Human Solid Tumors Contain High Endothelial Venules: Association with T- and B-Lymphocyte Infiltration and Favorable Prognosis in Breast Cancer

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Abstract

The mechanisms governing infiltration of lymphocytes into tumors remain poorly characterized, in spite of the critical impact of these cells on patient prognosis and therapeutic responses. High endothelial venules (HEV) are blood vessels found in lymphoid tissues, specialized in lymphocyte recruitment, but their implications in human cancer are unknown. In this article, we report the presence of MECA 79+ blood vessels displaying all the phenotypic characteristics of HEVs in most of the 319 human primary solid tumors, including melanomas, breast, ovarian, colon, and lung carcinomas, analyzed. Tumor HEVs were specifically located within lymphocyte-rich areas, and their density within the tumor stroma was a strong predictor of infiltration by CD3+ and CD8+ T cells as well as B cells. Large-scale flow cytometric and quantitative reverse transcriptase-PCR analyses in freshly operated breast tumors revealed that high densities of tumor HEVs correlated with increased naive, central memory and activated effector memory T-cell infiltration and upregulation of genes related to T-helper 1 adaptive immunity and T-cell cytotoxicity. Finally, in a retrospective cohort of 146 invasive breast cancer patients, we found that high densities of tumor HEVs independently conferred a lower risk of relapse and significantly correlated with longer metastasis-free, disease-free, and overall survival rates. Together, our findings suggest that tumor HEVs function as major gateways for lymphocyte infiltration into human tumors, and may represent attractive targets for cancer diagnosis and therapy. Cancer Res; 71(17); 1–10. ©2011 AACR.

Introduction

The immune system plays a critical role in tumor surveillance (1), patient’s clinical outcome (2), and therapeutic response (3, 4). The presence of high numbers of tumor-infiltrating lymphocytes, particularly T cells, has been found to be a major predictor of favorable clinical outcome in several solid cancers, including colorectal (5–7), lung (8), and ovarian carcinomas (9–11). However, the blood vessels and mechanisms governing the recruitment of lymphocytes into tumors remain poorly understood.

The first critical step in lymphocyte migration from circulation into tissue is the adhesion of lymphocytes to vascular endothelium (12). Evidence has recently been provided that overexpression of the endothelin B receptor on tumor blood vessels functions as a barrier that inhibits lymphocyte adhesion to endothelium and restricts lymphocyte infiltration into tumors (13). In addition, tumor angiogenesis has been shown to induce a downregulation of endothelial cell-adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1; ref. 14). In contrast, the characteristics of tumor blood vessels which facilitate large-scale influx of lymphocytes within human tumors are presently unknown.

High endothelial venules (HEV) are specialized post-capillary venules found in lymphoid tissues that support high levels of lymphocyte extravasation from the blood (15, 16). HEVs are composed of plump, cuboidal endothelial cells, expressing 6-sulfosialyl Lewis X ligands for the lymphocyte-homing receptor l-selectin (CD62L), that mediate the initial capture and rolling interactions of lymphocytes along the vessel wall (15–17). Although HEVs are generally restricted to lymphoid organs, evidence accumulated over the past 25 years indicates that blood vessels with HEV characteristics develop in nonlymphoid tissues in many chronic inflammatory diseases, including rheumatoid arthritis, inflammatory bowel diseases, chronic gastritis, and autoimmune thyroiditis (15, 18–20).

In the present study, we investigated the presence of HEVs within various human primary solid tumors and their association with lymphocyte infiltration, immune orientation, and clinical outcome in breast cancer.
Materials and Methods

Patients
This study was approved by the Scientific Review Board of the Institute Claudius Regaud (ICR; Toulouse, France). The prospective study on HEVs in human solid tumors was conducted on paraffin-embedded tumor blocks with representative tumor areas of 18 primary melanomas, 5 primary colon carcinomas, 5 primary lung carcinomas, 18 primary ovarian carcinomas, and 127 primary breast carcinomas, operated on between 2003 and 2010. The retrospective study was conducted with a cohort of 146 unselected, primary, nonmetastatic, invasive breast cancer patients operated at the ICR between 1997 and 1998. Patient characteristics are described in Supplementary Table S1. None of the patients analyzed in our study received chemotherapy or radiotherapy before surgery. They did not have previous history of cancer and did not present distant metastasis at the time of surgery. Postsurgical surveillance of patients was carried out at the ICR according to general standard practice for breast cancer patients. The median follow-up was 122 months, during which there were 61 relapses including 52 metastatic relapses and 50 deaths.

Immunohistochemistry and immunofluorescence staining
Immunohistochemistry was carried out on 5-μm-thick consecutive sections from paraffin-embedded tumor blocks using a TechMate Horizon slide processor (Dako). Details of the antibodies, fixatives, and antigen-retrieval methods used are provided in Supplementary Table S2. Briefly, slides were incubated with primary antibodies for 1 hour at room temperature. Antigen–antibody complexes were visualized using a peroxidase-conjugated polymer backbone coupled to a secondary antibody system (EnVision; Dako) and 3,3′-diaminobenzidine chromogen (Dako). For immunofluorescence detection, slides were incubated with secondary antibodies coupled to AF-488 or Cy3, diluted in PBS/bovine serum albumin (BSA) 1%, for 1 hour at room temperature and counterstained with 4′,6-diamidino-2-hemylindole (DAPI).

Method for cell quantification
Tumor slides stained with MECA-79, anti-CD3, anti-CD20, and anti-CD8 antibodies were scanned with a high-resolution scanner (NDP Slide scanner; Hamamatsu). Absolute numbers of MECA-79 vessels present within the tumor area (mm²) were quantified for each tumor slide, and the densities of tumor HEVs (HEV/mm²) were calculated. Because no cutoff point has been previously described for density of tumor HEVs, we discriminated patients with a high and a low density of tumor HEVs with the following cutoff points (highest tercile vs. 2 lowest terciles: 0.19 HEV/mm²). Automatic cell counts of CD3⁺, CD20⁺, and CD8⁺ cells were determined on 10 representative tumor fields (4.4 mm²; original magnification 5×; 2,560 × 2,048 resolution) with Image J Software (NIH, Bethesda, MD). Furthermore, CD3⁺, CD20⁺, and CD8⁺ cells were counted semiquantitatively (score: 0, 1, 2, and 3 for none, low, intermediate, and high density of positive cells) to validate automatic cell count. The density of CD34⁺ blood vessels was calculated by optical counting of vessel numbers on 10 representative tumor fields (0.5 mm²; original magnification 10×). Quantification of vessels and scoring were done by 3 independent observers who were blinded to the clinical outcome. We used the following cutoff points (highest tercile vs. 2 lowest terciles) to discriminate high and low densities of the different cell populations: CD3⁺ T cells, 270 cells/mm²; CD8⁺ T cells, 150 cells/mm²; and CD20⁺ B cells, 135 cells/mm².

Large-scale flow cytometric analysis
Freshly resected breast carcinoma samples were reduced to small fragments and incubated for 30 minutes at 37°C in sterile RPMI-1640 containing Collagenase IV (1 mg/mL; Sigma-Aldrich). Total cells were then extracted by mechanical dispersion and incubated for 30 minutes at 4°C with antibodies directed against markers for different immune cells or their isotype-matched controls (Supplementary Table S3). Analyses were carried out on a 6-color fluorescence-activated cell sorter (LSRII; Beckton Dickinson) with Diva Software (Beckton Dickinson). Overall, 120 combinations of surface and intracellular markers were used to identify the different populations of tumor-infiltrating immune cells (Supplementary Fig. S1). Heat-map representation of populations of immune cells expressed as a percentage of total cells extracted from tumor tissues was realized with the use of dChip Software (Harvard School of Public Health, Boston, MA).

Quantitative reverse transcriptase-PCR
An RNeasy Isolation kit (QiAGEN) was used to isolate total RNA from 20 cryopreserved breast tumor samples with a low or high density of tumor HEVs. The integrity and the quantity of the RNA were evaluated using 2100 Bioanalyzer (Agilent Technologies). cDNA was prepared by reverse transcription using superscript VILO cDNA Synthesis Kit (Invitrogen). Reverse transcriptase (RT)-PCR experiments were conducted using Power SYBR Green Master Mix with an ABI PRISM 7300HT (Applied Biosystems) according to the manufacturer’s instructions. All reactions were done in duplicate and normalized to the expression of glyceraldehyde phosphate dehydrogenase (GAPDH). For each gene, relative change in expression was calculated by the ΔΔct method with ΔΔct = average Δct from HEV-low tumors.

Statistical analysis
We analyzed 3 main endpoints: metastasis-free survival rate, which was defined as time from surgery to distant metastases (all other events were ignored for this endpoint); disease-free survival rate, that is, time from surgery to any recurrence (local or regional), second breast primary, distant metastasis, or death from any cause; and overall survival rate, which was defined as time from surgery to death from any cause. Categorical variables were reported by frequencies and percentages; continuous variables were presented by median and range. Comparative analyses between groups were done using the Mann–Whitney rank-sum test for continuous variables and the χ² test or Fisher’s exact test for categorical
variables. Correlations between continuous variables were evaluated using Spearman’s rank correlation test. The Kaplan–Meier product-limit estimator was used to display time-to-event curves for the 3 endpoints. The significance of various clinical characteristics and density of HEVs was assessed by univariate analysis with the use of the log-rank test. The Cox regression model was applied to determine whether a factor was an independent predictor of survival in multivariate analysis. Two-sided \( P \) values of less than 0.05 were considered statistically significant. Statistical analyses were done using the STATA 11.0 (STATA Corp) software.

Results

Human solid tumors contain blood vessels with HEV characteristics

To analyze the presence of HEVs within human solid tumors, we carried out immunohistochemistry on 173 primary tumor sections with MECA-79 (for details on the MECA-79 epitope, see Supplementary Fig. S2), an HEV-specific monoclonal antibody which recognizes sulfated ligands for lymphocytes and inhibits lymphocyte–HEV interactions \textit{in vivo} (17, 21, 22). Vessels expressing high levels of the MECA-79 epitope were observed in the majority of tumors analyzed (11 of 18 melanomas, 94 of 127 breast, 11 of 18 ovary, 4 of 5 lung, and 4 of 5 colon carcinomas; Fig. 1A). In contrast, MECA-79\(^+\) vessels were not detected in normal control tissues distant from the tumor site. Immunofluorescence staining of tumor sections revealed that MECA-79\(^+\) vessels are found in tumor areas infiltrated by both CD3\(^+\) T cells and CD20\(^+\) B cells (Fig. 1B). CD3\(^+\) T cells were frequently seen extravasating or attached to the luminal surface of the plump, cuboidal MECA-79\(^+\) endothelial cells, suggesting an active role of these vessels in lymphocyte recruitment (Fig. 1C). These endothelial cells expressed HEV markers HECA-452 (23) and DARC (24), the endothelial cell adhesion molecule ICAM-1, and pan-vascular markers CD31 and von Willebrand factor (vWB; Fig. 1D). Interestingly, tumor-associated MECA-79\(^+\) vessels were also labeled by...
Figure 2. The density of tumor HEVs predicts T- and B-lymphocyte infiltration in breast cancer. A, representative photograph from digitized tumor slide stained with MECA-79 antibody showing HEVs (arrows) within breast tumor stroma. B, absolute number and density of MECA-79+ HEVs in the tumor area for each patient of the retrospective breast cancer cohort (n = 146). The red bar indicates the cutoff value used to discriminate high and low densities of HEVs (highest tercile vs. 2 lowest terciles). C, the density of CD34+ blood vessels is similar in HEVlow and HEVhigh breast tumors. The line in the center of each box represents the median value of the distribution, and the upper and lower ends of the box are the upper and lower quartiles, respectively. D, tumors containing a high density of tumor HEVs exhibit high levels of infiltrating CD3+ T cells, CD8+ T cells, and CD20+ B cells. Consecutive breast tumor sections from representative HEVhigh and HEVlow tumors were analyzed by immunohistochemistry with MECA-79, anti-CD3, anti-CD8, and anti-CD20 antibodies. E, the density of CD3+ T cells, CD8+ T cells, and CD20+ B cells is significantly higher in HEVhigh than in HEVlow breast tumors. The line in the center of each box represents the median value of the distribution, and the upper and lower ends of the box are the upper and lower quartiles, respectively. Original magnification (A, 1.25×; B, 10×; C, 10×; and D, 5×).
patients (Fig. 2A). The number and the density of MECA-79-defined as HEVlow. Correlational analysis between the density cutoff) and tumors with the lowest density of HEVs were aimed. Links between HEV density and tumor angiogenesis, we adjuvant chemotherapy (Table 1). Aiming to examine potential differences in the density of tumor HEVs and clinicopathologic parameters for these patients revealed no statistical association between HEV density and tumor size, grade, nodal or hormonal receptor status, or adjuvant chemotherapy (Table 1). Aiming to examine potential links between HEV density and tumor angiogenesis, we quantified the microvessel density of breast tumor sections stained with antibodies against the pan-vascular marker CD34. We found no correlation between HEVs and CD34+ blood vessels present within breast tumor stroma (Fig. 2C), indicating that differences in the density of tumor HEVs are not related to differences in tumor angiogenesis.

Because HEVs are specifically located within lymphocyte-rich tumor areas, we then asked whether the density of tumor HEVs might correlate with lymphocyte infiltration. We quantified CD3+ T cells, CD8+ T cells, and CD20+ B cells using optical grading and automatic cell count of tumor sections of our breast cancer retrospective cohort (Supplementary Fig. S3). We observed a strong correlation between the density of tumor HEVs and tumor-infiltrating CD3+ T cells (p = 0.71; P < 0.001), CD8+ T cells (p = 0.66; P < 0.001), and CD20+ B cells (p = 0.73; P < 0.001; Fig. 2D and E; Supplementary Table S4). These results were confirmed with a prospective cohort of 42 breast cancer patients (Supplementary Fig. S4), indicating that tumor HEVs were associated with high levels of T-and B-lymphocyte infiltration in human breast tumors.

To further characterize the immune populations associated with tumor HEVs, we conducted large-scale flow cytometric analyses on 30 freshly resected breast tumors. Tumors were classified into HEVhigh or HEVlow group according to the density of MECA-79+ HEVs, quantified on adjacent paraffin-embedded breast tumor sections. Immune populations and T-cell differentiation, activation, and functions were analyzed using 120 combinations of surface and intracellular markers (Fig. 3A; Supplementary Fig. S5). We observed a strong increase in the percentage of total CD4+ and CD8+ T cells and CD20+ B cells in HEVhigh tumors as compared with HEVlow tumors (Fig. 3B). Naive T cells (e.g., CCR7+, CD44+, and CD45RA+CD62L+) and central memory T cells (e.g., CD45RA-CD62L+) that classically home toward secondary lymphoid organs through HEVs were greatly increased in HEVhigh tumors (Fig. 3B). In addition, effector memory T cells (e.g., CD45RA-CD62L-) and terminally differentiated T cells (e.g., TEMRA, CD45RA-CD62L-), T cells expressing activation markers (e.g., CD69, CD25, HLA-DR, CD86; refs. 5, 26), and cytotoxic CD8+ T cells containing granzyme A, granzyme B, and perforin were also present in larger proportion in HEVhigh breast tumors (Fig. 3B). Together, these data indicate that tumor HEVs are associated with increased numbers of both activated effector T cells and poorly differentiated T-cell populations, such as tumor-infiltrating naive and central memory T cells.

### Tumor HEVs correlate with T-helper 1 immune orientation

We then used quantitative real-time PCR to characterize the gene expression profile associated with the presence of tumor HEVs in breast cancer. Expression levels of genes related to lymphocyte migration, T-helper (Th1) cell orientation, cytotoxicity, and immune suppression were assessed in 10 cryopreserved breast tumor tissues with a high density of HEVs and 10 control tumors with a low density of HEVs, as determined by immunohistochemistry (Fig. 4). Genes encoding lymphoid chemokines (CCL19, CCL21, and CXCL13) and T-cell homing receptors (CCR7 and LSEL), which are

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**Table 1. Correlational analysis between the density of tumor HEVs and clinicopathologic data**

<table>
<thead>
<tr>
<th></th>
<th>Overall population</th>
<th>n (%)</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>HEVlow</td>
<td>HEVhigh</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>97</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0–0.189</td>
<td>0.197–10.658</td>
<td></td>
</tr>
<tr>
<td>Median</td>
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<td>0.614</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>Negative</td>
<td>74 (50.7)</td>
<td>51 (52.6)</td>
<td>23 (46.9)</td>
</tr>
<tr>
<td>Positive</td>
<td>72 (49.3)</td>
<td>46 (47.4)</td>
<td>26 (53.1)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>I-II</td>
<td>76 (52.1)</td>
<td>54 (55.7)</td>
<td>22 (44.9)</td>
</tr>
<tr>
<td>III</td>
<td>70 (47.9)</td>
<td>43 (44.3)</td>
<td>27 (55.1)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>&lt;2 cm</td>
<td>71 (48.6)</td>
<td>45 (46.4)</td>
<td>26 (53.1)</td>
</tr>
<tr>
<td>≥2 cm</td>
<td>75 (51.4)</td>
<td>52 (53.6)</td>
<td>23 (46.9)</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
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<td>0.75</td>
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<tr>
<td>Negative</td>
<td>25 (17.7)</td>
<td>16 (17.0)</td>
<td>9 (19.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>116 (82.3)</td>
<td>78 (83.0)</td>
<td>38 (80.9)</td>
</tr>
<tr>
<td>Missing</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
<td>0.77</td>
</tr>
<tr>
<td>No</td>
<td>53 (36.3)</td>
<td>36 (37.1)</td>
<td>17 (34.7)</td>
</tr>
<tr>
<td>Yes</td>
<td>93 (63.7)</td>
<td>61 (62.9)</td>
<td>32 (65.3)</td>
</tr>
</tbody>
</table>

Abbreviation: ER, estrogen receptor.
associated with naive and central memory T- and B-lymphocyte migration through HEVs, were greatly upregulated in HEV-high tumors as compared with HEV-low tumors. In contrast, no significant differences between 2 two groups of breast tumors were observed for housekeeping genes (HPRT, ACTB, and YWHAZ). Transcripts of genes related to T-cell–cytotoxic granule components (PERF1, GZMA, GZMB, and GNLY) and TH1 orientation (IFNG and TBX21) were also statistically more abundant in tumors with a high density of HEVs. In contrast, the expression of genes associated with TH2 (IL4 and GATA3) or

Figure 4. Expression of genes related to lymphocyte migration, cytotoxicity, TH orientation, and immune escape determined in 20 breast tumors according to the density of tumor HEVs (10 HEV-high vs. 10 HEV-low). Relative mRNA expression levels were adjusted to the level of GAPDH mRNA for each sample. The levels are represented as mean (SD) relative expression for HEV-high (black bars) and HEV-low (white bars) tumors calculated by the ∆∆ct cycling threshold (CT) method. *, P < 0.05; **, P < 0.01; Mann–Whitney U test.
T_{H17} (RORG and IL17A) phenotypes, and that of genes related to immune escape such as macrophage- (TGFBI, CD14, and CD68) and myeloid-derived suppressor cell (CD11b, CD33, and COX2)-associated genes did not vary between HEV_{high} and HEV_{low} tumors. Together, these results indicate that a high density of HEVs in breast tumors is associated with the upregulation of genes involved in lymphocyte migration, T_{H1} adaptive immune response, and cytotoxic effector functions.

**Density of tumor HEVs predicts clinical outcome in breast cancer**

Tumor HEVs are associated with cytotoxic T-cell infiltration and T_{H1} immune orientation, previously identified as critical for antitumor immunity both in human and mouse studies (1, 2); therefore, we next analyzed the clinical impact of HEVs in our retrospective cohort of breast cancer patients. Univariate analysis indicated that patients with a high density of tumor HEVs had a significantly longer disease-free survival rate ($P = 0.01$), metastasis-free survival ($P = 0.004$), and overall survival ($P = 0.02$) than patients with HEV_{low} tumors (Fig. 5A; Supplementary Table S5). Using the "minimum $P$ value" approach, we verified that the density of tumor HEVs was significantly correlated with metastasis-free survival rate for a large interval of cutoffs (Supplementary Fig. S6).

Density of tumor HEVs also showed a significant correlation with disease-free, metastasis-free, and overall survival rates in multivariate analysis after adjustments using prognostic factors previously identified (Table 2). The adjusted HRs of disease-free, metastasis-free, and overall survival rates for patients with HEV_{low} tumors versus HEV_{high} tumors were 2.68 (95% CI: 1.41–5.10; $P = 0.003$), 3.28 (95% CI: 1.56–6.87; $P = 0.002$), and 2.43 (95% CI: 1.16–5.06; $P = 0.018$), respectively.

Furthermore, we investigated the prognostic significance of tumor HEVs in the lymph node-positive group (N+ ) of breast tumors ($n = 72$). N+ patients with HEV_{high} tumors had significantly longer disease-free survival rate ($P = 0.02$) and metastasis-free survival rate ($P = 0.03$) than patients with HEV_{low} tumors (Fig. 5B; Supplementary Table S5). Multivariate analysis in N+ patients showed that a low density of tumor HEVs independently conferred a significantly higher risk of relapse with adjusted HRs of 2.48 (95% CI: 1.16–5.30; $P = 0.019$) and 2.43 (95% CI: 1.09–5.40; $P = 0.029$) for disease-free survival and metastasis-free survival rates, respectively (Supplementary Table S6). Absolute number of HEVs within tumor stroma, similar to HEV density, significantly predicted disease-free and metastasis-free survival rates for both global and N+ population of breast cancer patients (Supplementary Fig. S7).

**Figure 5.** The density of tumor HEVs predicts survival in breast cancer. A and B, Kaplan–Meier curves for disease-free, metastasis-free, and overall survival rates of 146 patients with primary breast cancer (A) or 72 node-positive breast cancer (B) patients, according to the density of tumor HEVs (HEV_{high}, black line; HEV_{low}, gray line).

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Discussion

Lymphocyte migration into tumors remains poorly understood, in spite of the critical impact of these cells on patients’ clinical outcomes (7–10). Our study shows, for the first time, that HEVs, blood vessels specialized in lymphocyte recruitment, are frequently found in human solid tumors, predict lymphocyte infiltration, immune orientation, and independently correlate with favorable clinical outcome in both global and N⁺ populations of breast cancer patients.

Our results suggest that vascular specialization plays a critical role in the control of lymphocyte infiltration into tumors. Tumor HEVs are structurally and phenotypically similar to HEVs from patients suffering from autoimmune or chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, and autoimmune thyroiditis (15, 18, 19). The presence of HEVs in these pathologies has been shown to participate in the development and the maintenance of chronic inflammation through the recruitment of large numbers of lymphocytes (27, 28). In contrast, HEVs have never been observed in normal nonlymphoid tissues. Therefore, HEVs may represent a local vascular response facilitating the rapid transfer of lymphocytes into both chronically inflamed tissues and tumor tissues.

We observed that HEV density within breast tumor stroma was not correlated with microvessel density. In addition, whereas microvessel density in breast tumors correlated with poor clinical outcome in breast cancer (29), a high density of HEVs within tumor stroma was significantly associated with longer disease-free survival rate of breast cancer patients. These results reinforce the importance of better identifying blood vessel subtypes present within tumor stroma to better discriminate between their pro- and antitumor properties.

The favorable impact of effector memory T-cell and cytotoxic T-cell infiltration on patients’ clinical outcomes has been showed in several types of cancer, including melanoma, colon, and ovarian carcinoma (5–8, 10, 11, 30, 31). The association between high densities of tumor HEVs and the presence of high numbers of these lymphocytes could, thus, explain the prognosis value of HEVs in breast cancer. In addition, recruitment of naive and central memory T cells through the specific expression of sulfated l-selectin ligands on tumor HEVs may contribute to the clinical impact of HEVs. Indeed, these undifferentiated populations of T cells, that have the ability to undergo self-renewal, have been shown to provide superior, long-term, antitumor response (32, 33).

We can speculate that recruitment of naive T cells through tumor HEVs and the generation, in the tumor vicinity, of high numbers of long-term, tumor antigen–specific central memory T cells and effector memory T cells with a TH1 orientation capable of re-circulating throughout the body, may limit the establishment of metastasis in distant organs. In agreement with this hypothesis, mouse tumor models that stimulate recruitment of naive T cells and priming at the tumor site have been shown to promote both primary tumor and distant metastasis regression (34, 35). Furthermore, high densities of tumor HEVs were associated with B-lymphocyte infiltration within tumor tissues. Activated B lymphocytes have been shown to critically impact T-cell activation and polarization through antigen presentation, costimulatory molecule

Table 2. Multivariate analysis using the Cox proportional hazard model

<table>
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<th>Disease-free survival</th>
<th>Overall survival</th>
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<tr>
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<td>HR [95% CI]</td>
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<tr>
<td>HEV/mm²</td>
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<tr>
<td>Low</td>
<td>2.68 [1.41–5.10]</td>
</tr>
<tr>
<td>High</td>
<td>1</td>
</tr>
<tr>
<td>Tumor size</td>
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</tr>
<tr>
<td>&lt;2 cm</td>
<td>1</td>
</tr>
<tr>
<td>≥2 cm</td>
<td>3.44 [1.82–6.47]</td>
</tr>
<tr>
<td>Lymph node invasion</td>
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</tr>
<tr>
<td>N⁺</td>
<td>2.49 [1.31–4.70]</td>
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<tr>
<td>I–II</td>
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</tr>
<tr>
<td>III</td>
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<td>Yes</td>
<td>1.64 [0.86–3.13]</td>
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Abbreviation: ER, estrogen receptor.
expression, and cytokine production (36). Recruitment of B lymphocytes through tumor HEVs could, thus, be critical for efficient antitumor T-cell responses.

Recent studies have established that the immune system actively participates in the clinical efficacy of conventional antitumor treatments such as chemotherapy and radiotherapy (3, 4). These observations give a rationale for therapeutic manipulation of immune components. Nonetheless, immune-based therapies tested so far have obtained limited results (37), mainly because of the inability of effector immune cells to reach tumor tissues. Several mechanisms that restrict lymphocyte migration into tumors have previously been proposed (13, 14).

In contrast, our study is the first to describe a mechanism which facilitates lymphocyte infiltration into tumors—the presence of HEVs within the tumor microenvironment. By their ability to recruit large numbers of circulating lymphocytes, tumor HEVs may represent new attractive targets for both cancer diagnosis and therapy. It will be important in future studies to further define the influence of tumor HEVs on clinical outcome, and to identify potential links between the density of tumor HEVs and the response of patients to chemotherapy.

Although HEV endothelial cells exhibit remarkable plasticity and rapidly lose their specialized characteristics outside their natural tissue microenvironment (38), the factors and mechanisms involved in the induction and maintenance of HEVs remain poorly understood. A better understanding of these factors may provide new opportunities to increase lymphocyte infiltration into tumors and enhance antitumor immune mechanisms.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

L. Martinet and I. Garrido designed research, carried out research, and analyzed data; T. Filleron, S. Le Guellec, and P. Rochaix carried out research and analyzed data; E. Bellard carried out research; J-J. Fournie analyzed data; and J-P. Girard designed research, analyzed data, and wrote the manuscript.

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