Coordination of Intratumoral Immune Reaction and Human Colorectal Cancer Recurrence

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Abstract
A role for the immune system in controlling the progression of solid tumors has been established in several mouse models. However, the effect of immune responses and tumor escape on patient prognosis in the context of human cancer is poorly understood. Here, we investigate the cellular and molecular parameters that could be present in situ immune responses in human colorectal cancer according to clinical parameters of metastatic lymph node or distant organ invasion (META- or META+ patients). Primary tumor samples of colorectal carcinoma were analyzed by integrating large-scale phenotypic (flow cytometry, 39 patients) and gene expression (real-time reverse transcription-PCR, 103 patients) data sets related to immune and protumoral processes. In META- colorectal cancer primary tumors with high densities of T cells, we observed significant positive correlations between markers of innate immune cells [tumor-associated macrophages, dendritic cells, natural killer (NK) cells, and NKT cells] and markers of early-activated T cells. Significant correlations were also observed between markers of cytotoxic and effector memory T-cell subpopulations. These correlation profiles were absent in tumors with low T-cell infiltrates and were altered in META+ tumors with high T-cell infiltrates. We show that the coexpression of genes mediating cytotoxicity (GNY) and Th1 adaptive immune responses (IRF1) accurately predicted patient survival independently of the metastatic status. High intratumoral mRNA expression of the proangiogenic mediator vascular endothelial growth factor was associated with significantly reduced survival rates in patients expressing high mRNA levels of GNY. Investigation of the colorectal cancer primary tumor microenvironment allowed us to uncover the association of favorable outcomes with efficient coordination of the intratumoral immune response. [Cancer Res 2009;69(6):2685–93]

Introduction
Cancer progression is a complex process involving host-tumor interactions through multiple molecular and cellular factors of the tumor microenvironment. Tumors may be vulnerable to immune destruction. As revealed by experiments in immune-deficient mice, immune responses mediated by IFNγ (2, 3) and cytotoxic mediators such as perforin (4, 5) secreted by lymphocytes are involved in cancer immunosurveillance (6, 7). In human cancer, complex tumor-host interactions are less well documented. However, lymphocytes were also shown to participate in anti-tumor responses (8). Consistent with findings in melanoma (9) and ovarian cancer (10, 11), tumor-infiltrating T cells were associated with improved clinical outcomes and survival in colorectal cancer patients (12–16).

We recently highlighted intratumoral memory T cells as the major immune effector cells significantly associated with the decrease of early metastatic events (tumor emboli) and the prevention of relapse in colorectal cancer patients (17). Furthermore, we revealed the importance to patient prognosis of the nature, functional orientation, density, and localization of immune cell populations within the primary tumor. Multivariate Cox analysis showed that immune patterns remained the unique parameter significantly associated with prognosis, whereas T stage, N stage, and differentiation of the tumor were not significant when adjusted to immune patterns (18). Patients with cancers at nonmetastatic stages had prognoses as bleak as patients with metastatic tumors, if presenting a low intratumoral adaptive immune reaction. Conversely, patients with metastatic tumors eliciting a high intratumoral immune reaction were of better prognosis. Thus, the amplitude of adaptive immune reaction within the primary tumor was a better predictor of survival than traditional clinical parameters (19).

However, the intrinsic capability of tumor cells to promote their own development (20) may allow tumors to overwhelm immune system activity. For instance, angiogenesis mediated by vascular endothelial growth factor (VEGF) is critical to the growth (by providing oxygen and nutrients) and malignant dissemination (providing a route for metastases) of solid tumors (21, 22). Furthermore, under the pressure of antitumoral immune activity, selection and outgrowth of variant tumor cells with reduced immunogenicity could occur (8, 22, 23). Thus, during cancer progression, tumor cells may acquire immune tolerance mechanisms by generating complex immunosuppressive networks at the tumor site (24, 25) involving interleukin (IL)-10 and transforming growth factor β (TGFβ); refs. 26, 27) as well as T-cell-specific coinhibitory molecules (CTLA-4 and PD-1; refs. 28, 29).

In this work, we attempted to describe in a comprehensive manner the immune reaction in primary colorectal tumors of patients with high or low densities of infiltrating T cells. Furthermore, we compared the immune microenvironment in patients presenting with invaded lymph nodes and/or distant metastases [META+ patients: Union Internationale Contre le
Cancer (UICC) tumor-node-metastasis (TNM) stages III–IV or without such metastases (META− patients: UICC-TNM stages I–II; ref. 30). We analyzed immune cell phenotypic clusters, or "phenoclusters" (31), obtained by grouping markers according to similar levels of expression. This allowed us to uncover functional marker patterns of efficient and coordinated antitumoral immune responses that represent powerful prognostic criteria for colorectal cancer clinical outcome.

At the cellular level, a high degree of functional coordination between intratumoral immune cells could be observed at the primary tumor sites of both META− and META+ colorectal cancer patients. At the molecular level, the coexpression of genes related to the Th1 immune response [IFN-regulatory factor 1 (IRF1) and cytotoxicity [granulysin (GNLY)] had strong prognostic values. Finally, we studied several tumor-promoting mechanisms including immunosuppression, angiogenesis, tumor survival, and local and metastatic invasion. Analysis of in situ gene expression of protumoral markers in combination with immune parameters revealed that angiogenesis (VEGF) was associated with increased risks of colorectal cancer relapse in patients nonetheless presenting evidence of strong intratumoral immune responses.

**Materials and Methods**

All details about Materials and Methods are available online.

**Patients and database.** Patients with colorectal cancer (n = 566) who underwent a primary resection at the Laennec/HEGP Hospital between 1986 and 2004 were randomly selected. Time to recurrence or disease-free time was defined as the time period from the date of surgery to confirmed tumor relapse date for relapsed patients and from the date of surgery to the date of last follow-up for disease-free patients.
Large-scale flow cytometric analysis. Cells were extracted from 39 fresh tumors, resuspended in PBS/0.5% bovine serum albumin and incubated for 30 min at 4°C with antibodies against immune cell markers for large-scale phenotypic analysis of T cells and with relevant isotype controls. Analyses were done with a four-color fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson) and CellQuest software (Becton Dickinson). Analyzed markers are presented in Supplementary Fig. S3. Complete-linkage hierarchical clustering was applied and the results were displayed with the use of the Genesis program (32, 33). Correlation matrices were constructed by calculation of Pearson correlation coefficients for all marker combinations, followed by unsupervised hierarchical clustering.

Real-time reverse transcription-PCR assay. Tissue samples were snap-frozen. Total RNA was extracted by homogenization with RNeasy isolation kit (Qiagen). The integrity and the quantity of the RNA were evaluated on Bioanalyzer-2100 (Agilent Technologies). Samples (n = 103) were assessed for gene expression analysis of the following 17 genes (see details about gene expression and name in Supplementary data): CDX2, CD4, CD8x, TBX21, IFI1, IFNγ, GNLX, GZMB, GATA3, FOXP3, CEACAM1, CEA, EBAG9, BIRC5, IL-10, TGFβ, and VEGF. Quantitative real-time TaqMan PCR was done using Low-Density-Arrays and the 7900 robotic real-time PCR system (Applied Biosystems). 18S primers and probes were used as internal controls.

Construction of tissue microarrays. Using a tissue microarray instrument (Beecher Instruments, Alphelys), we removed two representative areas of the tumor (center and invasive margin from paraffin-embedded tissue blocks). Tissue microarrays were cut into 5-μm sections for immunohistochemical staining.

Immunohistochemistry. After antigen retrieval and quenching of endogenous peroxidase activity, sections were incubated for 60 min at room temperature with monoclonal antibodies against CD3 (SP7), CD8 (4B11), CD1a (O10), Ki67 (SP6; Neomarkers), CD68 (PGM1; DAKO), FoxP3 (ab20034; Abcam), and M30 cytoDEATH (Alexis Biochemicals). The Envision+ system (enzyme-conjugated polymer backbone coupled to secondary antibodies) and 3,3′-diaminobenzidine chromogen were applied (DAKO). Tissue sections were counterstained with Harris's hematoxylin. Consecutive tissue sections were examined under a light microscope (Zeiss Axiosplan 2). Positive cells were defined as at least 50% of the cell population stained by the corresponding antibody. For confirmation, the expressions of CD3+ cells were represented (Fig. 1A).

Results

Intratumoral distribution of immune cell populations. We first investigated the immune cellular profiles of patients by flow cytometry with 39 freshly resected primary tumors. Percentages of positive cells for distinct markers were calculated among all cells (tumor and immune cells), thus reflecting the density of cells within tumors. Intratumoral T-cell densities were evaluated according to the mean percentage of double-positive cells for the T-cell–specific markers CD3 and CD5 among all samples. Patients presenting a percentage of CD3+CD5+ T cells superior to this mean (6.7% of all cells) were named “Hi patients” and otherwise “Lo patients.” As a control, the percentages of CD3+CD5− and CD3+TCRβ+ T cells were represented (Fig. 1A). In Hi patients, there were significant higher densities of T cells of both cytotoxic (CD3+CD8+) and helper (CD3+CD4+) phenotypes and of memory T cells. In Lo patients, these immune cell markers were significantly reduced compared with Hi patients. Patients with Lo T-cell density (1.7% of all cells) were reported to be less likely to survive long-term disease-free survival (< 0.05).

Figure 2. T-cell populations within primary colorectal tumors. A, T-cell memory differentiation markers (white squares, META–Hi; white triangles, META+ Hi; black squares, META–Lo; black triangles, META+ Lo). Cell populations were represented as the mean percentage of positive cells; bars, SE. P values (Mann-Whitney test) were presented in Supplementary Fig. S1 (*, P < 0.05). B, hierarchical clustering of 11 marker combinations among CD3+ cells with significant differential expression among the four groups of patients (P < 0.05). Combinations of surface markers were plotted from the minimal (blue) to the maximal (red) level of expression. Gray, not determined.
The distribution of global lymphoid cell populations (CD45 +) was studied in Lo patients, no differences were found between patients, metastases in lymph node (stage III) and/or distant organ tumor-associated macrophages and NKT cells were observed in contrast, in Hi patients, significantly lower percentages of dendritic cells, natural killer (NK) cells, NKT cells, or B cells. In immune cell populations according to the metastatic status of A, consistent with those of T cells (Fig. 1A). The relationships implied by these correlations were visualized by using unsupervised hierarchical clustering of correlation matrices of flow cytometry data. Figure 3.

Memory T-cell differentiation. No differences in the distribution of CD45RO + memory T-cell subpopulations were observed among the patient groups (Fig. 1D). However, we more precisely assessed the density of T cells along memory differentiation steps based on the differential expression of CCR7, CD45RA, CD27, and CD28 markers by CD3 + T cells (Fig. 2A). Very few naïve (CCR7 +) T cells were detected within primary tumors. In META− Lo (black squares) and META+ Lo patients (black triangles), despite low densities of T cells, similar levels of memory T-cell subpopulations from early (CD28 +) to late (CD45RA/CD27 +) memory were observed. META− Hi patients (white squares) presented high densities of all memory T-cell subpopulations. In contrast, META+ Hi patients (white triangles) presented a significant decrease in the densities of T cells at late stages of memory differentiation (CD27 +, CD45RA +) with percentages comparable to Lo patients and significantly inferior to META− Hi patients.

Eleven marker combinations expressed among CD3 + T cells were found significantly differentially expressed between META− Hi and META+ Hi patients. After hierarchical clustering of these markers, two major clusters (C.1 and C.2) were found (Fig. 2B). In C.1, CD4 + T-cell subpopulation markers (CD3+/CD4 +) and related memory markers (CD3+/CD8−/CD45RO +) grouped with early memory T-cell markers (CD3+/CD127−, CD3+/CD27−, CD3+/CD45RA−/CD27 +). In C.2, CD8 + T-cell subpopulation markers (CD3+/CD8 +) and related memory/effector T-cell markers (CD8+/CD45RO +/−) grouped with effector memory T-cell markers (CD3+/CD45RA−/CD27 +) and final effector T-cell markers (CD3+/CD45RO +, CD3+/CD62L−/CD27 +). Whereas no distinct pattern was observed in Lo patients, META− Hi patients presented a significant increase of CD8/effector memory T-cell subpopulations (red squares; C.2) compared with META+ Hi patients that had a majority of CD4/early memory T cells (C.1). These observations suggested that complete memory T-cell differentiation was associated with a higher proportion of cytotoxic T cells within highly infiltrated tumors and preferentially occurred in META− Hi patients compared with META− Hi patients. Conversely, B-cell density was significantly higher in META+ Hi patients compared with META− Hi patients (Fig. 1B). META+ Hi patients had significantly decreased densities of cytotoxic T cells (CD3+/CD8 +) compared with META− Hi patients, whereas no significant differences were observed for helper T cells (CD3+/CD4 +) or regulatory T cells (CD4+/CD25 +, Fig. 1C, left). Finally, CD8+/CD4 + and CD8+/CD3 + cell ratios were significantly higher in META− Hi patients compared with META+ Hi and META+ Lo patients (Fig. 1C, right).

Association between CD8 T cells and complete memory T-cell differentiation. Evaluation of intratumoral immune cooperation was assessed by analyzing the correlations between 62 combinations of cell surface markers of total intratumoral immune cell populations and T-cell subpopulations. For each patient group, pairwise comparisons of the markers were done by measuring Pearson correlation coefficients (r) and related P values (Supplementary Fig. S2). The relationships implied by these correlations were visualized by using unsupervised hierarchical clustering of r values (Fig. 3). The clustered markers were presented in Supplementary Fig. S3. Comparison of META− Hi patients (Fig. 3A, top) with other patients was assessed by the construction of META+ Hi (Fig. 3A, center), META− Lo (Fig. 3B, top), and META+ Lo (Fig. 3B, center) correlation matrices arrayed according to META− Hi matrix unsupervised clustering.

**Figure 3.** Correlation matrices of flow cytometry data. P values and Pearson correlation coefficients (r) were calculated between 62 marker combinations that were specific for T cells (markers in combination with CD3) and for major immune cell populations ("total" prefix), presented in Supplementary Fig. S2. r values were plotted from r = min (green) to r = max (red) in matrix representation, followed by unsupervised hierarchical clustering. Clustered markers were presented in Supplementary Fig. S3. Correlation matrices were independently clustered or arrayed according to the clustering of other correlation matrices (gray area). A, top, META− Hi patients; center and bottom, META+ Hi patients. B, top, META− Lo patients; center and bottom, META+ Lo patients.

phenotype (CD3+/CD45RO +) compared with Lo patients (Fig. 1A). The distribution of global lymphoid cell populations (CD45 +) was consistent with those of T cells (Fig. 1A).

We compared the distributions of the major intratumoral immune cell populations according to the metastatic status of the patients: META− patients, no metastases (stages I–II); META+ patients, metastases in lymph node (stage III) and/or distant organ (stage IV). In Lo patients, no differences were found between META− and META+ patients in the distribution of tumor-associated macrophages, immature dendritic cells, activated dendritic cells, natural killer (NK) cells, NKT cells, or B cells. In contrast, in Hi patients, significantly lower percentages of tumor-associated macrophages and NKT cells were observed in META+ Hi patients compared with META− Hi patients. Conversely, B-cell density was significantly higher in META+ Hi patients compared with META− Hi patients (Fig. 1B). META+ Hi patients had significantly decreased densities of cytotoxic T cells (CD3+/CD8 +) compared with META− Hi patients, whereas no significant differences were observed for helper T cells (CD3+/CD4 +) or regulatory T cells (CD4+/CD25 +, Fig. 1C, left). Finally, CD8+/CD4 + and CD8+/CD3 + cell ratios were significantly higher in META− Hi patients compared with META+ Hi and META+ Lo patients (Fig. 1C, right).
META—Hi patients displayed a correlation matrix with four major clusters (Fig. 3A, top). Cluster 1 contained markers of total T cells, CD4 T cells, B cells, NK cells, and activated dendritic cells, as well as CD4 T-cell subpopulation markers (CD4− or CD4+ in combination with CD25+/CD26+/CD103+/CCR7−, CD45RO−) and early memory T-cell markers (CD28−, CD27−, CD45RA−, CD27−). Significant positive correlations were found for the CD4 marker with CD27 and CD45RA, CD27 markers (P = 0.03 for both correlations). Cluster 2 contained naive CCR7+ T cells. Total CD8 T-cell marker from this cluster positively correlated with total T cells in cluster 1 (P = 0.03). In cluster 4, CD8 T-cell subpopulation markers (CD8− or CD8+ in combination with CD25+/CD26−, CD103−, CCR7+, CD45RA+) were grouped with markers of late-stage memory T-cell differentiation (CD45RA− CD27− CD127 CD62L−). Significant positive correlations were found between CD8 T-cell subpopulation markers and effector memory T-cell markers (CD45RA− CD27−/CD4− CD103+; P = 0.02) and final effector T-cell markers (CD127− CD62L−/CD8+ CCR7−; P = 0.02). As a control, a correlation between CD4 and CD8 T-cell subpopulation markers was negative (CD3+CD4+/CD8−; r = −0.871, P = 0.02). Interestingly, cluster 4 (CD8+/effector memory T cells) and cluster 1 (CD4/early memory T cells) were, globally, strongly inversely correlated. In cluster 3, early differentiated (CD45RA−CD27+) and activated (CD25−, CD26−, CD69) T-cell markers, as well as the global memory T-cell marker (CD45RO+), were grouped with markers of innate immune cell populations: tumor-associated macrophages, immature dendritic cells, and NK cells. Strong positive correlations were found between all markers of early T-cell activation (CD45RA−CD27−/CD25−/CD26−/CD69−; P < 0.05 for all combinations) and the markers of tumor-associated macrophages and immature dendritic cells (P = 0.01). These two functional groups of markers were positively correlated (tumor-associated macrophages/CD25−/CD26−/CD69− and immature dendritic cells/CD25−/CD26−; P < 0.05 for all combinations). Thus, cluster 3 may illustrate the role of the innate immune compartment for T-cell priming, activation, and memory differentiation required for efficient adaptive immune responses.

In contrast, cluster 3 was entirely disrupted in META−Hi patients (Fig. 3A, center). Indeed, the META−Hi clustered correlation matrix (Fig. 3A, bottom) displayed two inversely correlated groups of clusters (cluster 1 versus clusters 2 and 3). As observed in the META−Hi matrix, cluster 1 in META−Hi matrix contained the markers of CD4 and early memory T-cell subpopulations markers (CD4+/CD28−; P = 0.008), as well as markers of total T cells, CD4+ T cells, and B cells. In clusters 2 and 3, CD8 memory T-cell marker (CD8−CD45RO+) was significantly positively correlated with both effector memory (CD45RA−CD27−) and final effector (CD127−CD62L−) T-cell markers (P = 0.04 and P = 0.001, respectively). Markers of NK and NKT cells, dendritic cells (immature and activated), and tumor-associated macrophages were also grouped in clusters 2 and 3 (Fig. 3A, bottom).
Compared with Hi patients, META− Lo (Fig. 3B, top) and META+ Lo (Fig. 3B, center and bottom) patients had very distinct correlation profiles with a majority of noncorrelated markers (yellow). Furthermore, except for the only significant positive correlation between final effector and CD8$^+$ T-cell subpopulations (CD8$^+$/CD127$^-$/CD62L$^-$; $P = 0.03$) in the META+ Lo matrix, all patterns of significant positive correlations observed in Hi patients were lost in Lo patients (Supplementary Fig. S2).

This analytic approach allowed us to visualize the absence of immune coordination in patients with low intratumoral T-cell densities, whereas patients with high intratumoral T-cell densities presented correlation patterns consistent with continual recruitment and proliferation of activated CD8 T cells associated with complete memory T-cell differentiation at the primary tumor site (CD8 T-cell/effector memory T-cell correlations). This profile of efficient immune reaction is in balance (negative correlation) with patterns that could illustrate altered immune responses (CD8/early memory T-cell/B-cell correlations). Because in Hi patients the presence of metastases was associated with (a) a significant decrease of CD8 and late memory T cells and innate cells and (b) a significant increase of B cells (Figs. 1 and 2), our data suggest altered immune reactions in META+ Hi patients.

**Prognostic value of cellular immune coordination.** We next assessed the effect of immune coordination on the proliferation/apoptosis status of primary tumor cells by Ki67/M30 immunohistochemical stainings of cognate tumor samples (Supplementary Fig. S4). No differences were observed among the patient groups, suggesting that the effect on cancer progression of the immune system may be inefficient for the destruction of the primary tumor. Before surgery, immunestrength and coordination are in balance with mechanisms of tumor escape (tumor immunogenicity, inflammation, and angiogenesis) to control metastatic invasion from the primary tumor site. Four major immune coordination profiles within colorectal cancer primary tumors are found: (a) Strong and coordinated adaptive immune responses mediated by cytotoxic (blue cells; GNLY) and Th1 (yellow cells; IRF1) effector memory T cells (green cells) may contribute to the elimination of migrating tumor cells (red cells). (b) Angiogenic (VEGF) and inflammatory processes may facilitate metastatic invasion and (c) noncoordinate immune responses. (d) Weak (Lo) immune reactions (immune ignorance?). After surgical removal of clinically detectable tumors, the parameters defining this balance are significantly associated with the risks of cancer relapse [2-y disease-free survival (DFS)]. It could be postulated that the amount of invading occult tumors and the amount of circulating memory T cells, generated within distinct primary tumor microenvironment, are in balance to control cancer re-emergence in the periphery (after surgery).

We investigated the CD8/CD3 T-cell density ratio in relation to clinical outcome in TMA experiments. Increased densities of T-cell infiltrates exhibiting high proportions of CD8 cytotoxic T cells within the primary tumor of colorectal cancer patients were associated with a significant protection against tumor recurrence (Supplementary Fig. S5).

To better characterize the mechanisms involved in antitumoral activity at the tumor-host interface, we investigated the effect on clinical outcome of mRNA expression levels of 17 mediators involved in immune or tumoral mechanisms. For each gene, patients were defined as high or low according to median gene expression. Disease-free survival rates were then calculated for each patient group. The prognostic value of the expression levels of genes related to T-cell populations (CD3$\zeta$, CD4, CD8z), Th1 adaptive immune responses (TBX21/T-BET, IRF1, IFN$\gamma$), and cytotoxicity (GNLY, GZMB) were assessed. High expression of TBX21/T-BET, IFN$\gamma$, IRF1, and GNLY was associated with significantly improved disease-free survival rates ($P = 0.02$, $P = 0.02$, $P = 0.0003$, and $P = 0.0004$, respectively). Disease-free survival Kaplan-Meier curves according to GNLY and IRF1 gene expression were illustrated (Fig. 4A and B, respectively). Conversely to immune mediators, the expressions of cancer-promoting genes involved in tumor invasion (CEACAM1), metastasis spreading (EBAG9 and CEA), tumor cell antiapoptosis (BIRC5/Survivin), immune suppression (IL-10 and TGF$\beta$; data not shown), and angiogenesis (VEGF; Fig. 4C) had no prognostic values.

Immune coordination at the molecular level was assessed by analyzing combined expression of genes. We found significantly improved disease-free survival rates in patients with high combined gene expressions (Hi/Hi) of marker combinations related to CD4 T cells of Th1 phenotype (CD4/T-BET, CD4/IFN$\gamma$, CD4/IRF1 patients) and cytotoxic CD8 T cells (CD8/GNLY).
compared with patients expressing low levels of these genes (Lo/Lo; \(P = 0.04\), \(P = 0.002\), \(P = 0.002\), and \(P = 0.004\), respectively; Supplementary Fig. S6). High coexpression of IRF1 and GNLY genes (GNLY-Hi/IRF1-Hi) was essential for beneficial outcome with median disease-free survival >140 months, whereas patients expressing low levels of one of these genes or both (GNLY-Hi/IRF1-Lo, GNLY-Lo/IRF1-Hi, GNLY-Lo/IRF1-Lo) had median disease-free survival <15 months (Fig. 4D, top). These observations confirmed the importance of a strong coordination between immune mediators of cytotoxic and Th1 adaptive immune responses for favorable colorectal cancer outcome.

Finally, we assessed the effects of the tumor microenvironment on in situ antitumoral immune responses. We analyzed the prognostic value of the expression of protumoral mediators in combination with GNLY. Among all tested genes, only VEGF showed a profound effect on patient survival when expressed with GNLY. Patients expressing high levels of GNLY and VEGF genes (GNLY-Hi/VEGF-Hi) and patients expressing low levels of GNLY (GNLY-Lo/VEGF-Hi and GNLY-Lo/VEGF-Lo) had similar disease-free survival rates that were significantly lower than the disease-free survival rates of GNLY-Hi/VEGF-Lo patients (\(P < 0.004\) for all comparisons; Fig. 4D, bottom).

Discussion

The mechanisms controlling tumor progression and cancer relapse are not clearly characterized. Here, we investigated the quality of the immune reaction at the primary tumor site during cancer progression (i.e., according to the density of tumor-infiltrating T cells and the metastatic status of the patients). We showed that coordination of the immune response was drastically impaired in patients with low densities of intratumoral T cells compared with patients with high densities of such cells. Phenotypic correlation analyses showed matrices that were highly fragmented with no particular functional relevance in both META−Lo and META+ Lo patients. This suggested the absence of coordinated immune response independently of the metastatic status in Lo patients. Conversely, a high density of tumor-infiltrating T cells (Hi patients) was associated with strong immune coordination. Significant positive correlations between T cells of late memory and cytotoxic phenotypes indicated continual recruitment, activation, and memory differentiation of CD8 T cells at the primary tumor site. In larger cohorts of patients using tissue microarrays, we also showed that high densities of T cells associated with a high CD8/CD3 density ratio correlated with a very good prognosis. In contrast, low adaptive immune coordination was associated with very poor prognosis. Consistently, patients presenting high and coordinated intratumoral expression of the global Th1 immune response marker IRF1 and the cytotoxicity-specific marker GNLY had significantly better survival rates compared with patients expressing heterogeneous or low levels of these genes.

Yet, in Hi patients, the presence of metastases was associated with (a) a significant decrease of innate immune cells, (b) a significant decrease of CD8 T cells and fully differentiated memory T cells, (c) loss of the phenocluster of markers of innate cells and early activated T cells (illustrating innate/adaptive immune compartment interactions), and (d) a significant increase of B cells (suggesting immune deviation mechanisms; refs. 34, 35).

According to the coexpression of IRF1 and GNLY, the frequencies of strong immune coordination parameters were reduced in META+ patients (data not shown). META+ patients represented only 48% of GNLY-Hi/IRF1-Hi patients and 65% of GNLY-Lo/IRF1-Lo patients. Overall, these observations represent clues of altered immune responses when metastases are present. However, a significant number of META+ patients displayed a high degree of immune coordination preventing relapse events. This raises two hypotheses: Does the alteration of the immune reaction at the primary tumor site facilitate metastatic invasion? Is the immune system overwhelmed and affected by the presence of metastases? Interestingly, some patients without lymph node and/or distant organs (META− patients) have an absence of immune coordination and low densities of T cells. Thus, local immune escape mechanisms may exist in the primary tumor even before metastatic spread. Because proliferation and apoptosis rates of tumor cells were not significantly different between the patient groups, the outgrowth of the primary tumor may overcome the destruction by the immune system. Whichever hypothesis on the mechanisms of long-term relapse prevention after surgery may be related to the quality of the immune reaction at the primary tumor site in patients with advanced colorectal cancer.

Among the distinct factors potentially involved in immune escape at the primary site, several mechanisms or cell types may participate, such as immature dendritic cells, regulatory T cells, Th1/Th2 immune response switch, immunosuppression, local metastatic invasion, inflammation, and angiogenesis. In patients with metastases or low intratumoral T-cell densities, there was no increased expression of markers of regulatory T cells, tumor-associated macrophages, and immature dendritic cells by flow cytometry (CD4+CD25\(^{hi}\), CD45\(^{+}\)CD14\(^{+}\), and CD45\(^{+}\)CD1a\(^{+}\)CD14\(^{–}\)CD83\(^{+}\), respectively) and by tissue microarray (Foxp3\(^{+}\), CD68\(^{+}\), and CD1a\(^{+}\), respectively) experiments (data shown). This may suggest the existence of immune ignorance or reduced tumor immunogenicity mechanisms for differential immune cell recruitment among patients. Interestingly, we found that only the proangiogenic factor VEGF had a deleterious effect on relapse prevention mechanisms associated with strong antitumoral immune reaction. VEGF expression levels had no prognostic value per se, in agreement with immunohistochemical-based studies (36, 37). Our data indicate that cytotoxic Th1 adaptive immune responses may be necessary, but not sufficient, to prevent tumor recurrence. At the primary tumor site, inflammatory cytokines (such as IL-1A, IL-6, IL-8, oncostatin M, and tumor necrosis factor \(\alpha\) can enhance tumorigenic processes by up-regulating important mediators of angiogenesis, such as VEGF (38). In this context, the effect on colorectal cancer outcome of the balance between GNLY/IRF1 and VEGF expressions may reflect beneficial cytotoxic Th1 adaptive immune responses versus deleterious inflammatory reaction (39–41). However, other roles of angiogenesis may affect cytotoxic Th1 adaptive immune responses. In the primary tumor site, the role of angiogenesis in promoting nutrient supply (21) may not explain the obliteration of the beneficial role of strong immune responses. In contrast, the induction of vascular exit paths for migrating tumor cells (42) could result in increased metastatic dissemination favoring relapse occurrence. In this case, even strong in situ cytotoxic Th1 adaptive immune responses may not be sufficient to counteract metastatic invasion. Thus, in the periphery, great number of occult tumor cells may overwhelm immunosurveillance mechanisms during the equilibrium phase. Furthermore, if the migrating tumor cells inherit the strong angiogenic properties of their resident counterparts, occult tumor outgrowth may be further enhanced (43). This idea that angiogenesis
Based on these data, it could be proposed that the immune system cells within primary tumors and that both criteria were powerful evidence of early metastatic invasiveness within lymphovascular outgrowth. We previously showed that the absence of microscopic condition of "equilibrium." In these experiments, loss of either controlled by the adaptive immune system of the host in a surveillance (45). Recently, Koebel and colleagues (46) showed that T cells reacting at the primary tumor site might also, after surgical two antitumoral functions of the immune system could be tightly reducing the number of disseminated occult tumors, and (b) in the periphery by controlling occult tumor evolution from dormancy state to cancer re-emergence (equilibrium phase). Moreover, these two antitumoral functions of the immune system could be tightly associated. As suggested in mice (47), cytotoxic effector memory T cells reacting at the primary tumor site might also, after surgical removal of tumors, be in charge of long-term antitumoral immunity in colorectal cancer.

In conclusion, our study argues for the involvement of immune coordination and late memory and cytotoxic T-cell populations in antitumoral activity against human colorectal cancer (Fig. 5). First, due to their enhanced cytotoxic capabilities, effector memory T cells may be involved in the control of metastatic invasion at the primary tumor site. Second, due to their memory properties, effector memory T cells may provide long-term protection against outgrowth of disseminated occult tumor cells potentially involved in relapse events. Depending on the strength and coordination of antitumoral immune responses elicited in primary tumor microenvironments (level of immunogenicity and angiogenesis), populations of T cells with distinct quantity (number of clones) and quality (memory differentiation state) could be generated. Subsequently, distinct potentials for long-lived antitumoral immunity may be maintained after surgical resection of primary and secondary tumors. Future comparative studies of tumors according to immune parameters and angiogenesis may reveal biological mechanisms involved in emergence and cancer progression. More adapted treatment and therapeutic strategies may ultimately be proposed to cure colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

24. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven angiogenesis may reveal biological mechanisms involved in emergence and cancer progression. More adapted treatment and therapeutic strategies may ultimately be proposed to cure colorectal cancer.


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