombinant vaccinia virus promotes the generation of CD8(+) central memory T cells. Eur J Immunol 2016;46:420–31.
29. Ackermann MF, Lamm KR, Wiegand GW, Luster MI. Antitumor activity of murine neutrophils demonstrated by cytometric analysis. Cancer Res 1989;49:528–32.
30. Pelletier M, Micheletti A, Cassatella MA. Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. J Leukoc Biol 2010;88:1163–70.
31. McKinney EF, Lee JC, Jayne DR, Lyons PA, Smith KG. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. Nature 2015;523:612–6.
32. Granot Z, Fridlender ZG. Plasticity beyond cancer cells and the "immunosuppressive switch". Cancer Res 2015;75:4441–5.
33. Granot Z, Henke E, Comen EA, King TA, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell 2011;20:300–14.
34. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, et al. Granulocyte-colony-stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. Proc Natl Acad Sci U S A 2010;107:21248–55.
35. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature 2015;522:345–8.
36. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. Cell Rep 2015;10:562–73.
37. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun 2016;7:12150.
38. Granot Z, Jablonska J. Distinct functions of neutrophil in cancer and its regulation. Mediators Inflamm 2015;2015:701067.
39. Richards CH, Flegg KM, Roxburgh CS, Going JJ, Mohammed Z, Horgan PG, et al. The relationships between cellular components of the peritumoural inflammatory response, clinicopathological characteristics and survival in patients with primary operable colorectal cancer. Br J Cancer 2012;106:2010–5.
40. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol 2011;11:519–31.
41. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. Immunity 2014;40:785–800.
42. Schwab L, Goroncy L, Palaniyandi S, Gautam S, Triantafyllopoulou A, Mocsai A, et al. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. Nat Med 2014;20:648–54.
43. Fridlender ZG, Albelda SM, Granot Z. Promoting metastasis: neutrophils and T cells join forces. Cell Res 2015;25:765–6.
44. Liang W, Ferrara N. The complex role of neutrophils in tumor angiogenesis and metastasis. Cancer Immunol Res 2016;4:83–91.
45. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2012;12:298–306.
46. Frey DM, Droeser RA, Viehl CT, Zlobec I, Lugli A, Zingg U, et al. High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. Int J Cancer 2009;126:2635–43.
47. Nebiker CA, Han J, Eppenberger-Castori S, Iezzi G, Hirt C, Amicarella F, et al. GM-CSF production by tumor cells is associated with improved survival in colorectal cancer. Clin Cancer Res 2014;20:3094–106.
48. Abreu MT, Peek RM Jr. Gastrointestinal malignancy and the microbiome. Gastroenterology 2014;146:1534–46.
49. Zitvogel L, Ayyoub M, Routy B, Kroemer G. Microbiome and anticancer immunosurveillance. Cell 2016;165:276–87.
50. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–7.

Published OnlineFirst January 20, 2017; DOI: 10.1158/1078-0432.CCR-16-2047

Clinical Cancer Research

The Interplay Between Neutrophils and CD8$^+$ T Cells Improves Survival in Human Colorectal Cancer

Valeria Governa, Emanuele Trella, Valentina Mele, et al.

Clin Cancer Res 2017;23:3847-3858. Published OnlineFirst January 20, 2017.