# The Adaptive Immunologic Microenvironment in Colorectal Cancer: A Novel Perspective

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## Abstract

**Colorectal cancer is one of the most common malignancies, often presenting with a poor prognosis. To date, the anatomic extent of disease has been by far the most important prognostic factor. Recently, we obtained evidence that the type, density, and location of immune cells in colorectal cancer could provide a prognostic factor superior and independent to that of criteria related to the anatomic extent of the tumor. Here, we discuss the meaning and potential implications of this novel finding.** [Cancer Res 2007;67(5):1883–6]

## Background

Cancer is a major public health problem worldwide. At present, surgery remains the primary form of therapy for solid tumors. The pathologic assessment of the resection specimen describes the anatomic extent of the tumor [tumor-node-metastasis (TNM) categories; ref. 1]. TNM stage groupings estimate the postoperative outcome and rationale for adjuvant therapy. Despite the prognostic power of this staging system, determining the outcome for patients is imprecise. In colorectal cancers, staging accuracy has remained largely unchanged since 1932 Dukes' original classification (2).

The detection of micrometastasis and occult tumor cells in the blood, bone marrow, and lymph nodes might improve staging accuracy. However, whether these variables offer a prognostic marker for recurrence in colorectal cancer is controversial. The cancer research community anticipates that an improved understanding of the genetic and epigenetic mechanisms driving the tumor will provide reliable prognostic factors and effective therapeutics. Chromosomal instability, deficiency of the DNA mismatch repair system, and gene silencing by hypermethylation of CpG-rich promoters constitute destabilizing pathways of the genome in colorectal cancer (3). These genotypes, as well as some molecular, protein, and carbohydrate markers, have been shown to influence clinical outcome. None of these factors have been validated yet as robust independent prognostic markers for patient care (4). Recently, DNA microarray-based gene expression profiling technology has identified a signature of tumor markers for colorectal cancer prognosis (5). Additional studies will be required to define the reliability of these markers. In summary, although improvements in our understanding of colorectal cancer biology have provided significant benefit to the development of new therapeutics [e.g., epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) inhibitors], these improvements have yet to increase the accuracy of prognosis for planning appropriate adjuvant therapy.

The absence of evidence to support the clinical use of markers of the oncogenic process suggests that increased accuracy of prediction of relapse might be obtained by nontumoral variables. Importantly, primary tumors and metastasis develop within a microenvironment. Tumor cells live a complex milieu of cellular components comprising fibroblasts, endothelial cells, and immune cells. The innate immune system can influence tumor development. Inflammatory mediators promote tumor development by inducing tumor mutations, resistance to apoptosis, angiogenesis, and tumor growth and favoring metastasis (6, 7). There is also now clear evidence in support of the conclusion that the immune system does indeed protect the host against tumor development through immunosurveillance mechanisms (8). Increased susceptibility of immunodeficient mice to carcinogen-induced and spontaneous tumors showed the role of innate and adaptive immunity in the control of tumor development (8). Transfer of immune T lymphocytes protects mice from tumor challenge. Elimination of $CD8^+$ T cells abrogates protective and therapeutic antitumor effects. Antitumor immunity, however, leads to immunoediting, a process favoring the outgrowth of tumor cells with reduced immunogenicity (8).

Solid tumors are commonly infiltrated by immune cells (e.g., T and B lymphocytes, natural killer cells, dendritic cells, macrophages, neutrophils, eosinophils, and mast cells). All of them are variably scattered within the tumor and loaded with an assorted array of cytokines, chemokines, and inflammatory and cytotoxic mediators. This complex network reflects the diversity in tumor biology and tumor-host interactions. Data from previous studies suggest that antitumor T-cell immune responses may take place *in vivo* in patients with solid tumors (9–11), influencing prognosis and shaping the tumor immunologic profile. To gain an understanding of tumor-host interaction in colorectal cancer, we developed a comprehensive analysis of the immune reaction at tumor sites based on the nature, functional orientation, density, and localization of immune cell populations within distinct tumor regions. We further analyzed these variables in relation to tumor evolution and clinical outcome.

## Findings

In a large series of colorectal cancer, we assessed the immune component of the tumor microenvironment by a combination of high-throughput genotypic and phenotypic analyses and evaluated its possible influence on tumor dissemination.

The presence of tumor emboli in lymphovascular and perineural structures is considered as early steps of metastatic invasion. We

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found an association between evidence of an immune reaction within the tumor and the absence of tumor emboli. In particular, colorectal cancer without tumor emboli was associated with an enhanced infiltration by immune cells and an increase of mRNA expression of adaptive T-helper 1 ($T_{H1}$) effector T-cell markers [CD8, T-box transcription factor 21 (T-bet), IFN regulatory factor-1 (IRF-1), IFN-γ, granulysin, and granzyme B]. Large-scale flow cytometric analysis of T-cell subpopulations infiltrating colorectal cancer revealed a significant difference between tumors with and without tumor emboli for 65 different combinations of markers. Hierarchical clustering showed that markers of T-cell migration, activation, and differentiation were increased in colorectal cancer without sign of early metastatic invasion. These patients presented with significant increases in T-cell subpopulations from early memory to effector memory CD8 T cells ($T_{EM}$). Using tissue microarrays, we confirmed the association between a high number of $CD45RO^+$ memory T cells infiltrating colorectal cancer (415 tumors studied) and the absence of lymphovascular and perineural invasion. Interestingly, a high density of *in situ* memory T cells was also associated with tumors without lymph node involvement and distant metastases.

The use of high-throughput quantitative measurement of cellular and molecular differences among colorectal cancer allowed a detailed characterization of the tumor microenvironment. This revealed that the immune reaction of the host might influence the tumor dissemination from the early steps of the metastatic process to the established metastasis in lymph node and distant organs. Notably, we evidenced a central role of a recently characterized immune cell subpopulation, the $T_{EM}$. In mice, protective immunity against colon cancer is mediated in part by long-lived memory T cells (12). These cells may be responsible for long-lasting protection against tumors. Accordingly, we showed, in human colorectal cancer, a longer disease-free survival and overall survival among patients with tumors containing a high density of $CD45RO^+$ cells than among patients whose tumors had a low density of such cells ($P < 0.0001$, log-rank test). These data may outline the delayed antitumor activity of $T_{EM}$ through a systemic immunosurveillance, as $T_{EM}$ decrease the incidence of relapse and strongly improve patient's survival (13).

In a first attempt to do integrative biology approaches of the host response in colorectal cancer, we combined the assessment of the nature, functional orientation, density, and localization of immune cell populations within distinct regions of the tumor. All data were entered into a dedicated Tumoral MicroEnvironment Database (TME.db). Results showed a major importance of the natural antitumor adaptive immunity against colorectal cancer.

Immune genes related to inflammation, $T_{H1}$ adaptive immunity, and immunosuppression showed variable expression levels in the tumors studied. Correlation matrix followed by unsupervised clustering revealed highly significant combinations of comodulated genes, isolating clusters referring to known biological functions. Strikingly, a sole cluster for $T_{H1}$ adaptive immunity (T-bet, IRF-1, IFN-γ, CD3ζ, CD8, granulysin, and granzyme B) correlated with protection against relapse. These data indicated that $T_{H1}$ adaptive immunity could have a beneficial effect on clinical outcome.

We did immunohistochemical analyses for adaptive immune markers (CD3, CD8 granzyme B, and CD45RO) of tissue microarrays prepared from cores of the center of the tumor (CT) and the invasive margin (IM) of 415, 119, and 75 colorectal cancer (three independent series). The corresponding 7,384 immunostainings were quantified with the use of a dedicated image analysis workstation. In each tumor region (CT and IM) and for each adaptive immune marker, there was a statistically significant correlation between immune cell density and patient outcome. Further, the combined analysis of tumor regions (CT plus IM) improved the accuracy of prediction of survival for the different patient groups compared with single-region analysis. Univariate and multivariate analyses done in multiple parallel ways (correction factors, median cutoff, cross-validation methods, leave-one-out, and bootstrap methods) led into similar conclusions. Natural adaptive immune reaction influenced clinical outcome at all stages of the disease. The immune reaction at tumor site determined cancer evolution and clinical outcome regardless of the local extent and spread of the tumor. A weak adaptive immune reaction correlated with a very poor prognosis even in patients with minimal tumor invasion. Conversely, a high density of adaptive immune cells correlated with a highly favorable prognosis whatever the local extent of the tumor and the invasion of regional lymph nodes. Beneficial *in situ* adaptive immune reaction was not restricted to patients with minimal tumor invasion, indicating that *in situ* immunologic forces may persist along with tumor progression. Multivariate Cox analysis revealed that the immune criteria remained the unique variable significantly associated with prognosis. The histopathologic staging system (T stage, N stage, and differentiation) did not present a significant predictive value anymore.

In summary, the adaptive immune reaction within the tumor seemed to be the sole variable influencing outcome after surgical treatment with curative intent (14). The strength of the immune reaction identified in our studies could advance our understanding of cancer evolution with important consequences in clinical practice.

## Implications

We first provided evidence of a higher proportion of strong immune infiltrates in tumors without perineural or lymphovascular emboli. In addition, we did not observe a higher density of the lymphoid nodules surrounding the tumor (Crohn's-like reaction), an inflammatory-associated lymphoid structure, in tumors with perineural or lymphovascular emboli. No significant difference was found in the expression of proinflammatory and angiogenic mediators in tumors according to the presence of tumor emboli. Together, this suggests that the inflammatory process does not play a critical role in the early invasion-metastasis process in colorectal cancer. In contrast, an *in situ* adaptive immune reaction may control tumor dissemination from the early stages of the metastatic process to fully established metastases in lymph node and distant organs.

A note of caution is useful when interpreting the highly significant correlation we found between the quality of the *in situ* immune reaction and the signs of tumor dissemination and clinical outcome. This indicates a strong but indirect evidence of an immune-mediated control of colorectal cancer progression. It cannot be excluded that intratumoral lymphocytes modify tumor stroma or tumor cells, or both, in such a way that they attenuate the metastatic capacity of tumor cells. In mice, targeted disruptions of genes that encode critical components of the immune system [e.g., mice lacking IFN-γ, IFN-γ receptor, or the transcription factor signal transducers and activators of transcription 1 (STAT1) important in mediating IFN-γ receptor signaling, perforin, the recombination activating gene *RAG-2*, αβ T cells, and

interleukin (IL)-12] induce an increased susceptibility of the host to tumors (8). These key findings show an immune-mediated control of tumor development partly supported by the adaptive compartment. In human colorectal cancer, we also observed that the expression level of a $T_{H1}$ adaptive cluster of genes influenced tumor recurrence. Finally, evidence is accumulating that colorectal cancer expresses tumor-associated antigens (e.g., K-ras mutations, p53, carcinoembryonic antigen, Ep-CAM, and SART3) that can induce tumor-specific T-cell responses in patients (11). Recently, systematic analysis of genetic alterations identified 751 somatic mutations in human colorectal cancer and derived cell lines (15), emphasizing a wide spectrum of putative peptides for T-cell recognition. All these experimental data provide strong arguments in favor of the immune-mediated control of colorectal cancer.

We showed that a $T_{H1}$ polarization with cytotoxic and memory T cells in the CT and the IM of the tumor maximally influenced clinical outcome. Previous studies focusing on the host response in colorectal cancer indicated a possible positive prognostic role for the presence of lymphoid infiltrates (11). To the best of our knowledge, the prognostic value we observed is far beyond that mentioned in previous investigations of immune markers in colorectal cancer and also in other solid tumors. To explain this difference, we hypothesize that the combination of immune variables associating the nature, density, functional orientation, and distribution of immune cells within the tumor would all be essential to accurately define the effect of the local host immune reaction in colorectal cancer. We have proposed to define these immune criteria as "immune contexture" (16).

Compared with immune contexture, as we have defined it, no tumor variable associated with survival has been reported to achieve a similarly high level of significance in colorectal cancer. The absence of evidence to support the clinical use of markers of the oncogenic process suggests that these markers are closely associated with primary tumor extension and spreading. Furthermore, assessment of tumor infiltration by cytotoxic and memory T lymphocytes could reflect a level of antitumor immunity shaped by multiple tumor variables, such as altered expression level of HLA molecules (11) or tumor antigens (11), the identity of specific

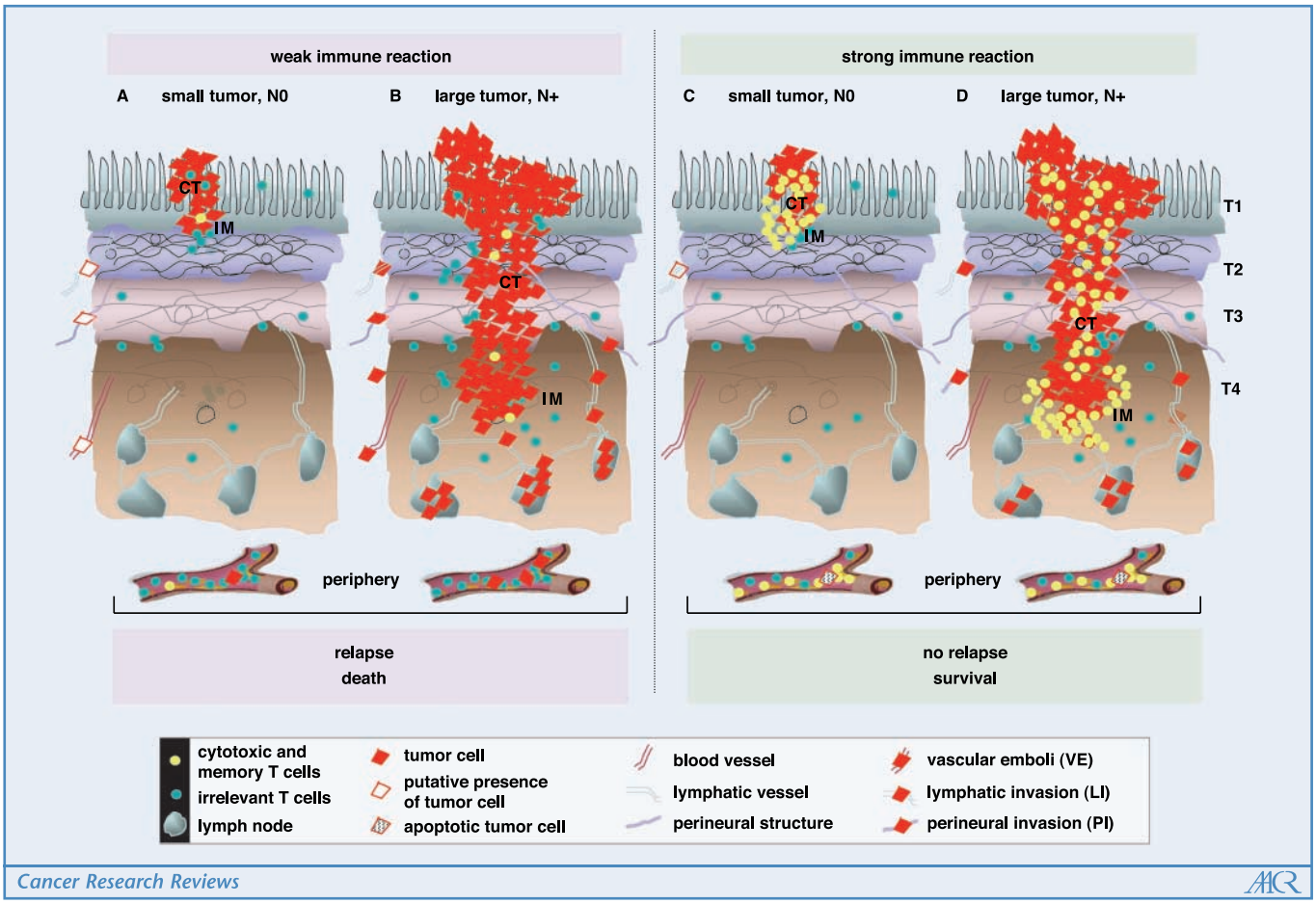


**Figure 1.** *In situ* immune reaction and colorectal cancer. A proposed model of the mechanisms by which the immune system may control tumor dissemination and influence clinical outcome. The main observations supporting this model can be summarized as follows. (*a*) Correlation between a high density of $CD45RO^+$ memory T cells and $CD8^+$ T cells infiltrating colorectal cancer and the absence of lymphovascular and perineural invasion (*C* versus *A* and *D* versus *B*). (*b*) A weak adaptive immune reaction is associated with a very poor prognosis even in patients with minimal tumor invasion (*A* and *B*). (*c*) A high density of adaptive immune cells correlates with a favorable prognosis whatever the local extent of the tumor and the invasion of regional lymph nodes (*C* and *D*). (*d*) The combined analysis of infiltrating T cells within tumor regions (CT plus IM) improves the accuracy of prediction of survival for the different patient groups compared with single-region analysis. (*e*) Effector and memory T cells *in situ* limit tumor dissemination. Effector and memory T cells could reflect a quality of systemic effectors for recognition and killing of circulating cancer cells. (*f*) The histopathologic variables (T stage, N stage, and differentiation) are no longer informative for the evaluation of the clinical outcome when analyzed together with adaptive immune variables (*A* and *B* versus *C* and *D*). Adaptive immune reaction within the tumor may be an ultimate variable influencing clinical outcome following surgical treatment with curative intent.

mutational pathways (i.e., chromosomal instability, deficiency of the DNA mismatch repair system, and promoter hypermethylation; ref. 3), and microenvironmental factors (17). Altogether, the *in situ* adaptive immune reaction could be an ultimate point of convergence for prognosis of colorectal cancer.

Because the primary tumor is removed by surgery, the prognostic value associated with the host response in colorectal cancer may reflect a capacity of the *in situ* effectors to limit tumor dissemination as discussed above. This host response may also reflect a quality of systemic effectors for recognition and killing of circulating cancer cells in peripheral blood, peritoneal cavity, bone marrow, or lymph nodes. Together, these processes may lead to the elimination of circulating cancer cells or to an equilibrium phase [i.e., a subclinical phase in which the tumor persists but is prevented from expanding by immune pressure (18)]. As part of our work, we defined a central role for memory T cells in prognosis. Immunologic memory is defined as the ability to "remember" previously encountered antigens leading to faster response on reexposure. Following a primary response to antigen, memory T cells disseminate and are maintained in the body for long periods (19). Thus, the critical trafficking properties and the long-lasting antitumor capacity of memory T cells may help control circulating tumor cells as has been illustrated in a mouse model (12). Various cellular and molecular factors originating in the tumor microenvironment (such as IL-10, transforming growth factor-β, and VEGF) contribute to a local defective immune attack. This phenomenon might no longer be effective for circulating tumor cells, facilitating their destruction before these cells grow into metastases and reconstitute an immunosuppressive network.

Unexpectedly, our immunologic criteria not only had a prognostic value that was superior and independent of those of the TNM staging system but also correlated with survival regardless of the extent of tumor invasion. The histopathologic staging system was therefore no longer informative in patients with colorectal cancer.

In summary, the presence of an adaptive immune reaction within the tumor seemed to be the critical sole variable influencing the outcome after surgical treatment with curative intent. This finding suggests that time to recurrence and overall survival times generally are governed in large part by the state of the local adaptive immune response (Fig. 1), challenging our understanding of cancer progression and how to treat colorectal cancer in clinic.

## Future Questions

Two essential questions that must be resolved before a prognostic factor can be incorporated into tumor staging are the following (20); first, does the factor have an effect on outcome independent of TNM? second, is there a reliable measurement methodology available for routine practice? The immune criteria we defined seem to address both these questions. However, before initiating extensive use of this model for selecting patients at risk of relapse, it will be important of course to confirm the results in independent data sets. Provided our findings can be validated successfully at other centers, the immunologic criteria that we have used may lead to revision of the current indicators of clinical outcome and may help identify the high-risk patients who would benefit the most from adjuvant therapy. Future work may determine whether this general approach may be useful for the investigation of other tumor types beyond colorectal cancer.

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