The interplay between neutrophils and CD8+ T cells improves survival in human colorectal cancer

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Composition of tumor microenvironment impacts on cancer progression and clinical course. Regarding colorectal cancer (CRC), infiltration by CD8+ T lymphocytes is associated with improved survival, but the role of myeloid cells is unclear. We have observed that CRC 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 CRC-derived tumor associated and peripheral blood neutrophils from patients with CRC and healthy donors enhance CD8+ lymphocyte responsiveness to T-cell receptor complex triggering. Most importantly, the prognostic significance of CD8+ T cell infiltration in CRC is significantly improved by a concomitant neutrophil infiltration. These data powerfully support the use of a refined analysis of CRC immune contexture for clinical decision-making and identify neutrophils as important players in anti-tumor immune responses in CRC.
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 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 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.
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 anti-tumor immune responses of 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 (6;7). Furthermore, tumor “educated” neutrophils were shown to elicit anti-metastatic 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 anti-tumor activities (9). Earlier studies indicated that production by tumor cells of granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF), promoting neutrophil survival and activation, could induce adaptive anti-tumor 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 anti-tumor responses has been demonstrated (12;13). However, their prognostic significance was not addressed.

Colorectal cancer (CRC) represents the third cause of cancer-related mortality worldwide. TNM staging, routinely used to identify patients eligible for different treatments is frequently ineffective in predicting CRC clinical course (14).

Clinical relevance of the composition of tumor infiltrate in CRC has been extensively investigated. CRC 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 neo-antigens (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 CRC 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 CRC (21).
The role of neutrophils has not been comparably explored. We previously observed that CRC 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 CRC. In a cohort of East Asian patients (n=229) neutrophil infiltration was associated with severe prognosis (24). Moreover, neutrophil infiltration in CRC-derived lung metastases has been suggested to be associated with severe prognosis following surgical excision (25). However, neutrophil infiltration in CRC was reported to be associated with responsiveness to 5-fluorouracil (5FU) treatment (26). Thus, clinical significance of tumor associated neutrophils (TANs) infiltrating CRC is still unclear and underlying functional mechanisms remain to be elucidated.

We have analyzed the prognostic significance of CRC infiltrating CD66b+ neutrophils by using a clinically annotated tissue microarray (TMA) including >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 specifically addressed. Finally, the prognostic relevance of the association of neutrophils with CD8+ T cells infiltrating CRC microenvironment was explored.
Materials and methods

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) (18;22). A semi-automated 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-pathological data for patients included in the TMA are summarized in Supplementary Tables 1-2. Use of clinical information was approved by local ethical authorities.

Immunohistochemistry

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

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 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, and Ospedale Civico, Lugano, 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 200U/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 (27).

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 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, 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 PBMC obtained by gradient centrifugation or digested tumor specimens, respectively, by using anti CD8-coated magnetic beads (Miltenyi Biotec), as previously described (28).

CRC/N co-cultures

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

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, CXCR1, CXCR2 (BD Biosciences). Alternatively, cells were fixed and intracellular staining was performed with antibodies specific for MPO (23). Stained cells were analyzed by FACS Calibur flow cytometer (BD Biosciences), using FlowJo software (Tree Star).
**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.

**Neutrophil and CD8+ T cell co-cultures**

PBNs and TANs, obtained from HD and CRC patients, were co-cultured with CD8+ T cells from autologous peripheral blood or tumor specimens (see above). For co-stimulation experiments, 96-well flat bottom culture plates were coated overnight with anti CD3 mitogenic mAb (TR66, a gift of Dr. Lanzavecchia, Bellinzone, 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 x 10⁶/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 (1 μg/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 (28). 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.

**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-phenylidole (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).
Statistical analysis

Statistical significance of differential expression of activation markers, cytokine release and cell proliferation was 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.

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.
Results

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 (23). 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 (26). CRC infiltrating CD66b+ cells could be efficiently detected in punches from fixed, paraffin embedded tissues (Fig. 1A-B). In this cohort of patients high CRC 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 cut-off (\(P=0.0003\) and \(P=0.001\), respectively) or by using non-dichotomized 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 Tab. 1).

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 \((P=0.0009)\) 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 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 (22;23), 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 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 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, phenotypic profiles of PBNs from patients with CRC and HD are similar (Supplementary Fig. S1).

**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 (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 CRC and HD. Co-culture in the presence of granulocytes did not decrease proliferation (Supplementary Fig. S2A-B) nor induced apoptosis (data not shown) in a panel of CRC cell lines. Prior treatment of neutrophils with IFN-γ or fMLP also failed to impact on viability and proliferation potential of co-cultured CRC 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 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 to associate with favorable prognosis (15), although their antigen specificity is largely unclear. Cytokines released by activated T cells, including GM-CSF and IFN-γ 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 co-localize with CD8+ and CD45RO+ T lymphocytes within tumor tissues (Fig. 3A-B). Based on these findings, 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. 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, IFN-γ 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 IFN-γ 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. 3B and cumulative data are reported in Fig. 3D-E. T cells proliferation, as assessed by CFSE dilution at 72 hours, was also significantly enhanced. Representative flow-cytometry profiles are shown in Supplementary Fig. 3D, whereas cumulative data are reported in Fig. 3E. In contrast, these co-stimulatory effects were undetectable in T cells activated with optimal mitogenic concentrations of anti CD3 mAb (supplementary Fig. S3A).

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 (figure 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 TAN and PBN are able to co-stimulate CD8+ T cells, and that these effects are detectable in sub-optimal activation conditions.

**Neutrophil-mediated co-stimulation results in increased memory CD8+ T cell numbers**

Favorable prognosis in CRC has been repeatedly associated with tumor infiltration by “memory” T lymphocytes (15;16). 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 five days, e.g. beyond time points usually considered for detection of maximal proliferation (31). Remarkably, peripheral blood CD8+ T cells 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 out 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 by phenotypic profiles different from autologous peripheral blood CD8+ T cells. In particular, significantly higher percentages of CD8+ TIL, as compared to 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 to cultures performed in their absence, in CD8+ TIL 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-C.

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 (Supplementary Table 2), 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 ($P=0.038$) (Supplementary Table 2).

Several models with additive inclusion of single clinical-pathological 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, CRC infiltration by CD66b+ and CD8+ cells lost its independent prognostic value (data not shown).
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 myeloid-derived suppressor cells (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 towards pro- or anti-tumor functional states (38), possibly characterized by different physical and functional profiles (36). Remarkably, depending on anatomical locations and histological 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 CRC 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+ CRC infiltrating cells are neutrophils. 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 agreement with data regarding early lung cancer infiltrating neutrophils (12;13), TAN appear to express CD54 to higher extents as compared to neutrophils infiltrating autologous adjacent healthy mucosa, consistent with an “activated” phenotypic profile.

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 may promote the expansion of a lymphocyte subset characterized by the expression of “memory” markers. 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. High neutrophil infiltration “per se”, in the absence of concomitant CD8+ lymphocytes, provided a more modest, but still highly significant, prognostic advantage as compared to CRC 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 anti-tumor effects of neutrophils.

These data may suggest that the favorable prognostic significance of infiltration by neutrophils in CRC might, at least in part rely on their interaction with CD8+ T cells, possibly based on co-stimulatory 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 CRC 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 co-stimulatory 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 CRC and healthy donors 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 CRC frequently exceeding 50% at five 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 CRC 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 healthy donors 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 (see above). 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 CRC, 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 CRC. The microenvironment of these tumors presents a variety of peculiar characteristics. Similarly to other cancers (45), CRC 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 histological origin (20) CRC infiltration by macrophages is also associated with favorable prognosis (21). Most remarkably, CRC 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 CRC 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. CRC 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 anti-tumor 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 CRC. Within this context, our data also 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 an additional example of clinically relevant interaction between non-cancerous cells from the tumor microenvironment. Furthermore, it clearly identifies neutrophils as key players in CRC immunobiology.

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**Contributors:** VG and GS conceived and designed the experiments, analyzed and interpreted results, obtained funding, wrote the manuscript. ET and VM contributed experimental design data analysis, and revision of the manuscript. GI contributed to conception of research, experimental design and interpretation of data. FA, EC, LT, HX, MGM performed the experiments. SE-C performed statistical analysis of data. MB, SD, RR contributed to the data collection. RAD, DO contributed to data collection and provided financial support. LMT contributed to conception of research, data collection and financial support.

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Reference List


Legends to the figures

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.

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 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.

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.

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 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.
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 5 days. Representative flow-cytometry plots and cumulative data regarding the expression of CD62L central memory marker in CD45RO+ cells are reported in panel (A). Similar experiments were also performed by using freshly derived CD8+ TIL and autologous TAN from CRC specimens. Panel B reports representative flow-cytometry plots and cumulative data regarding the expression of CD62L central memory marker in CD8+ TIL stimulated in the presence or absence of autologous TAN. In unstimulated cultures, CD62L was expressed in <4% of CD8+ TIL. Cumulative data regarding IFN-γ release are also reported (C).

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
Figure 1.
Figure 3.
Figure 4

A

B

C

D

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Legend:

- NT
- αCD3/αCD28
- αCD3/αCD28 Transwell

- aCD3/αCD28
- αCD3/αCD28 + αCD11a

- PBN
- PBN+CD8 NT
- PBN+CD8+αCD3/αCD28

- PBN
- PBN+CD8 NT
- PBN+CD8+αCD3/αCD28

Statistical significance:

- *
Figure 5.
Figure 6

![Graph showing the probability of OS over time for different groups based on CD66b and CD8 expression levels.]
The interplay between neutrophils and CD8+ T cells improves survival in human colorectal cancer

valeria governa, Emanuele Trella, Valentina Mele, et al.

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