

## Prognostic and Predictive Impact of Intra- and Peritumoral Immune Infiltrates

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### Abstract

Leukocyte infiltrates into or around tumor cell nests are found in the context of protumorigenic inflammation and anticancer immunosurveillance. Hence, the detailed composition, density, architecture, and function of leukocyte infiltrates must be analyzed to understand their prognostic impact. The ectopic presence within tumors of high endothelial venule cells, which are normally characteristic for secondary lymphoid organs, correlates with a more pronounced infiltration by T lymphocytes and has a positive predictive impact on local advanced breast cancer treated with neoadjuvant chemotherapy. Recent progress in the field indicates that immune infiltrates of the primary tumors, as well as of metastases, are not only independent prognostic biomarkers but can also constitute predictive factors, suggesting that the pretherapeutic immune response can determine the efficacy of conventional chemotherapies. Moreover, accumulating evidence indicates that chemotherapy can stimulate anticancer immune responses coupled with an increased intratumoral lymphoid infiltration, which correlates with tumor mass reduction and patient survival. Improved methods for the automation of immunohistochemistry and digitalized image analyses will pave the way to an improved understanding of the complex interplay between cancer parenchyma, stroma, and immune effectors, as well as to the routine evaluation of immune-related parameters to the clinical management of cancer patients. *Cancer Res*; 71(17); 5601–5.  2011 AACR.

### Introduction

Before the introduction of sophisticated molecular tools for their analysis, inflammatory and immune infiltrates in tumor lesions were viewed in a partisan way by advocates of the inflammatory etiology of tumors and defenders of the immunosurveillance theory. Whereas the former focused on the protumorigenic effects of inflammation, the latter proffered the capacity of immune effectors to eliminate early (pre) cancerous lesions or to maintain small tumors in an equilibrium state, before their eventual escape from immune-mediated control (1).

With the development of more accurate methods for analyzing the immune infiltrates, it is becoming clearer that distinct infiltrating cell types have distinct prognostic and

predictive significance. Dendritic cells (DC), M<sub>1</sub> macrophages, Th1 CD4<sup>+</sup> T cells, cytotoxic CD8<sup>+</sup> T cells, and natural killer (NK) cells present in the tumor bed tend to reduce cancer growth. In contrast, M<sub>2</sub> macrophages, myeloid-derived suppressor cells (MDSC), neutrophils, Th2 and Th17 CD4<sup>+</sup> T cells, and Foxp3<sup>+</sup>CD4<sup>+</sup> regulatory T (Treg) cells are suspected to stimulate cancer growth, although this functional distinction has to be placed into context for each tumor type (2, 3). Moreover, beyond just the composition, the intra- and peritumoral distribution, architecture, and functional articulation of the immune infiltrate, the immune context, must be analyzed to comprehend its significance and elaborate accurate prognostic or predictive biomarkers (Fig. 1; ref. 4).

Large clinical studies, involving more than 1,000 patients each, revealed the prognostic and predictive impact of the immune infiltrate for colorectal and breast cancer, respectively (5–8). In terms of immune parameters, colorectal cancer is one of the best-studied tumors. Colorectal cancer prognosis is influenced by the presence of an mRNA expression profile indicative of a type 1 adaptive immune response (antigen presentation, IFN- and T-cell receptor signaling, cytotoxic T cells). Moreover, colorectal cancer prognosis is influenced by the presence of T cells in the stroma, within the invasive front, and in the parenchyma, in an intraepithelial localization (5–7). The cell type with the most favorable prognostic impact is the memory effector T cell (CD45RO<sup>+</sup>CCR7<sup>-</sup>, CD28<sup>-</sup>CD27<sup>-</sup>; refs. 6, 9). The chemokines with the most significance are CXCL10 and CXCL9, which may attract CD45RO<sup>+</sup> T cells into

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	Pro-tumorigenic inflammation	Anticancer immunosurveillance
Cell types	M2 macrophages Myeloid-derived suppressor cells Neutrophils Foxp3 <sup>+</sup> T reg, Th17 cells	Dendritic cells M1 macrophages Cytotoxic CD8 <sup>+</sup> T cells with a memory effector phenotype
Cytokine profiles	Th2 Th17	Th1 CX3CL1 CXCL9, CXCL10
Distribution	Peritumoral	Intratumoral, close to cancer cells, as well as in the invasive front
Associated features	Stat3 phosphorylation	High endothelial venules
Functional impact	Negative prognostic impact	Positive prognostic and predictive impact

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Figure 1. Hallmarks of the immune infiltrates associated with inflammation versus anticancer immunosurveillance. See text for details.

the tumor bed, as well as CX3CL1 (also known as fractalkine), which may attract Th1 cells into the tumor (10). If appropriately analyzed, the immune infiltrate furnishes more accurate prognostic information on colorectal cancer than the currently used Union for International Cancer Control (UICC) tumor–node–metastasis (TNM) and Dukes classifications, as has been determined in several retrospective trials (6, 11).

Here, we review recent progress in the field, placing special emphasis on the mechanisms that account for tumor infiltration by lymphocytes, the unexpected finding that even metastases may be under the control of the immune system, as well as perspectives to standardize and automate the analysis of tumor immune infiltrates for clinical practice.

### Variable Causes and Consequences of Immune Infiltration

Although lymphoid infiltration may constitute a mechanism or sign of tumor progression, antitumor immune responses are suspected to control tumor growth. Obviously, the underlying causes that attract lymphoid cells are distinct in chronic inflammation and immunosurveillance, determining major differences in the composition and "architecture" of leukocyte infiltrates. Accordingly, the expression of genes associated with proinflammatory Th17 cells (IL17A and the Th17-specific transcription factor ROR.C) has a negative prognostic impact on colorectal cancer prognosis, whereas expression of genes associated with Th1 cells (such as IRF1, IL12R.2, STAT4, and the Th1-specific transcription

factor TBet) is associated with prolonged disease-free survival (12).

Phosphorylation of the transcription factor STAT3 (*p*-STAT3) constitutes a sign of inflammation. Indeed, among 724 colorectal cancers, *p*-STAT3 expression correlated with significantly higher cancer-specific mortality. *p*-STAT3 was also associated with peritumoral lymphocyte reactions (13) but not with tumor-infiltrating lymphocytes, which in turn constitute a positive prognostic factor (11).

High-mobility group protein B1 (HMGB1) is one of the most abundant nonhistone chromatin-binding proteins and is normally contained in the nucleus. However, upon cell stress or death, HMGB1 can be released through the cytoplasm to the pericellular microenvironment, where it acts as a danger signal and activates inflammatory cells. In stage IIIB colorectal cancers, the abnormal coexpression of HMGB1 in both nuclei and the cytoplasm, which occurs in a subset of tumors, inversely correlated with the density of tumor-infiltrating CD45RO<sup>+</sup> T cells and predicts poor survival (14). It is tempting to speculate that the presence of local proinflammatory factors such as STAT3 and HMGB1 may have a negative impact on the recruitment of anticancer effectors.

Calreticulin (CRT) is the most abundant luminal protein of the endoplasmic reticulum (ER). It is required for optimal loading of class I MHC proteins with antigen peptides. Moreover, CRT can be translocated to the cell surface upon ER stress, an event that is thought to be essential for the immunogenicity of cancer cell death, allowing dying tumor cells to be engulfed by DCs (15). A subset of colorectal cancers lack

CRT expression, and this correlates with a reduction of tumor-infiltrating CD45RO<sup>+</sup> T cells and poor prognosis (16). These latter findings are reminiscent of a recent study in acute myeloid leukemia showing that surface expression of CRT on malignant myeloblasts correlates with enhanced anti-cancer Th1 responses and improved survival in acute myeloid leukemia (17). It remains to be investigated whether these findings reflect events of immunogenic apoptosis (18) that spontaneously occur in tumor lesions. Indeed, events of local cell death are frequently found in cancers, perhaps as a result of oncogenic stress, of the incapacity of the cells to cope with dwindling resources, or of a lack of trophic support as they proliferate and invade the tissue. However, whether apoptosis can indeed stimulate immunosurveillance mechanisms has not been shown. Moreover, the prognostic impact of infiltrating inflammatory cells associated with foci of necrosis, adjacent to and/or within the tumor, remains to be studied.

### The Microanatomy of Lymphoid Infiltration

High endothelial venules (HEV), which stain with the molecular marker MECA 79, are specialized postcapillary blood vessels that are present in secondary lymphoid organs and that support the recruitment and extravasation of lymphocytes. HEVs can also develop in nonlymphoid tissues at sites of chronic inflammation, for instance, in the context of autoimmune diseases such as rheumatoid arthritis or inflammatory bowel disease. Importantly, MECA 79<sup>+</sup> HEVs (which also express other HEV markers, such as HECA-452, DARC, the endothelial cell adhesion protein ICAM-1, as well as the pan-vascular markers CD31 and vWB) have been detected in a substantial fraction of primary human solid cancers, in particular in lymphocyte-rich areas (19). Indeed, in a retrospective cohort of 146 primary invasive, nonmetastatic breast cancers, the density of tumor-associated MECA 79<sup>+</sup> HEVs was found to predict the frequency of infiltrating CD3<sup>+</sup>CD8<sup>+</sup> T cells and B cells, as determined by immunohistochemistry. Moreover, the density of HEVs was found to constitute an independent prognostic marker of metastasis-free, disease-free, and overall survival (19). As compared with HEV<sup>low</sup> tumors, HEV<sup>high</sup> breast cancers were more heavily infiltrated by naïve (CCR7<sup>+</sup>CD44<sup>-</sup>CD45RA<sup>+</sup>CD62L<sup>+</sup>), central memory (CD45RA<sup>+</sup>CD62L<sup>+</sup>), effector memory (CD45RA<sup>-</sup>CD62L<sup>-</sup>), and terminally differentiated (TEMRA<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>-</sup>) T cells, as well as by T cells expressing markers of activation (CD25, CD69, CD86, HLA-DR) and cytotoxicity (granzyme A, granzyme B, perforin), as determined by cytofluorometric methods. Gene expression profiling by quantitative reverse transcriptase PCR revealed that HEV<sup>high</sup> cancer upregulated genes related to T-cell cytotoxic granule components (such as granzymes and perforin), Th1 orientation (IFN-, TBX21), lymphoid chemokines (CCL19, CDL21, CXCL13), and T-cell homing receptors (CCR7 and LSEL). Altogether, these results underscore the correlation between HEV density and intratumoral T-cell infiltration and suggest that the presence of HEV may convert tumors *de facto* into secondary lymphoid organs, thus permitting the extravasation of T cells into the tumor bed. Intriguingly, antiangiogenic agents, including

anti-VEGF antibodies, can increase the infiltration of tumors by lymphocytes (20), and it will be interesting to discover how such agents affect intratumoral HEVs *in vivo*.

As mentioned above, HEVs are hallmarks of secondary lymphoid organs and apparently are found in multiple distinct human cancers, including melanomas, breast, ovarian, colon, and lung carcinomas (19). Tertiary lymphoid structures have been identified thus far only in non-small cell lung cancers (NSCLC) as so-called tumor-induced bronchus-associated lymphoid tissues (Ti-BALT). The presence of Ti-BALTs, which are surrounded by HEVs and composed of DC-Lamp/CD208<sup>+</sup> DCs close to T-cell clusters often adjacent to B-cell follicles, is associated with a favorable clinical outcome in NSCLC (21). It remains to be seen whether such tertiary lymphoid structures influence the prognosis of other human cancers as well. In view of the fact that secondary or tertiary lymphoid structures are needed to mount adaptive immune responses, it seems that "structured" lymphoid responses (be they secondary or tertiary) can be interpreted as signs of an organized local immune response, which have a positive prognostic (and perhaps predictive) value. This finding would contrast with "unstructured" responses that occur in the context of non-specific inflammation and negatively influence cancer morbidity and mortality.

### The Immune Infiltrate: From Prognostic to Predictive

Immune infiltrates may influence prognosis of cancers (2–4). Few studies, however, have unraveled the predictive impact of such infiltrates on the efficacy of conventional cancer therapies. Colorectal metastases in the liver (UICC stage IV, Dukes stage D) are detected when the immune infiltrate in the primary tumor tends to be scarce (22). However, at this late stage, an immune control of the neoplastic disease may persist, because those rare cases, in which memory effector T cells still infiltrate both the center of the primary tumors and their invasive margin, have a relatively favorable prognosis (22).

Surprisingly, this general rule even applies to the immune infiltration of hepatic metastases (23). Indeed, an automated microscopic analysis of the densities of CD3<sup>+</sup>, CD8<sup>+</sup>, or granzyme B<sup>+</sup> lymphocytes found in the invasive margins of liver metastases permitted the development of an accurate predictive scoring score (23). In this analysis, CD3 densities above a threshold (>600 cells/mm<sup>2</sup>) received 2 points, and CD8 or granzyme B densities above their thresholds (>200 and >25 cells/mm<sup>2</sup>, respectively) received 1 point each, and patients with a score from 0 to 2 were predicted to be nonresponders. This scoring system was not improved by including additional covariates [age, gender, chemotherapy, treatment with epidermal growth factor receptor (EGFR)-specific antibodies], underscoring that it constituted an independent prognostic marker. Thus, it seems that the composition of the immune infiltrate *before* chemotherapy may have an impact on the efficacy of the treatment of colorectal cancer.

In locally advanced breast cancer treated with neoadjuvant chemotherapy, the presence of tumor-infiltrating lymphocytes, in particular T cells, in the primary biopsy predicts

complete pathologic responses as an independent biomarker (8). In one recent study, the tumor infiltration by CD8<sup>+</sup> and FOXP3<sup>+</sup> T lymphocytes was compared before and after neoadjuvant chemotherapy (24). The association of high CD8<sup>+</sup> and low FOXP3<sup>+</sup> cell infiltrates postchemotherapy was associated with improved relapse-free survival and overall survival. Moreover, this association outperformed classical predictive factors in multivariate analyses, including the hormone receptor status and HER2 expression (24). Thus, it is possible that chemotherapy-induced anticancer immune responses have a decisive impact on the success or failure of anticancer chemotherapies.

Anthracycline-based chemotherapies induce a vigorous infiltration of anticancer immune effectors, both in mice (25) and, as discussed above, at least in a fraction of patients with breast cancer (24). Anthracycline- and oxaliplatin-based chemotherapies are more efficient when they are administered to immunocompetent rather than to immunodeficient mice transplanted with similar tumors (26, 27). Indeed, the mere absence of TLR4 from the mouse genome reduces the efficacy of these chemotherapies, presumably because TLR4 signaling is required for optimal presentation of dead cell antigens by DCs (28), and patients bearing loss-of-function alleles of TLR4 show reduced efficacy of adjuvant chemotherapy of breast cancer (with anthracyclines) and colorectal cancer (with oxaliplatin; refs. 28, 29).

These observations have given rise to the hypothesis that anticancer immune responses are indispensable for the achievement of optimal therapeutic results (30). As discussed above, the infiltration of tumors *before* chemotherapy can predict the therapy-induced reduction of tumor mass and may determine patient survival (8). Moreover, changes in the composition of the infiltrate may have an impact on the therapeutic response as well (24). The question is, then, to what extent does reactivation of a preexisting immunity or the neo-induction of novel anticancer immune responses contribute to the efficacy of anticancer therapies? Do chemotherapies subvert immunosuppressive mechanisms and hence reactivate a latent immune response? Or does the induction of immunogenic tumor cell death convert the tumor into a sort of therapeutic vaccine that (re)activates the immune control of "escaped" cancers?

### Future Directions: Automation of Molecular Pathology

Traditionally, anatomic pathologists have examined histologic sections to diagnose histologic cancer cell types in

merely qualitative terms, without attributing numeric values to the degree of cancer cell heterogeneity (which denotes increasing malignancy) or the infiltration by immune effectors. At best, they used semiquantitative terms (+, ++, +++, etc.) to express their appreciation. As a result of the chronic absence of rigorous and systematic quantification, it has been possible that the obvious correlation between lymphocyte infiltration (as revealed by hematoxylin and eosin staining) and complete pathologic responses in locally invasive breast cancer has been overlooked for decades (8).

Progress in image analysis, coupled with automation of immunohistochemical procedures, now opens the way to refine anatomic pathology to more accurately determine the density and distribution of immune effectors within and around malignant cells. It seems more than plausible that efforts in standardization, robotization, computerization, and ever-more sophisticated image analysis algorithms will render the immunohistochemical analysis of tumor infiltrates more robust and more objective. Thorough analysis of immune infiltrates is possible by technologies that allow quantification of thousands of parameters, including those that might easily escape the pathologist's eye, such as the cytoplasmic and/or nuclear ratio of each infiltrating lymphocyte (which is an indirect parameter of activation); the shape of antigen-presenting cells (which become increasing complex when DCs mature); the distance between dying tumor cells and immune effectors; the presence of synaptic contacts between different cell types; and the 3-dimensional reconstruction of groups of cells that form secondary and tertiary lymphoid structures. It is our hope that such methodologic improvements will generate the scientific resources to further explore immune contexture and finally open the avenue for successful prospective trials, hence facilitating their implementation in the clinical management of malignant diseases.

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No potential conflicts of interest were disclosed.

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