

ORIGINAL ARTICLE

Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer

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ABSTRACT

BACKGROUND

The role of tumor-infiltrating immune cells in the early metastatic invasion of colorectal cancer is unknown.

METHODS

We studied pathological signs of early metastatic invasion (venous emboli and lymphatic and perineural invasion) in 959 specimens of resected colorectal cancer. The local immune response within the tumor was studied by flow cytometry (39 tumors), low-density-array real-time polymerase-chain-reaction assay (75 tumors), and tissue microarrays (415 tumors).

RESULTS

Univariate analysis showed significant differences in disease-free and overall survival according to the presence or absence of histologic signs of early metastatic invasion ($P < 0.001$). Multivariate Cox analysis showed that an early conventional pathological tumor–node–metastasis stage ($P < 0.001$) and the absence of early metastatic invasion ($P = 0.04$) were independently associated with increased survival. As compared with tumors with signs of early metastatic invasion, tumors without such signs had increased infiltrates of immune cells and increased levels of messenger RNA (mRNA) for products of type 1 helper effector T cells (CD8, T-BET [T-box transcription factor 21], interferon regulatory factor 1, interferon- γ , granulysin, and granzyme B) but not increased levels of inflammatory mediators or immunosuppressive molecules. The two types of tumors had significant differences in the levels of expression of 65 combinations of T-cell markers, and hierarchical clustering showed that markers of T-cell migration, activation, and differentiation were increased in tumors without signs of early metastatic invasion. The latter type of tumors also had increased numbers of CD8+ T cells, ranging from early memory (CD45RO+CCR7–CD28+CD27+) to effector memory (CD45RO+CCR7–CD28–CD27–) T cells. The presence of high levels of infiltrating memory CD45RO+ cells, evaluated immunohistochemically, correlated with the absence of signs of early metastatic invasion, a less advanced pathological stage, and increased survival.

CONCLUSIONS

Signs of an immune response within colorectal cancers are associated with the absence of pathological evidence of early metastatic invasion and with prolonged survival.

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ACCUMULATING EVIDENCE SUGGESTS that tumor progression is governed not only by genetic changes intrinsic to cancer cells¹ but also by epigenetic and environmental factors. Inflammation is also a factor, and there is experimental evidence to support the idea that the innate immune system can promote tumor development through inflammation-dependent mechanisms.^{2,3} Recently, increased production of inflammatory mediators, including tumor necrosis factor α (TNF- α), by stromal cells was found during cancer progression in mouse models of colorectal cancer.⁴⁻⁶ These results are consistent with the association between an increased number of inflammatory cells in tumors and tumor progression.⁷⁻¹⁰

By contrast, mice that are deficient in one or more components of the adaptive immune system have an increased susceptibility to spontaneous tumors.^{11,12} Immune surveillance can not only eliminate tumors but also select variant tumor cells that resist the immune-surveillance mechanism, a process called “immunoediting.”^{11,12} In humans, the presence of lymphocytes within the tumor can be a favorable prognostic sign.¹¹⁻¹⁵ The elimination phase of cancer immunoediting is thought to be a continuous process, and local control of metastatic invasion by the immune system may be critical for survival.

Little is known about the role of the immune system in the early steps of the metastatic processes, which include vascular emboli, lymphatic invasion, and perineural invasion (collectively referred to as “VELIPI”). We aimed to determine whether VELIPI-positive colorectal cancers are associated with inflammatory or immunosuppressive mediators, or both, and whether the absence of VELIPI is associated with an adaptive immune response.

METHODS

PATIENTS AND DATABASE

The records of 959 patients with colorectal cancer who underwent a primary resection of the tumor at the Laënnec–Georges Pompidou European Hospital between 1986 and 2004 were reviewed (Table 1). The observation time in this unselected cohort was the interval between diagnosis and last contact (death or last follow-up). Data were censored at the last follow-up for patients who had not relapsed and for those who had died. The mean duration of follow-up was 44.5 months. Six patients lost to follow-up were excluded from the analysis. Histo-

pathological and clinical findings were scored according to the tumor–node–metastasis (TNM) staging system of the Union Internationale contre le Cancer¹⁶ (Table 1 and the Supplementary Appendix, available with the full text of this article at www.nejm.org). Early metastatic invasion was defined by the presence of components of VELIPI, alone or in combination. A VELIPI-positive tumor had at least one of these pathological findings, whereas a VELIPI-negative tumor had none of the three findings. The TNM stage and VELIPI status of the tumors were determined from the histopathological reports obtained at the time of resection. A secure, Web-based database, Tumoral MicroEnvironment Database (TME.db, available on request), with a three-tier architecture was assembled with the use of Java-2 Enterprise edition software to integrate clinical data sets and the results of high-throughput techniques.

HISTOPATHOLOGICAL ANALYSIS

For each patient, all sections of tumor that had been stained with hematoxylin and eosin were reassessed in a blinded fashion by two pathologists or two investigators trained to identify the pathological features of colonic cancer. Each specimen was examined for the following: lymphoid infiltrates within the tumor and a lymphoid reaction at the invasive margin (10 to 20 fields analyzed per patient). The densities of these immune infiltrates were scored independently by the investigators, as weak (score of 1), moderate (score of 2), or strong (score of 3) (details are provided in the Supplementary Appendix).

REAL-TIME POLYMERASE-CHAIN-REACTION ASSAY

For the polymerase chain reaction (PCR), total RNA was extracted from 100 randomly selected frozen tumor specimens from the cohort of 959 patients; 75 samples of sufficient quality and quantity were analyzed for gene expression with the use of quantitative real-time TaqMan PCR with low-density arrays and a robotic PCR system (model 7900, Applied Biosystems) (details are provided in the Supplementary Appendix).

LARGE-SCALE FLOW-CYTOMETRIC ANALYSIS

Cells were extracted by mechanical dispersion from 39 fresh tumor samples. All cells (including tumor cells) were analyzed by flow cytometry. Cells from normal mucosa from a site that was distant from the fresh tumor were also analyzed. Cells were in-

Table 1. Disease-free and Overall Survival among 959 Patients with Colorectal Cancer.

Characteristic	No. of Patients	Disease-free Survival			Overall Survival		
		5 yr %	Median mo	P Value*	5 yr %	Median mo	P Value*
Tumor (T) stage†				<0.001			<0.001
pTis	39	48.7	55.7		48.7	55.7	
pT1	54	42.6	52.2		44.4	53.8	
pT2	156	40.4	43.6		44.2	49.1	
pT3	502	23.7	16.5		26.7	25.8	
pT4	208	16.8	1.6		17.8	16.8	
Nodal (N) status				<0.001			<0.001
Negative	568	35.4	34.6		38.6	43.1	
Positive	384	15.1	4.3		16.7	16.9	
Nx‡	7						
Distant metastases (M)				<0.001			<0.001
None detected	747	34.5	32.6		37.6	41.1	
Present	212	0.5	0.1		0.9	12.3	
Dukes' stage				<0.001			<0.001
A	84	47.0	55.6		47.0	55.6	
B	438	37.2	39.2		41.1	46.8	
C	228	24.7	19.5		27.3	28.1	
D	209	0.5	0.1		1.0	12.1	
Sex				0.38			0.47
Male	494	25.9	16.4		28.5	29.4	
Female	465	28.2	19.3		30.5	27.3	
Location				0.20			0.14
Right side of colon	242	23.9	14.5		24.7	19.7	
Transverse colon	50	7.8	9.2		9.8	22.2	
Left side of colon	83	28.6	15.3		31.0	27.2	
Sigmoid colon	297	26.8	14.7		29.5	29.5	
Rectum	287	32.4	32.1		36.5	40.4	
Differentiation				0.26			0.09
Well	737	30.7	21.7		33.6	33.2	
Moderate	187	14.4	9.3		15.5	17.8	
Poor	35	17.1	2.6		17.1	11.6	
Mucinous (colloid) adenocarcinoma				0.087			0.27
No	766	28.2	19.5		30.9	30.9	
Yes	193	22.3	14.9		23.8	21.8	

cubated for 30 minutes at 4°C with antibodies against immune-cell markers (details are provided in the Supplementary Appendix). Analyses were performed with a four-color fluorescence-activated cell sorter (FACScalibur, Becton Dickinson) and CellQuest software (Becton Dickinson). Immune

subpopulations were measured as a percentage of the total number of all cells and a percentage of the total number of CD3+ cells. Average-linkage hierarchical clustering was applied, and the results were displayed with the use of the Genesis program^{17,18} (software available at www.genome.tugraz.at).

Table 1. (Continued.)							
Characteristic	No. of Patients	Disease-free Survival			Overall Survival		
		5 yr %	Median mo	P Value*	5 yr %	Median mo	P Value*
No. of lymph nodes analyzed				0.11			0.69
<8	426	34.0	31.0		37.1	40.0	
≥8	533	21.4	12.9		23.5	23.2	
Vascular emboli				<0.001			<0.001
No	797	31.0	23.6		33.9	34.1	
Yes	162	7.4	1.4		8.0	13.9	
Lymphatic invasion				<0.001			<0.001
No	803	29.5	21.6		32.1	32.0	
Yes	156	14.1	0.5		16.0	16.1	
Perineural invasion				<0.001			<0.001
No	860	29.3	20.7		32.0	32.0	
Yes	99	7.1	0.1		8.1	16.2	
VELIPI [§]				<0.001			<0.001
No	702	32.4	26.9		35.5	35.5	
Yes	257	12.1	3.3		13.2	16.8	
Vascular emboli or lymphatic invasion				<0.001			<0.001
No	716	31.6	24.4		35.2	35.0	
Yes	243	13.6	3.7		12.8	16.3	
Vascular emboli and lymphatic invasion				<0.001			<0.001
No	884	28.3	19.7		31.2	31.0	
Yes	75	12.0	0.2		9.3	11.9	
Vascular emboli, lymphatic invasion, and perineural invasion				<0.001			<0.001
No	911	28.0	19.5		30.7	30.5	
Yes	48	8.3	0.1		6.3	9.5	

* The log-rank test was used.

† The stage was determined by pathological (p) examination. Tis denotes carcinoma in situ, T1 tumor invading submucosa, T2 tumor invading muscularis propria, T3 tumor penetrating muscularis propria and invading subserosa, and T4 tumor invading other organs or structures or perforating visceral peritoneum.

‡ It was not possible to determine the nodal status of seven patients.

§ VELIPI is a surrogate for early metastatic invasion and denotes the presence of vascular emboli, lymphatic invasion, and perineural invasion, alone or in combination.

CONSTRUCTION OF TISSUE MICROARRAYS

Using a tissue-microarray instrument (Beecher Instruments, Alphelys), we removed two representative areas of the tumor (center and invasive margin, 0.6 mm and 1 mm in diameter, respectively) from paraffin-embedded tissue blocks that had been prepared at the time of resection. Tissue microarrays containing the tissue cores were then cut into 5- μ m sections for staining with Harris's hematoxylin and immunohistochemical staining. Of the colonic car-

cinomas that were resected between 1990 and 2003, 50 percent (415) were randomly selected for construction of tissue microarrays. On the basis of the TNM stage and VELIPI pathological findings, the 415 patients with these tumors were representative of the entire cohort.

IMMUNOHISTOCHEMISTRY

After antigen retrieval and quenching of endogenous peroxidase activity, sections were incubated

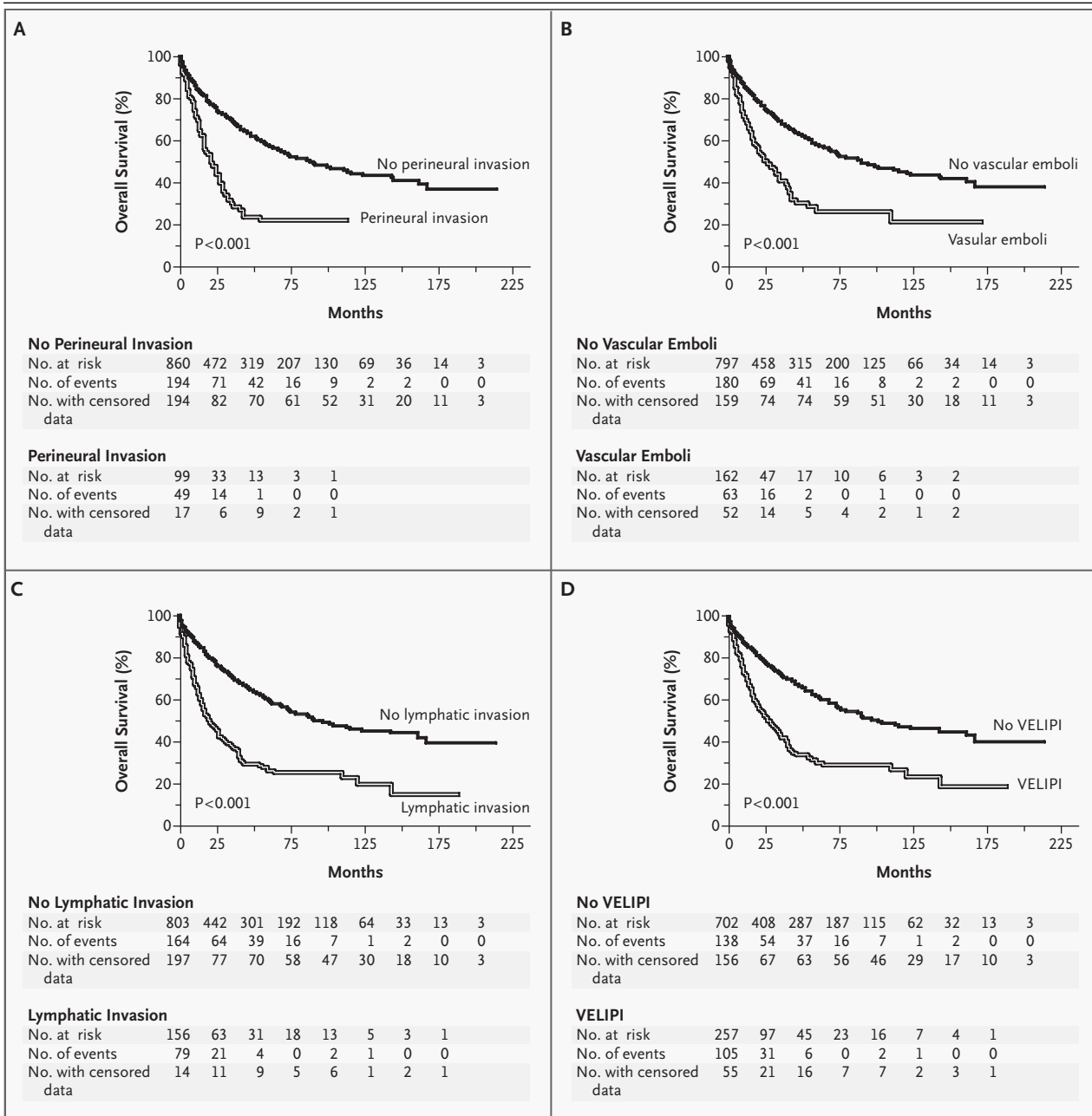


Figure 1. Kaplan–Meier Curves for Overall Survival According to the Presence or Absence of Perineural Invasion (Panel A), Vascular Emboli (Panel B), Lymphatic Invasion (Panel C), or Any Sign of Early Metastatic Invasion (Panel D) among 959 Patients with Colorectal Cancer.

Early metastatic invasion was defined by the presence of vascular emboli, lymphatic invasion, and perineural invasion (collectively referred to as VELIPI), alone or in combination. P<0.001 for all comparisons by the log-rank test.

for 60 minutes at room temperature with monoclonal antibodies against CD45RO and CD3 (Neomarkers). The Envision+ system (enzyme-conjugated polymer backbone coupled to secondary antibodies) and 3,3'-diaminobenzidine chromogen were ap-

plied (Dako). Tissue sections were counterstained with Harris's hematoxylin. Isotype-matched mouse monoclonal antibodies were used as negative controls. Slides were analyzed with the use of an image-analysis workstation (Spot Browser, Alphelys).

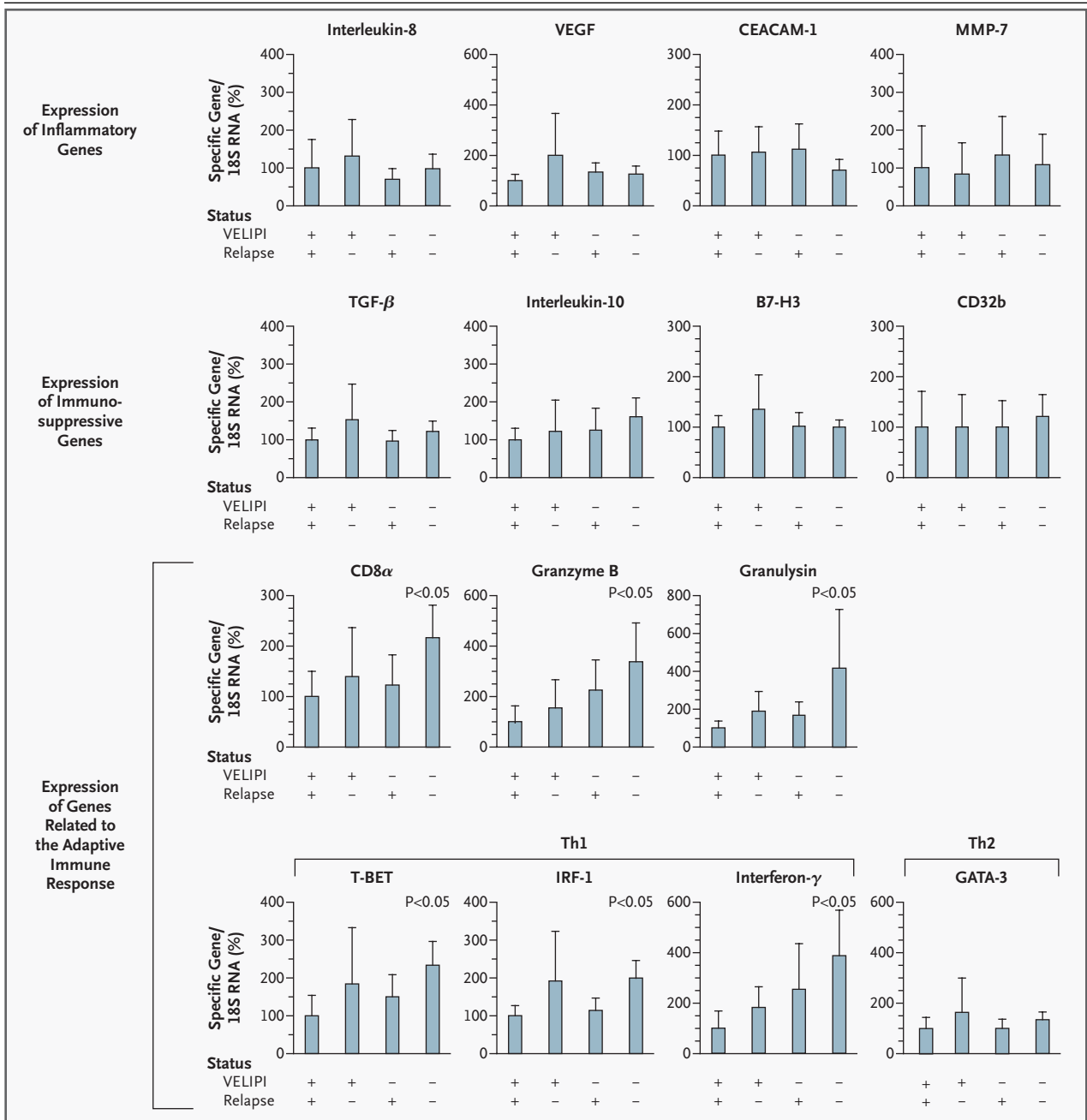


Figure 2. Expression of Inflammatory Genes, Immunosuppressive Genes, and Genes Related to the Adaptive Immune Response in a Series of 75 Colorectal Cancers, According to the Presence or Absence of Any Sign of Early Metastatic Invasion and Relapse.

Early metastatic invasion was defined by the presence of vascular emboli, lymphatic invasion, and perineural invasion (collectively referred to as VELIPI), alone or in combination. Relative levels of expression of mRNA were adjusted for the level of 18S mRNA for each sample. The levels are represented as mean percentage (+SE) increases as compared with levels in the group of patients with VELIPI-positive tumors who had a relapse, and P values are for the comparison with the reference group. VEGF denotes vascular endothelial growth factor, CEACAM-1 carcino-embryonic-antigen-related cell-adhesion molecule, MMP-7 matrix metalloproteinase 7, TGF-β transforming growth factor β, T-BET T-box transcription factor 21, and IRF-1 interferon regulatory factor 1.

Polychromatic high-resolution spot images (740 by 540 pixels; resolution, 1.181 μm per pixel) were obtained (magnification, $\times 100$). Measurements were recorded as the number of positive cells per unit of tissue surface.

STATISTICAL ANALYSIS

Kaplan–Meier curves were used to assess the influence of pathological signs of early metastatic invasion (VELIPI) on overall and disease-free survival. The significance of various clinical characteristics was assessed by univariate analysis with the use of the log-rank test (Table 1). We used a Cox proportional-hazards model to test the simultaneous influence on overall and disease-free survival of all covariates found to be significant in the univariate analysis. The same tests were used to assess the effect of the density of CD45RO+ cells (the number of cells per square millimeter) on overall and disease-free survival, alone or together with the TNM-stage covariates. The analysis-of-variance t-test and the Wilcoxon–Mann–Whitney test, respectively, were the parametric and nonparametric tests used to identify markers with significantly different levels of expression among VELIPI-positive and VELIPI-negative tumors. The normality of the logarithm of the gene-expression levels and of the densities of CD45RO+ cells was determined with the use of the Shapiro test. The Wilcoxon test was used to assess the significance of the difference in median survival across different groups of patients. All tests were two-sided. A P value of less than 0.05 was considered to indicate statistical significance. All P values are reported without adjustments for multiple corrections. All analyses were performed with the use of R and StatView, two types of statistical software.

RESULTS

EARLY METASTATIC INVASION AND CLINICAL OUTCOME

The prognostic significance of the presence of VELIPI, which delineated early metastatic invasion, was investigated by univariate analysis of data from the 959 patients with colorectal cancer. The presence or absence of VELIPI as well as the TNM stage significantly influenced disease-free and overall survival ($P < 0.001$ for all comparisons) (Table 1).

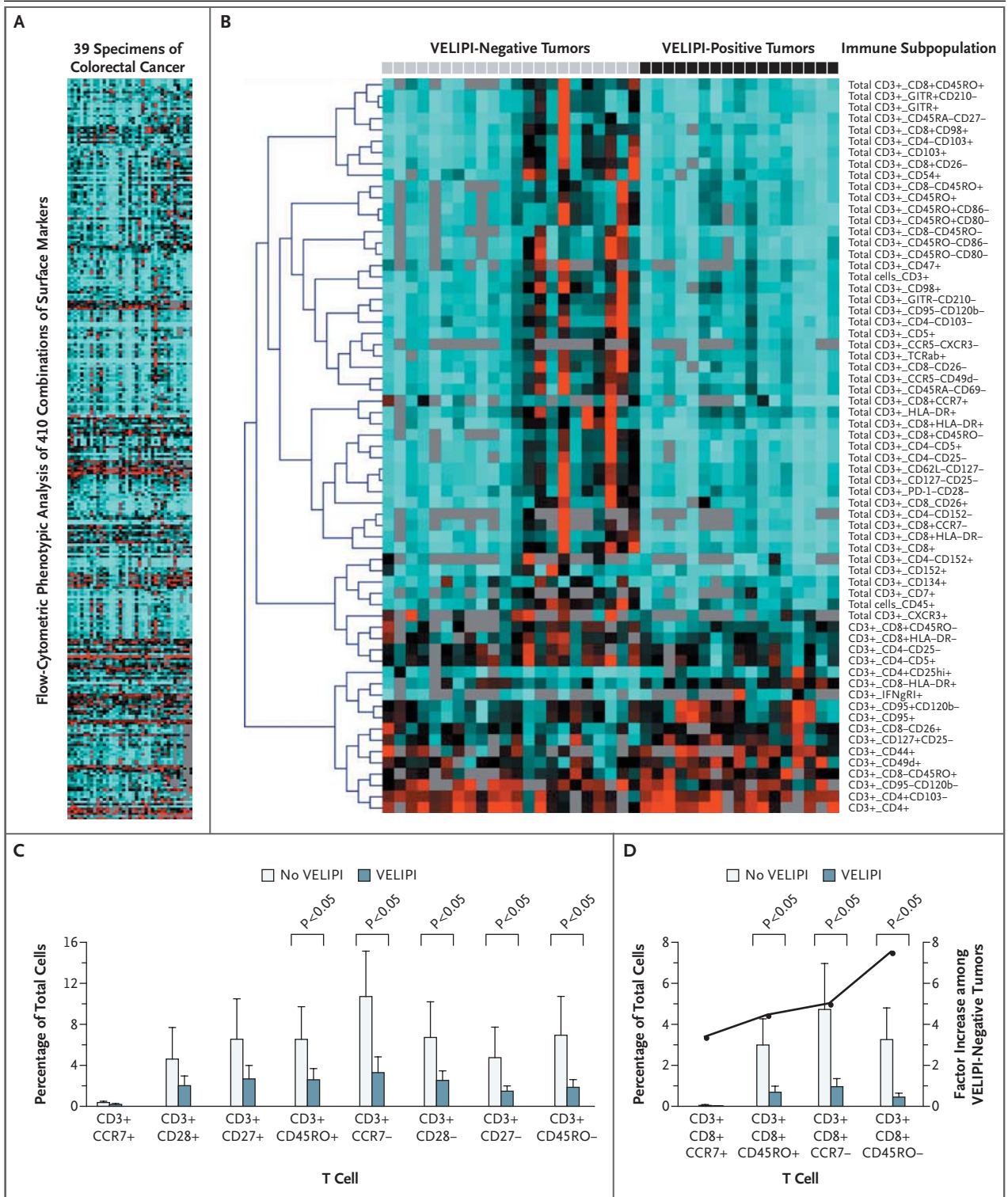
The five-year disease-free survival rates were 32.4 percent among patients with VELIPI-negative tumors and 12.1 percent among patients with VELIPI-positive tumors (Table 1). There were also signifi-

Figure 3 (facing page). Large-Scale Four-Color Flow-Cytometric Analysis of the Cell Populations and Immune-Cell Subpopulations in Freshly Resected Tumors from 39 Patients with Colorectal Carcinoma.

Early metastatic invasion was defined by the presence of vascular emboli, lymphatic invasion, and perineural invasion (collectively referred to as VELIPI), alone or in combination. Panel A shows the 410 combinations of surface markers measured by means of a fluorescence-activated cell sorter and subsequently plotted from the minimal level of expression (blue) to the maximal (red); gray areas represent analyses that were not done. Panel B shows the hierarchical clustering of the 65 combinations of markers that differed significantly between VELIPI-negative and VELIPI-positive tumors ($P < 0.05$). Panel C shows the T-cell differentiation process with the use of the markers CD45RO, CCR7, CD28, and CD27. Cells were analyzed in VELIPI-positive tumors and VELIPI-negative tumors and expressed as the mean (\pm SE) percentage of the total cells present within the tumor. Panel D shows the CD8+ T-cell subpopulations from naive to effector T cells with the use of the markers CD3, CD8, CCR7, and CD45RO, represented as the mean (\pm SE) percentage of the total cells within the tumor and as the factor increase among VELIPI-negative tumors, as compared with VELIPI-positive tumors. Immune-cell subpopulations (percentage of positive cells in the total population isolated from the tumor and with the CD3+ T-cell population) analyzed as a percentage of total cells reflect their density within the tumor. The Mann–Whitney test was used for statistical analyses.

cant differences in the median duration of disease-free survival between patients with VELIPI-positive tumors and patients with VELIPI-negative tumors (3.3 months vs. 26.9 months, $P < 0.001$) (Table 1). A similar pattern was found for overall survival (Table 1). Furthermore, the presence of more than one sign of early metastatic invasion conferred a worse prognosis than the presence of a single sign (Fig. 1 and the Supplementary Appendix). Kaplan–Meier curves suggested longer overall survival (Fig. 1) and disease-free survival (data not shown) among patients with VELIPI-negative tumors than among patients with VELIPI-positive tumors ($P < 0.001$ by the log-rank test). The VELIPI status correlated with the N and M stages ($P < 0.001$ for all comparisons) (data not shown).

The influence of all significant covariates on survival was simultaneously tested with the use of a Cox proportional-hazards model. After adjustment for TNM stage, multivariate analysis confirmed that the absence of VELIPI was significantly and independently associated with a better prognosis ($P = 0.04$ for overall survival and $P = 0.01$ for disease-



free survival). After adjustment for the Dukes' stage, the absence of VELIPI was independently associated with a better prognosis ($P=0.007$ for overall survival and $P=0.002$ for disease-free survival) (details are provided in the Supplementary Appendix).

IMMUNE-CELL INFILTRATION, INFLAMMATION, EARLY METASTATIC INVASION, AND PROGNOSIS

A total of 377 colorectal tumors were assessed histopathologically for an immune-cell infiltrate within the tumor and in the invasive margin (details are provided in the Supplementary Appendix). The presence of a strong immune infiltrate (indicated by a score of 3) was associated with VELIPI-negative tumors (see the Supplementary Appendix). We used quantitative real-time PCR with a low-density array to measure the levels of messenger RNA (mRNA) for inflammatory and immunosuppressive molecules in 75 colorectal tumors. No significant association between the content of mRNA for inflammatory mediators (interleukin-8, vascular endothelial growth factor, carcinoembryonic-antigen-related cell-adhesion molecule 1, matrix metalloproteinase 7, cyclooxygenase 2, and thrombospondin-1) or for immunosuppressive molecules (transforming growth factor β [TGF- β], interleukin-10, B7-H3, and CD32b) and VELIPI status or relapse was found (Fig. 2 and data not shown).

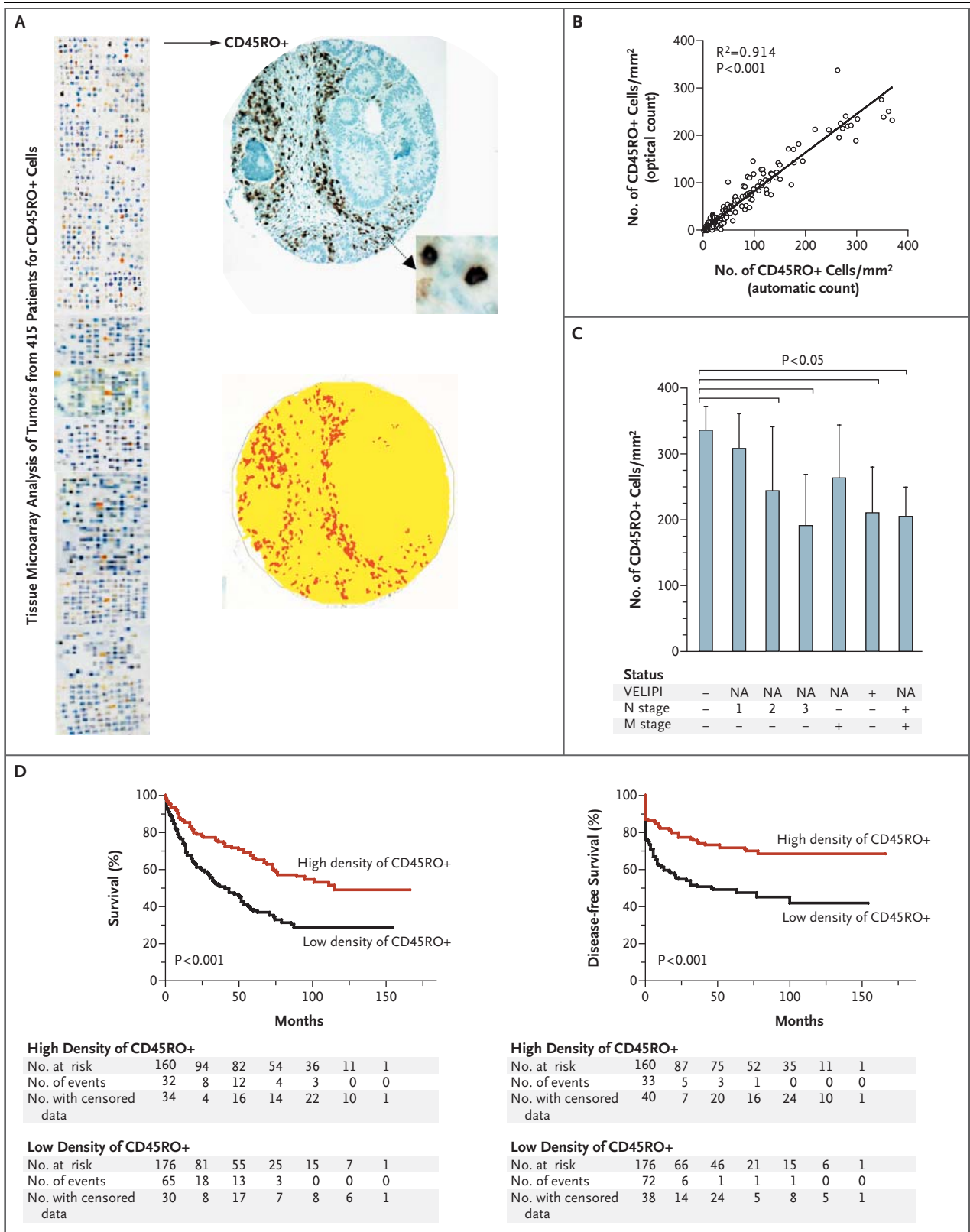
T cells differentiate into type 1 or type 2 helper T cells (Th1 and Th2, respectively) after the expression of T-BET (T-box transcription factor 21) or GATA-3, respectively.¹⁹ Protective immune responses are mediated by effector memory T cells with the phenotype CD8+, CD45RO+, CCR7- (negative for CC chemokine receptor 7), CD62L- (negative for CD62 ligand), perforin+, granzyme B+, and granulysin+. Stimulation with an antigen induces these cells to exert an immediate effector function²⁰ by releasing cytotoxic mediators.^{21,22} As shown in Figure 2, levels of CD8 α , granzyme B, and granulysin were increased in VELIPI-negative tumors and were further increased in such tumors from patients who had not relapsed, as compared with levels in VELIPI-positive tumors from patients who had relapsed ($P<0.05$). Moreover, VELIPI-negative tumors from patients who had not relapsed had a significant increase in the Th1 mediators T-BET, interferon regulatory factor 1, and interferon- γ , as compared with VELIPI-positive tumors from patients who had relapsed ($P<0.05$). In contrast, levels of the Th2 transcription factor GATA-3 were not increased in either group of patients (Fig. 2).

Figure 4 (facing page). Tissue Microarray (Panel A); Correlation between Optical and Automatic Counts of CD45RO+ Cells (Panel B); Mean (\pm SE) Density of CD45RO+ Cells According to the Presence or Absence of Early Metastatic Invasion, Nodal (N) Stage, and Metastasis (M) Stage (Panel C); and Overall and Disease-free Survival According to the Presence of a High or Low Density of CD45RO+ Cells within the Tumor (Panel D).

Early metastatic invasion was defined by the presence of vascular emboli, lymphatic invasion, and perineural invasion (collectively referred to as VELIPI), alone or in combination. Panel A shows the tissue microarrays. Four cores were obtained (two from the center and two from the invasive margin of the tumor) from the tumor specimens from 415 patients with colorectal cancer. Panel A shows an enlargement of a spot and an enlargement of CD45RO+ cells, as well as the digital image captured by the camera and analyzed by the image software (Spot Browser), with tissue represented in yellow and CD45RO+ cells represented in red. Panel B shows the correlation between optical and automatic counts of CD45RO+ cells in 100 randomly selected patients. Panel C shows the mean (\pm SE) numbers of CD45RO+ cells in the various groups of patients according to the VELIPI, N, and M status. N and M stages were determined according to the TNM staging system of the Union Internationale contre le Cancer.¹⁶ NA denotes not applicable. The Mann-Whitney test was used for statistical analyses. Panel D shows Kaplan-Meier curves for overall survival and disease-free survival among 160 patients with tumors that had a high density of CD45RO+ cells (at least 250 per square millimeter) and 176 patients with tumors that had a low density of CD45RO+ cells (fewer than 250 per square millimeter).

PHENOTYPES OF TUMOR-INFILTRATING IMMUNE CELLS

We used large-scale flow cytometry to analyze subpopulations of immune cells from 39 freshly resected colon cancers. To refine the analysis, 410 combinations of surface markers were measured by means of flow cytometry, and the results were plotted from the minimal (blue) to the maximal (red) level of expression (Fig. 3A). T cells, B cells, natural killer cells, natural killer T cells, and macrophages were analyzed in relation to the VELIPI status of the tumors. CD3+ T cells were the most prevalent tumor-infiltrating immune cells. The levels of CD3+, CD3+CD4+, and CD3+CD8+ T cells were significantly increased (by a factor of 2.6, 2.5, and 4.9, respectively; $P<0.05$) in VELIPI-negative tumors as compared with VELIPI-positive tumors (see the Supplementary Appendix). Large-scale analysis of phenotypic and functional markers of T-cell subpopulations (percentage of positive cells in the total



population isolated from the tumor and within the CD3+ T-cell population) revealed a significant difference ($P < 0.05$) between VELIPI-negative and VELIPI-positive tumors for 65 different combinations of markers. Hierarchical clustering²³ showed a homogeneous pattern in VELIPI-positive tumors, whereas two subgroups of VELIPI-negative tumors could be distinguished (Fig. 3B). All markers (CD45RO, CD45RA, CD27, CD28, CCR7, and CD127) of the T-cell differentiation process, from naive to effector memory T cells, were present in the cluster of differentially expressed markers. Markers of T-cell migration (CD62L-, CC chemokine receptor 7 [CCR7-], CD103, CD49d, and CXC chemokine receptor 3 [CXCR3]) and activation (HLA-DR, CD98, CD80, CD86, and CD134) were also differentially expressed between VELIPI-negative and VELIPI-positive tumors. Figure 3C shows that naive T cells (CD3+CCR7+) were rare in the tumors. By contrast, in the differentiation pathway from early memory T cells (CD45RO+CCR7-CD28+CD27+) to effector memory T cells (CD45RO+CCR7-CD28-CD27-), all subpopulations were detected. As compared with VELIPI-positive tumors, VELIPI-negative tumors had significantly more of these T cells ($P < 0.05$). Figure 3D shows the high proportion of mature CD8+ T cells in VELIPI-negative tumors. In contrast to tumors, distant normal mucosa from the same patients did not have significant differences in the CD8+ T-cell subpopulations according to VELIPI status (data not shown).

EFFECTOR MEMORY T CELLS AND SURVIVAL

We performed immunohistochemical analysis of tissue microarrays prepared from 415 colorectal cancers. Staining with an antibody against CD3 revealed the presence of T cells both within and at the invasive margin of the tumor (data not shown). We used automatic-image software to count CD45RO+ cells (Fig. 4A). A validation study showed a close correlation between optical and automatic cell counts ($R^2 = 0.914$, $P < 0.001$) (Fig. 4B).

VELIPI-negative tumors contained high numbers of CD45RO+ cells as compared with VELIPI-positive tumors ($P = 0.02$). In addition, a high density of memory T cells was associated with tumors without lymph-node involvement and metastases ($P < 0.001$). Advanced stages of lymph-node invasion (N2 and N3) were associated with low densities of CD45RO+ cells in tumors (Fig. 4C). Multivariate Cox proportional-hazards analysis showed that the M stage

($P < 0.001$), the N stage ($P = 0.002$), and the T stage ($P = 0.004$) as well as the CD45RO+ status ($P = 0.02$) were independent prognostic factors for overall survival (details are provided in the Supplementary Appendix). Kaplan–Meier curves suggested longer overall survival and disease-free survival (Fig. 4) among patients with tumors containing a high density of CD45RO+ cells than among patients whose tumors had a low density of such cells ($P < 0.001$ by the log-rank test). Patients whose tumors had a high density of CD45RO+ cells had a median disease-free survival of 36.5 months and a median overall survival of 53.2 months, as compared with 11.1 months and 20.6 months, respectively, among patients with tumors that had a low density of CD45RO+ cells ($P < 0.001$ for all comparisons) (Fig. 4D). The respective five-year overall and disease-free survival rates were 46.3 percent and 43.1 percent among patients with tumors containing a high density of CD45RO+ cells and 23.7 percent and 21.5 percent among patients with tumors containing a low density of CD45RO+ cells (Fig. 4D).

DISCUSSION

Our studies demonstrate a relation between the pathological signs of early metastatic invasion — vascular emboli, lymphatic invasion, and perineural invasion, collectively termed “VELIPI” — and the outcome in 959 colorectal cancers. We also found an association between the VELIPI status of the tumor and evidence of an immune response within the tumor. In particular, an analysis of 39 colorectal cancers showed that the presence of effector memory T cells within the tumor, defined by the presence of CD3, CD8, CD45RO, CCR7, CD28, and CD27 markers, was associated with VELIPI-negative tumors. Analysis of 415 colorectal tumors showed that a high density of infiltrating CD45RO+ cells correlated with a good clinical outcome.

The influence of early metastatic invasion on the course of colorectal cancer has been reported previously, but there are disparities in the literature, owing to inherent problems in the histopathological analysis of this phenomenon.^{24,25} In our series of 959 colorectal cancers, emboli detected by routine pathological examination showed a significant, independent association between VELIPI status and overall survival. Nevertheless, caution is warranted in interpreting these results, because of possible false negative cases. The identification of tumor em-

boli could be improved by the use of immunohistochemical staining of endothelium or neural structures. Such an approach is not routine.

Some tumors acquire the ability to sabotage inflammatory responses and exploit them to promote the proliferation, survival, and invasiveness of tumor cells.^{2,3,26,27} For this reason, the presence of leukocytes within a tumor may be a consequence of an inflammatory response that favors either dissemination of tumor cells or a protective host response.

Infiltrates of immune cells were frequent in tumors without perineural or lymphovascular emboli but rare in tumors with perineural or lymphovascular emboli, suggesting a beneficial effect of the host's immune response. We found no significant differences in the levels of mRNA for inflammatory and immunosuppressive molecules between VELIPI-positive and VELIPI-negative tumors or between tumors from patients who did not have a relapse and tumors from patients who had a relapse. These findings suggest that inflammation is not a factor in early metastatic invasion. In contrast, there were increased levels of mRNA for products and markers of Th1 effector T cells (CD8, T-BET, interferon regulatory factor 1, interferon- γ , granzyme B), and this increase was associated with prolonged survival and the absence of pathological signs of early metastatic invasion. Previous reports have shown that the presence of lymphocytes within the tumor and Th1-related cytokines such as interleukin-18 can be favorable prognostic signs.^{11,28}

In mice, protective immunity against colon cancer is mediated in part by long-lived memory T cells.²⁹ These cells may be responsible for long-

lasting protection against tumors. In our study of colorectal cancers from patients, we showed that, as compared with VELIPI-positive tumors, VELIPI-negative tumors contained significantly more memory T cells. All stages of T-cell differentiation were represented in VELIPI-negative tumors, with a pronounced increase in mature T cells, suggesting a process of T-cell differentiation. These findings are inconsistent with infiltration of the tumor by inactive, anergic T cells. Using tissue microarrays, we confirmed the association between a high number of CD45RO+ T cells and the absence of lymphovascular and perineural invasion ($P < 0.02$). Tumors that had a high density of effector memory T cells were associated with longer disease-free and overall survival than tumors without such cells ($P < 0.001$). The presence of CD45RO+ memory T cells in the tumor was an independent prognostic factor.

Our use of high-throughput quantitative measurement of cellular and molecular differences among colorectal cancers allowed us to make a detailed characterization of the tumor microenvironment and to identify associations with clinical outcomes. Our data suggest that the tumor microenvironment and the host's immune response are of major importance in tumor progression.

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