Coordination of Intratumoral Immune Reaction and Human Colorectal Cancer Recurrence

Matthieu Camus,[1,2,3] Marie Tosolini,[1,2,3] Bernhard Mlecnik,[1,2,3] Franck Pagès,[1,2,3,4] Amos Kirilovsky,[1,2,3] Anne Berger,[5] Anne Costes,[1,2,3] Gabriela Bindea,[1,2,3,7] Pornpimol Charoentong,[7] Patrick Bruneval,[6] Zlatko Trajanoski,[7] Wolf-Herman Fridman,[1,2,3,4] and Jérôme Galon[1,2,3]

[1]Integrative Cancer Immunology INSERM AVENIR Team 15, INSERM U872; [2]Cordeliers Research Centre, Université Pierre et Marie Curie Paris 6; [3]Université Paris-Descartes; Departments of [4]Immunology, [5]General and Digestive Surgery, and [6]Pathology, Georges Pompidou European Hospital, Paris, France; and [7]Institute for Genomics and Bioinformatics, Graz University of Technology, Graz, Austria

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 describe *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 (*GNLY*) 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 *GNLY*. 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 (1). 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 antitumoral responses (8). Consistent with findings in melanoma (9) and ovarian cancer (10, 11), tumor-infiltrating T cells were associated with improved clinical outcome 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

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

Requests for reprints: Jérôme Galon, AVENIR Team 15, Cordeliers Research Centre, INSERM U872, 15 rue de L'Ecole de Médecine, 75006 Paris, France. Phone: 33-1-5310-0404; Fax: 33-1-4051-0420; E-mail: jerome.galon@crc.jussieu.fr.

doi:10.1158/0008-5472.CAN-08-2654

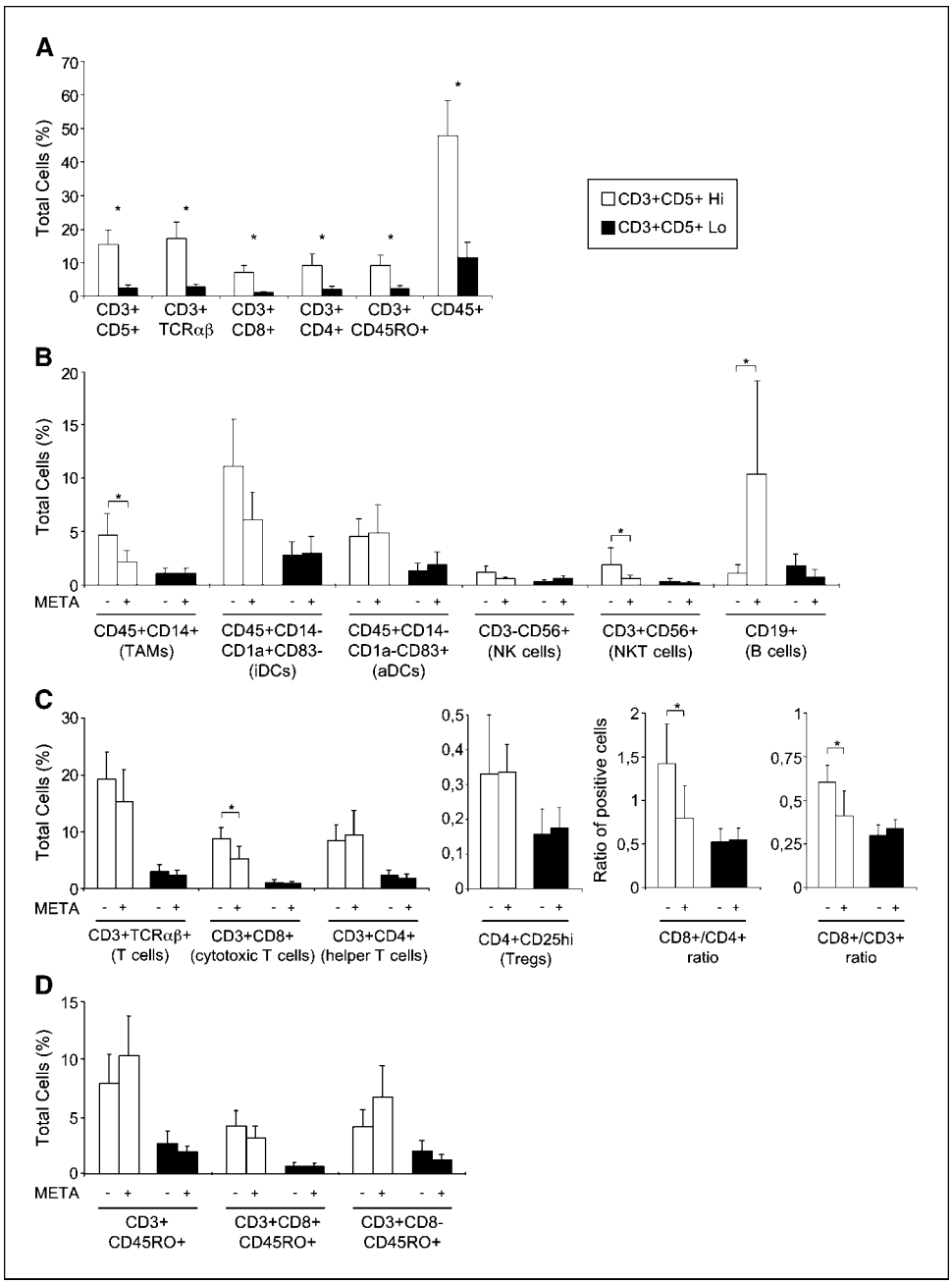

Figure 1. Immune cell populations within primary colorectal tumors. Patients (n = 39) were classified according to the mean percentage of $CD3^+CD5^+$ cells among total cells within tumors (*white columns,* $CD3^+CD5^+$Hi; *black columns,* $CD3^+CD5^+$Lo) and the metastatic status (META− Hi, n = 6; META− Lo, n = 10; META+ Hi, n = 7; META+ Lo, n = 16). Cell populations were represented as the mean percentage of positive cells; *bars,* SE. *, $P < 0.05$, Mann-Whitney test. *A,* T cells ($CD3^+CD5^+$, $CD3^+TCR\alpha\beta^+$), cytotoxic T cells ($CD3^+CD8^+$), helper T cells ($CD3^+CD4^+$), memory T cells ($CD3^+CD45RO^+$), and lymphoid cells ($CD45^+$). *B,* tumor-associated macrophages (*TAMs*; $CD45^+CD14^+$), immature dendritic cells (*iDCs*; $CD45^+CD1a^+CD14^-CD83^-$), activated dendritic cells (*aDCs*; $CD45^+CD1a^-CD14^-CD83^+$), NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+CD56^+$), and B cells ($CD19^+$). *C, left,* T cells ($CD3^+TCR\alpha\beta^+$), cytotoxic T cells ($CD3^+CD8^+$), helper T cells ($CD3^+CD4^+$), and regulatory T cells (*Tregs*: $CD4^+CD25^{hi}$). *Right,* ratios of $CD8^+/CD4^+$ and $CD8^+/CD3^+$ cell subpopulations. *D,* memory T cells ($CD3^+CD45RO^+$), cytotoxic memory T cells ($CD3^+CD8^+CD45RO^+$), and helper memory T cells ($CD3^+CD4^+CD45RO^+$).

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): *CD3ζ, CD4, CD8α, TBX21, IRF1, IFNγ, GNLY, 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. Isotype-matched mouse monoclonal antibodies were used as negative controls.

Statistical analysis. Kaplan-Meier curves were used to assess the influence of immune and tumoral parameters on disease-free survival. The significance of these parameters was assessed by univariate analysis with the use of the log-rank test. To identify markers with significant different levels of expression among tissues, Wilcoxon-Mann-Whitney and t tests (ANOVA) were used. $P < 0.05$ was considered to indicate statistical significance. All analyses were done with the use of statistical software programs R and StatView.

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 $CD3^+TCR\alpha\beta^+$ T cells were represented (Fig. 1*A*). In Hi patients, there were significant higher densities of T cells of both cytotoxic ($CD3^+CD8^+$) and helper ($CD3^+CD4^+$) phenotypes and of memory

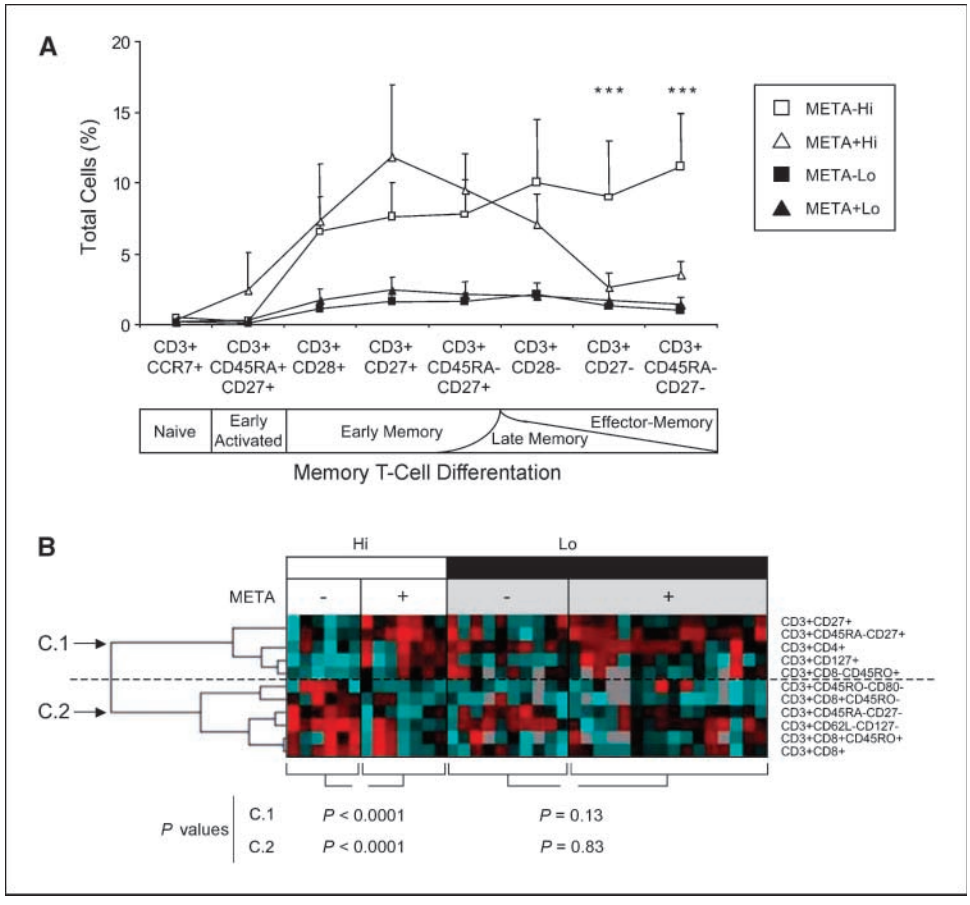

Figure 2. T-cell populations within primary colorectal tumors. *A,* T-cell memory differentiation markers (*white squares,* META− Hi; *white triangles,* META+ Hi; *black squares,* META− Lo; *black triangles,* META+ Lo). Cell populations were represented as the mean percentage of positive cells; *bars,* 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.

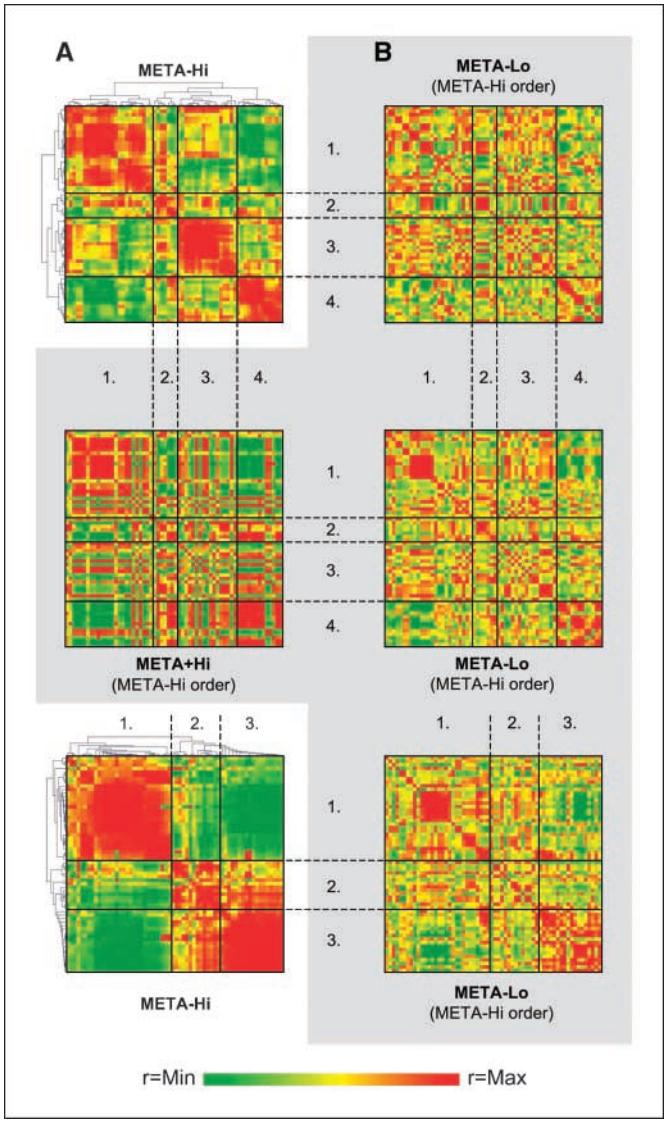

Figure 3. Correlation matrices of flow cytometry data. *P* values and Pearson correlation coefficients (*r*) were calculated between 62 marker combinations that were specific for T cells (markers in combination with CD3) and for major immune cell populations ("total" prefix), presented in Supplementary 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. 1*A*). The distribution of global lymphoid cell populations ($CD45^+$) was consistent with those of T cells (Fig. 1*A*).

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. 1*B*). 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 ($CD4^+CD25^{hi}$; Fig. 1*C, 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. 1*C, right*).

Memory T-cell differentiation. No differences in the distribution of $CD45RO^+$ memory T-cell subpopulations were observed among the patient groups (Fig. 1*D*). 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. 2*A*). Very few naive ($CCR7^+$) 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. 2*B*). 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 ($CD3^+CD45RO^-$, $CD3^+CD62L^-CD127^-$). 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.

Association between CD8 T cells and complete memory T-cell differentiation. Evaluation of intratumoral immune coordination 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. 3*A, top*) with other patients was assessed by the construction of META+ Hi (Fig. 3*A, center*), META− Lo (Fig. 3*B, top*), and META+ Lo (Fig. 3*B, center*) correlation matrices arrayed according to META− Hi matrix unsupervised clustering.

META− Hi patients displayed a correlation matrix with four major clusters (Fig. 3*A, 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 $CD8^-$ 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 $CD4^-$ in combination with $CD25^{+/-}$, $CD26^-$, $CD103^+$, $CCR7^-$, $CD45RO^+$) 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, correlation between CD4 and CD8 T-cell subpopulation markers was negative ($CD3^+CD4^+/CD3^+CD8^+$; $r = -0.871$, $P = 0.02$). 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 NKT 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. 3*A, center*). Indeed, the META+ Hi clustered correlation matrix (Fig. 3*A, 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. 3*A, bottom*).

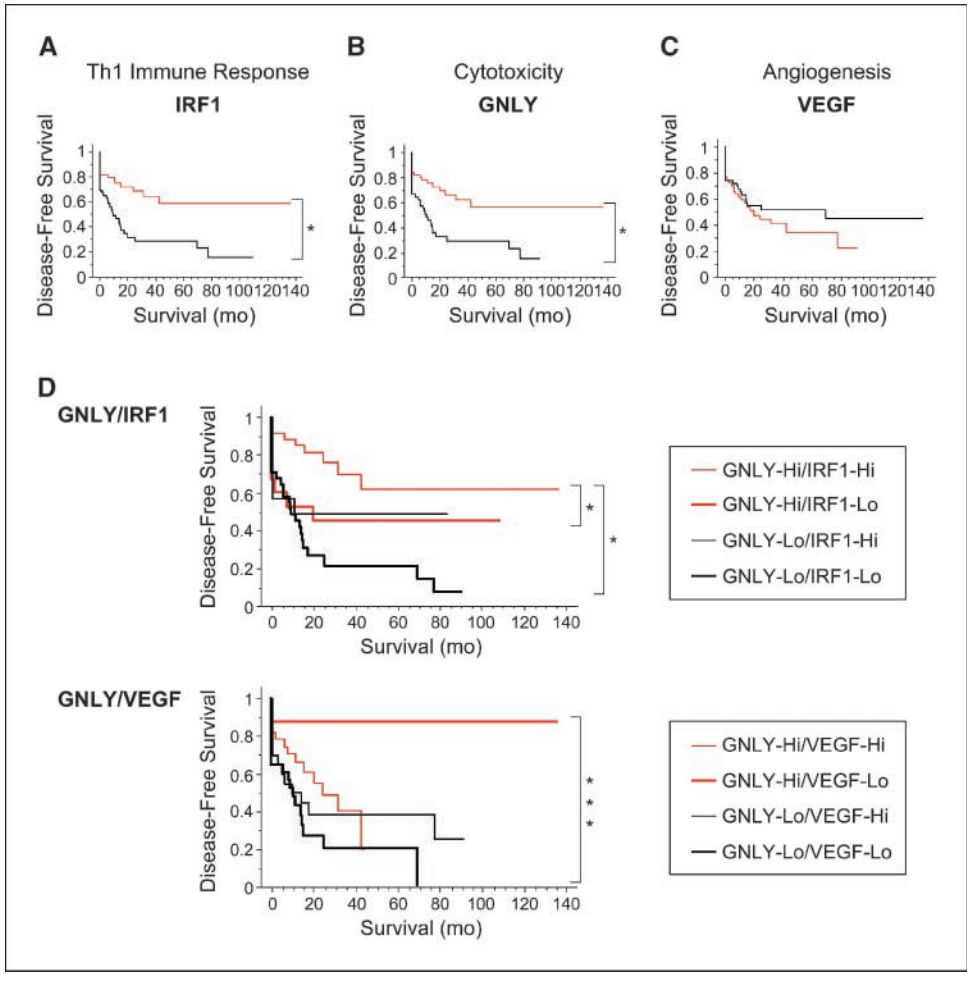

Figure 4. Disease-free survival of colorectal cancer patients according to expression of genes. *A* to *C,* disease-free survival of 103 patients according to high (*red lines*) or low (*black lines*) mRNA expression levels of IRF1 (*A*), GNLY (*B*), and *VEGF* (*C*) genes. *D,* disease-free survival of patients according to the expression levels of the *GNLY* gene in combination with *IRF1* (*top*) and *VEGF* (*bottom*) genes (*thin red lines,* GNLY-Hi/IRF1-Hi, GNLY-Hi/VEGF-Hi; *bold red lines,* GNLY-Hi/IRF1-Lo, GNLY-Hi/VEGF-Lo; *thin black lines,* GNLY-Lo/IRF1-Hi, GNLY-Lo/VEGF-Hi; *bold black lines,* GNLY-Lo/IRF1-Lo, GNLY-Lo/VEGF-Lo). The cutoff value for the expression of each gene was defined at the median of the cohort. *, $P < 0.05$, log-rank test.

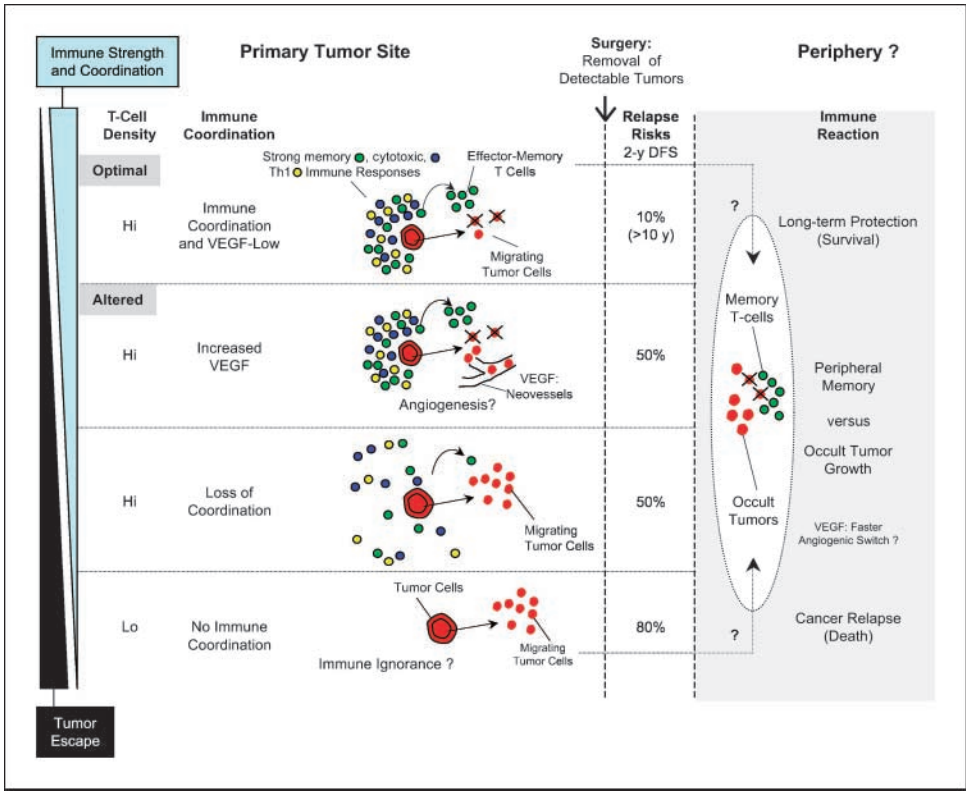

Figure 5. Proposed model: control of colorectal cancer outcome by the immune system. Before surgery, immune strength and coordination are in balance with mechanisms of tumor escape (tumor immunogenicity, inflammation, and angiogenesis) to control metastatic invasion from the primary tumor site. Four 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).

Compared with Hi patients, META− Lo (Fig. 3*B, top*) and META+ Lo (Fig. 3*B, 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 (CD4/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. To validate the effect of the coordination of *in situ* immune response on colorectal cancer prognosis, we evaluated the density of intratumoral immune T cells in a large cohort of 435 patients. 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 (*CD3ζ, CD4, CD8α*), Th1 adaptive immune responses (*TBX21/T-BET, IRF1, IFNγ*), and cytotoxicity (*GNLY, GZMB*) were assessed. High expression of *TBX21/T-BET, IFNγ, 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. 4*A* 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 *TGFβ*; data not shown), and angiogenesis (*VEGF*; Fig. 4*C*) 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/IFNγ, 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. 4*D, 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. 4*D, 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 even 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 α) 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

and adaptive immune responses are strongly linked in cancer recurrence should be taken into account when considering therapeutic options.

We were able to describe four major immune coordination profiles within colorectal cancer primary tumors depending on the balance between tumor escape and immune coordination: (*a*) strong and coordinate cytotoxic Th1 immune responses (*GNLY/IRF1*) without or (*b*) with tumor angiogenesis (*VEGF*), (*c*) noncoordinate immune responses, and (*d*) weak (Lo) immune reactions (immune ignorance?). These distinct immune profiles are associated with significant distinct cancer outcome (relapse risks), as summarized in Fig. 5.

It is suspected that metastatic invasion can lead to the dissemination of tumor cells that can remain in an asymptomatic and nondetectable state of dormancy (i.e., not expanding in mass) for long periods of time before cancer re-emergence (44). Control of cancer dormancy involves various mechanisms such as cellular dormancy (G_0-G_1 arrest), angiogenic dormancy, and immunosurveillance (45). Recently, Koebel and colleagues (46) showed that stable lesions of transformed immunogenic cells in mice were controlled by the adaptive immune system of the host in a condition of "equilibrium." In these experiments, loss of either immunocompetence or immunogenicity could lead to tumor outgrowth. We previously showed that the absence of microscopic evidence of early metastatic invasiveness within lymphovascular vessels was associated with high densities of effector memory T cells within primary tumors and that both criteria were powerful indicators of improved prognosis in human colorectal cancer (17). Based on these data, it could be proposed that the immune system exerts its protective role against cancer relapse (*a*) at the primary tumor site by eliminating migrating tumor cells, subsequently 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.

Acknowledgments

Received 7/10/2008; revised 12/11/2008; accepted 1/2/2009; published OnlineFirst 3/3/09.

Grant support: Association pour la Recherche sur le Cancer (ARC), the National Cancer Institute (INCa), the Canceropole Ile de France, Ville de Paris, Immucan, INSERM, the Austrian Federal Ministry of Science and Research (GEN-AU project Bioinformatics Integration Network) and Academic Cooperation and Mobility Unit (fellowship to P Charoentong), and the European Commission (7FP, Geninca Consortium, grant no. 202230).

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.

References

1. Finn OJ. Cancer immunology. N Engl J Med 2008;358:2704–15.
2. Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced *in vivo* growth and resistance to rejection of tumor cells expressing dominant negative IFNγ receptors. Immunity 1994;1:447–56.
3. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A 1998;95:7556–61.
4. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med 2000;192:755–60.
5. van den Broek ME, Kagi D, Ossendorp F, et al. Decreased tumor surveillance in perforin-deficient mice. J Exp Med 1996;184:1781–90.
6. Shankaran V, Ikeda H, Bruce AT, et al. IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;410:1107–11.
7. Zhou G, Lu Z, McCadden JD, Levitsky HI, Marson AL. Reciprocal changes in tumor antigenicity and antigen-specific T cell function during tumor progression. J Exp Med 2004;200:1581–92.
8. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329–60.
9. Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 1996;77:1303–10.
10. Sato E, Olson SH, Ahn J, et al. Intraepithelial $CD8^+$ tumor-infiltrating lymphocytes and a high $CD8^+$/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005;102:18538–43.
11. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003;348:203–13.
12. Baier PK, Wimmenauer S, Hirsch T, et al. Analysis of the T cell receptor variability of tumor-infiltrating lymphocytes in colorectal carcinomas. Tumour Biol 1998;19:205–12.
13. Dalerba P, Maccalli C, Casati C, Castelli C, Parmiani G. Immunology and immunotherapy of colorectal cancer. Crit Rev Oncol Hematol 2003;46:33–57.
14. Diederichsen AC, Hjelmborg JB, Christensen PB, Zeuthen J, Fenger C. Prognostic value of the $CD4^+$/$CD8^+$ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. Cancer Immunol Immunother 2003;52:423–8.
15. Naito Y, Saito K, Shiiba K, et al. $CD8^+$ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 1998;58:3491–4.
16. Prall F, Duhrkop T, Weirich V, et al. Prognostic role of $CD8^+$ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. Hum Pathol 2004;35:808–16.
17. Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005;353:2654–66.
18. 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;313:1960–4.
19. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. Cancer Res 2007;67:1883–6.
20. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
21. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401–10.
22. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol 2001;2:293–9.
23. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol 2006;6:836–48.
24. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res 2006;66:5527–36.
25. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5:263–74.

26. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 2002;3:999–1005.

27. Mocellin S, Wang E, Marincola FM. Cytokines and immune response in the tumor microenvironment. J Immunother 2001;24:392–407.

28. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. Nat Immunol 2002;3:611–8.

29. Okazaki T, Honjo T. The PD-1-PD-L pathway in immunological tolerance. Trends Immunol 2006;27:195–201.

30. Sobin LH, Greene FL. Global TNM advisory group. Cancer 2004;100:1106.

31. Boulton SJ, Gartner A, Reboul J, et al. Combined functional genomic maps of the *C. elegans* DNA damage response. Science 2002;295:127–31.

32. Galon J, Franchimont D, Hiroi N, et al. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J 2002;16:61–71.

33. Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics 2002;18:207–8.

34. Shah S, Divekar AA, Hilchey SP, et al. Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells. Int J Cancer 2005;117:574–86.

35. Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. Curr Opin Immunol 2007;19:209–16.

36. Doger FK, Meteoglu I, Tuncyurek P, Okyay P, Cevikel H. Does the EGFR and VEGF expression predict the prognosis in colon cancer? Eur Surg Res 2006;38:540–4.

37. Zheng S, Han MY, Xiao ZX, Peng JP, Dong Q. Clinical significance of vascular endothelial growth factor expression and neovascularization in colorectal carcinoma. World J Gastroenterol 2003;9:1227–30.

38. Angelo LS, Kurzrock R. Vascular endothelial growth factor and its relationship to inflammatory mediators. Clin Cancer Res 2007;13:2825–30.

39. Balkwill F, Coussens LM. Cancer: an inflammatory link. Nature 2004;431:405–6.

40. Moore RJ, Owens DM, Stamp G, et al. Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. Nat Med 1999;5:828–31.

41. Voronov E, Shouval DS, Krelin Y, et al. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A 2003;100:2645–50.

42. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 2002;29:15–8.

43. Naumov GN, Akslen LA, Folkman J. Role of angiogenesis in human tumor dormancy: animal models of the angiogenic switch. Cell Cycle 2006;5:1779–87.

44. Vessella RL, Pantel K, Mohla S. Tumor cell dormancy: an NCI workshop report. Cancer Biol Ther 2007;6:1496–504.

45. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer 2007;7:834–46.

46. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature 2007;450:903–7.

47. Xiang R, Lode HN, Gillies SD, Reisfeld RA. T cell memory against colon carcinoma is long-lived in the absence of antigen. J Immunol 1999;163:3676–83.