High Endothelial Venule Blood Vessels for Tumor-Infiltrating Lymphocytes Are Associated with Lymphotoxin β–Producing Dendritic Cells in Human Breast Cancer

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High Endothelial Venule Blood Vessels for Tumor-Infiltrating Lymphocytes Are Associated with Lymphotoxin β–Producing Dendritic Cells in Human Breast Cancer

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Blood vessels and tumor angiogenesis are generally associated with tumor growth and poor clinical outcome of cancer patients. However, we recently discovered that some blood vessels present within the tumor microenvironment can be associated with favorable prognosis (3). These specialized blood vessels, designated tumor high endothelial venules (HEVs), are normally found in lymph nodes where they mediate the extravasation of large numbers of lymphocytes from the blood (4–6). In human breast tumors (3) and primary melanomas (7), the density of tumor HEVs was highly correlated with the density of tumor-infiltrating CD3+ T cells, CD8+ cytotoxic T cells, and CD20+ B cells, indicating that, like in lymph nodes, HEVs may function as major gateways for lymphocyte infiltration into solid tumors (3, 7–9). In contrast, we found no correlation between tumor HEVs and density of blood vessels (HEVhigh and HEVlow tumors had the same numbers of CD34+ tumor blood vessels), indicating that differences in the density of tumor HEVs are not related to differences in tumor angiogenesis (3).

By allowing infiltration of naïve, memory, cytotoxic, and activated T cells (3, 8, 9), tumor HEVs may facilitate destruction of tumor cells and generation of memory T cells that limit metastasis of tumor cells at distant sites. Indeed, in breast cancer, we found that high densities of tumor HEVs were associated with longer disease-free and metastasis-free survival of the patients (3). Tumor HEVs were also associated with good clinical characteristics in primary melanomas, including thin Breslow thickness and low Clark level of invasion and tumor regression (7). Therefore, although blood vessels are generally believed to promote tumor growth, our studies showed that the phenotype of blood vessels is important and that some types of blood vessels present in the tumor microenvironment (i.e., tumor HEVs) can contribute to tumor suppression rather than tumor growth. Tumor HEVs have been observed in many different types of human solid tumors, including primary and metastatic melanomas, breast, lung, ovarian, and colon carcinomas (3, 7, 10, 11). A better understanding of tumor HEVs could thus have broad applications for human cancer.

Interestingly, tumor HEVs have been detected in murine B16 melanoma tumors after targeting of lymphotoxin α (LTα) within the tumor (12, 13), and more recently in methylcholanthrene-induced fibrosarcomas upon depletion of regulatory T cells (Tregs) in FoxP3-DTR transgenic mice (14). In both cases, the presence of tumor HEVs in the tumors was associated with T cell infiltration and tumor regression (12–14). It is currently unknown whether Tregs...
regulate HEV differentiation directly. In contrast, CD11c⁺ dendritic cells (DCs) were recently shown to play a critical role in this process (15). In vivo depletion of DCs in adult mice resulted in a profound downregulation of the HEV phenotype in mouse lymph nodes. Coculture experiments revealed that lymphotixin B receptor (LTβR) signaling is involved in the dialog between DCs and HEV endothelial cells (15). DCs expressed lymphotixin ligands for LTβR, and DC-derived lymphotixin was found to be important for HEV-mediated extravasation of lymphocytes in lymph nodes (15). DCs and LTβR signaling have also been implicated in the regulation of HEVs in murine inflamed lymph nodes and chronically inflamed nonlymphoid tissues (16–23). Despite these important advances in mouse models, little is known yet about the mechanisms governing the development of HEVs in human solid tumors.

In this study, we investigated the mechanisms regulating HEV blood vessels in human breast cancer. We show that high densities of tumor HEVs in breast tumors were associated with high expression levels of lymphotixin β (LTβ) and high densities of DC-LAMP⁺ DC clusters. LTβ expression was strongly correlated with expression of DC-LAMP, and CD11c⁺ DCs were found to be the major producers of membrane-bound LTβ (Lta1β2) in the breast tumor microenvironment. Tumor HEVs were often surrounded by Fascin⁺ and DC-LAMP⁺ DCs within breast tumor stroma, and the density of DC-LAMP⁺ DC clusters was strongly correlated with the density of tumor HEVs, T and B cell infiltration, and favorable clinical outcome of breast cancer patients. Strikingly, a progressive loss of tumor HEVs and DC-LAMP⁺ DCs was observed during breast cancer progression from in situ to invasive carcinoma. Finally, densities of Tregs and tumor HEVs were correlated in human breast tumors. However, the Tregs/CD3⁺ T cells ratio was significantly reduced in HEV⁺ high breast tumors.

Materials and Methods

Patients

Fresh, frozen, and paraffin-embedded breast tumor samples were all obtained from breast cancer patients undergoing surgery at the Institut Claudius Regaud (ICR) in Toulouse, France. Approval of the study was obtained from the Scientific Review Board of the ICR. A written informed consent was obtained from the patients before inclusion in the study. The retrospective study was conducted with a cohort of 146 unselected primary invasive breast cancer patients operated at ICR between 1997 and 1998 (3). Patient characteristics have been previously described (3) and are summarized in Supplemental Table I. Experiments also involved tissue samples from 110 patients operated at ICR between 2004 and 2012, including invasive ductal carcinomas (IDC; n = 66), ductal carcinomas in situ (DCIS; n = 29), and nonmalignant breast tissues (n = 15).

Immunohistochemistry and immunofluorescence staining

Immunohistochemistry was performed on 5-μm consecutive sections from RCL2, formalin- or Dubosq-fixed, paraffin-embedded tumor blocks using a Technate Horizon slide processor (Dako, Trappes, France) as previously described (3). Details of the Abs, fixatives, and Ag retrieval methods used are provided in Supplemental Table II. For immunofluorescence detection, tumor slides were incubated with fluorochrome- or biotin-coupled secondary Abs (MAB1684; R&D, Minneapolis, MN) for 30 min at 4°C, followed by incubation with FITC-conjugated goat anti-mouse IgG secondary Abs (Jackson Immunoresearch, Suffolk, U.K.) for 30 min at 4°C. Fluorochrome-conjugated mAbs directed against CD3, CD20, CD56, CD11c, and CD45 were then added for 30 min at 4°C. Analyses were performed on a six-color fluorescein-activated cell sorter (LSRII; Becton Dickinson) with Diva software (Becton Dickinson) and FlowJo softwares (Tree Star, Ashland, OR).

For cell sorting, total cells from freshly resected HEV⁺ high breast tumor samples (n = 7), extracted as described earlier, were pooled together and

![Image](http://www.jimmunol.org/)

**FIGURE 1.** Expression of LTβ is increased in HEV⁻ high breast tumors and highly correlated with expression of HEV-associated chemokines. (A) Graphs showing the relative mRNA levels of LTβR signaling pathway genes in HEV⁻ high (n = 10, black bars) and HEV⁻ low (n = 11, white bars) breast tumor samples. (B) Graphs showing the correlation between the relative mRNA levels of LTβ coding for LTβ (B) and HEV-associated chemokines (CCL19, CCL21, or CXCL13) in breast tumor samples, and the absence of correlation between expression of LTβ and expression of chemokines CCL2, CCL12, or CXCL12. *p < 0.05, **p < 0.01, Mann–Whitney U test.
incubated for 30 min at 4°C with fluorochrome-conjugated mAbs directed against different immune and tumor cell markers. Tumor cells (EpCAM+CD45+), T lymphocytes (CD45+CD3+), B lymphocytes (CD45+CD20+), NK cells (CD3+CD56+), and DCs (CD45+, lin−, HLA-DR+, CD11c+, CD1A+) were isolated by flow cytometry using a FACSARia II cell sorter.

Quantitative RT-PCR

An RNase isolation kit was used to isolate total RNA (Qiagen, Valencia, CA) from 22 cryopreserved breast tumor samples (12 HEVlow versus 10 HEVhigh, identified by immunohistochemistry with MECA-79) and cells isolated by cell sorting as described earlier. The integrity and the quantity of the RNA were evaluated using a bioanalyzer-2100 (Agilent Technologies, Palo Alto, CA). cDNA was prepared by reverse transcription using superscript VILO cDNA Synthesis Kit (Invitrogen, Paisley, U.K.). Quantitative RT-PCR (qRT-PCR) experiments were performed using Power SYBR Green mix with an ABI PRISM 7300HT (Applied Biosystems, Warrington, U.K.) according to manufacturer’s instructions. All reactions were done in triplicate and normalized to the expression of GAPDH. Heat map representation of gene expression in tumor tissues containing a high (HEVhigh) or a low density of HEVs (HEVlow) was performed with the use of Genesis software (Institute for Genomics and Bioinformatics, Graz, Austria) (24, 25). The expression of each gene was obtained by the Δ cycling threshold (CT) method as 2^−ΔΔCTsample, and the relative change in expression between HEVhigh and HEVlow tumor samples was calculated as 2^−ΔΔCTsample = average ΔCT from HEVlow tumors/average ΔCT from HEVhigh tumors.

Statistical analysis

Categorical variables were reported by frequencies and percentages; continuous variables were presented by median and range. Comparisons between groups were performed using the Mann–Whitney rank sum test for continuous variables and χ^2 test for categorical variables. Correlations between continuous variables were evaluated using Spearman rank correlation test.

We analyzed two main end points: disease-free survival (DFS), which was defined as time from surgery to any recurrence (local or regional or distant metastasis) or death, and overall survival (OS), which was defined as time from surgery to death from any cause. The Kaplan–Meier product-limit estimator was used to display time-to-event curves for the two end points. Comparisons between groups were performed using log rank test. Cox regression model was applied to determine whether a factor was an independent predictor of survival in multivariate analysis (with backward variable elimination). All p values were two sided, and statistical significance was defined as p < 0.05. Statistical analyses were performed using the STATA 11.0 (STATA Corp, College Station, TX) software.

Results

LTβ is overexpressed in HEVhigh breast tumors

Previous studies in mouse have demonstrated that maintenance of HEVs depend on LTβR signaling initiated by its cell-associated ligand LTα1β2, an heterotrimer formed by the membrane-associated subunit LTα, and LTβ, which is critical for cell-surface expression of the complex (15, 16, 18). We thus analyzed the mRNA levels of LTBR and its ligands LTA (encoding LTα1), LTB (encoding LTβ), and LIGHT (an alternative LTBR ligand) by qRT-PCR in HEVhigh and HEVlow breast tumor samples. Levels of LTBR and LIGHT mRNA were not significantly different between HEVhigh and HEVlow tumors. A small but significant increase in LTA was detected in HEVhigh tumors (Fig. 1A), whereas LTB was specifically and strongly overexpressed (>10-fold) in HEVhigh breast tumors (Fig. 1A). Interestingly, LTB relative expression within breast tumor...
samples was highly correlated to expression of chemokines associated with HEV-mediated lymphocyte extravasation (4, 10, 14, 26, 27), including CCL21 (Spearman r = 0.78, p < 0.001), CCL19 (Spearman r = 0.91, p < 0.001), and CXCL13 (Spearman r = 0.78, p < 0.001) (Fig. 1B). In contrast, LTβ mRNA levels were not correlated (p > 0.05) to mRNA levels of chemokines implicated in myeloid cell, neutrophil, and lymphocyte migration independently of HEVs (CCL2, CXCL8, CXCL12; Fig. 1B).

**DCs are the major producers of LTβ within the breast tumor microenvironment**

To identify the cells that produce membrane-associated LTβ (LTβ1β2) within breast tumors, we used flow cytometry to analyze cell-surface expression of LTβ on cells isolated from freshly resected breast tumor samples containing high densities of tumor HEVs and large numbers of tumor-infiltrating lymphocytes (HEVhigh breast tumors). Control experiments were performed on blood mononuclear cells from healthy donors stimulated or not with anti-CD3/anti-CD28–coated beads to induce LTβ expression (Supplemental Fig. 2). In HEVhigh breast tumors, ~30% of CD11c+ DCs expressed membrane-bound LTβ (Fig. 2A, 2B). In contrast, very few CD45+ tumor cells, CD3+ CD56+ NK cells, CD3+ T cells, and CD20+ B cells had detectable LTβ expression. To confirm these results, we used qRT-PCR to analyze LTβ mRNA expression in tumor cells, NK cells, T cells, B cells, and DCs isolated from HEVhigh breast tumor samples by cell sorting (Supplemental Fig. 2). We found that LTβ mRNA levels were significantly higher in DCs than in other immune cell populations (Fig. 2C) and were strongly correlated to the levels of mature DC marker DC-LAMP in breast tumor samples (Fig. 2D). Similarly to LTβ, DC-LAMP expression in breast tumor samples was correlated (p < 0.001) to expression of HEV-associated chemokines CCL19, CCL21, and CXCL13, but not

**FIGURE 3.** Tumor HEVs are associated with DC-LAMP+ DCs clusters in human breast tumors. (A) DC-associated genes are upregulated in HEVhigh breast tumors. Expression levels of the indicated genes were determined by quantitative PCR and compared between 22 human breast tumor samples (10 HEV high versus 12 HEVlow). Heat map representations of the DC and “angiogenesis” gene clusters are shown. Genes are plotted from the minimal level of expression (green) to the maximal level (red). The Mann–Whitney U test was used to compare the expression levels of each gene between tumor groups. n.s., p > 0.05. (B and C) Immunofluorescence staining of breast tumor sections with Abs against HEV (MECA-79) and mature DC markers Fascin (B) and DC-LAMP (C). DNA was stained with DAPI (B) and T cells with an anti-CD3 Ab (C). Original magnifications x100. (D) Immunohistochemical detection of DC-LAMP in breast tumors containing either low (DC-LAMPlow) or high (DC-LAMPhigh) densities of DC-LAMP+ cells. (E and F) Serial breast tumor sections (n = 146) were stained with Abs directed against DC-LAMP or HEV (MECA-79), and the density of the two cell types was calculated as described in Materials and Methods. Representative pictures of the spatial association between HEVs and DC-LAMP+ DCs clusters are shown (E). The density of tumor HEVs is significantly higher in breast tumors with a high density of DC-LAMP+ DC clusters (F). Original magnification (D) ×20; (E) ×10. ***p < 0.001, Mann–Whitney U test.
FIGURE 4. Density of DC-LAMP+ DCs correlates with HEV density, lymphocyte infiltration, and favorable clinical outcome of breast cancer patients. (A) Representative images from immunohistochemistry with DC-LAMP, MECA-79, CD3, and CD20 Abs showing the spatial association between DCs, HEVs, T cells, and B cells within breast tumor stroma. Original magnification \( \times 10 \). (B) The density of DC-LAMP+ DC clusters is correlated with the density of HEVs, CD3+ T cells, and CD20+ B cells in breast tumor samples. Serial breast tumor sections (n = 146) were stained with Abs directed against DC-LAMP, HEV (MECA-79), CD3, and CD20, and the density of the different cell types was calculated as described in the Materials and Methods. (C) Kaplan–Meier curves for DFS and OS rates of 146 patients with primary breast cancer, according to the density of DC-LAMP+ DCs. The rapid decrease in the DC-LAMPhigh DFS curve after 144 mo is due to the small number of patients at risk at this time.

We then analyzed DC-LAMP and MECA-79 markers by immunohistochemistry in a retrospective cohort of 146 primary invasive breast cancer patients, previously used to study the impact of tumor HEVs on clinical outcome (3). The density of DC-LAMP+ cells was variable within breast tumor microenvironment, allowing us to define tumors containing either low (DC-LAMPlow) or high (DC-LAMPhigh) densities of DC-LAMP+ cells (Fig. 3D). Tumor HEVs were mainly found in DC-LAMP+ cell–rich areas within tumor stroma (Fig. 3E), and significantly higher densities of tumor HEVs (Fig. 3F) were observed in DC-LAMPhigh breast tumors. We concluded that tumor HEVs were associated with DC-LAMP+ DCs within breast tumor stroma.

DC-LAMP+ DCs correlate with density of tumor HEVs, lymphocyte infiltration, and favorable clinical outcome of breast cancer patients

Significantly higher densities of CD3+ T cells and CD20+ B cells were found in breast tumors containing high densities of DC-LAMP+ DC clusters (Fig. 4A, Supplemental Fig. 1B). Indeed, the density of DC-LAMP+ DC clusters was strongly correlated with the density of tumor HEVs (Spearman \( r = 0.63, \) (p > 0.05) to that of chemokines CCL2, CXCL8, and CXCL12 (Fig. 2E). Together, these results indicated that CD11c+ DCs represent the main source of membrane-associated LTβ within human breast tumors.

Tumor HEVs are associated with DC-LAMP+ DCs within breast tumor microenvironment

When we compared the expression of genes related to immune subpopulations and angiogenesis, by qRT-PCR in cryopreserved breast tumor samples containing either low (HEVlow) or high (HEVhigh) densities of tumor HEVs (12 HEVlow versus 10 HEVhigh), we observed that the "DCs" cluster was significantly upregulated in HEVhigh breast tumors (Fig. 3A). In contrast, the "angiogenesis" cluster was not significantly different between tumors with a low or a high density of tumor HEVs (Fig. 3A). To further define the association between tumor HEVs and DCs within the breast tumor microenvironment, tissue sections were double stained with the HEV-specific mAb MECA-79 and Abs against two markers of mature DCs, Fascin and DC-LAMP. These immunofluorescence analyses revealed that tumor HEVs were often surrounded by mature Fascin+ DCs (Fig. 3B) and DC-LAMP+ DCs (Fig. 3C).

We used the Kaplan–Meier method to estimate the DFS and OS of patients with primary breast cancer according to the density of DC-LAMP+ DCs. The rapid decrease in the DC-LAMPhigh DFS curve after 144 mo is due to the small number of patients at risk at this time.

**Table I. Multivariate analysis using Cox proportional hazard model**

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<th>DFS</th>
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<td></td>
<td>HR 95% CI p (Wald)</td>
<td>HR 95% CI p (Wald)</td>
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<td>DC-Lamp</td>
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<tr>
<td>Low</td>
<td>3.56 1.42–8.94 0.007</td>
<td>4.14 1.28–13.32 0.017</td>
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<td>Tumor size, cm</td>
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<tr>
<td>&lt;2</td>
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<td>1</td>
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<td>( \geq 2 )</td>
<td>3.32 1.82–6.06 &lt;0.001</td>
<td>3.18 1.58–6.38 0.001</td>
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<td>Nodal status (N)</td>
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<tr>
<td>N*</td>
<td>2.15 1.22–3.79 0.005</td>
<td>2.38 1.23–4.61 0.010</td>
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HR, Hazard ratio.
p < 0.001), CD3+ T cells (Spearman r = 0.72, p < 0.001), and CD20+ B cells (Spearman r = 0.58, p < 0.001; Fig. 4B), consistent with the link between tumor HEVs and tumor-infiltrating lymphocytes (3).

We then analyzed the prognostic value of DC-LAMP marker in the retrospective cohort. In univariate analysis, we observed that a high density of DC-LAMP+ cells was significantly associated with a longer DFS (p < 0.02) and OS (p < 0.02) as compared with tumors with a low density of DC-LAMP+ cells (Fig. 4C, Supplemental Table I). DC-LAMP density also showed a significant correlation with DFS and OS in multivariate analysis after adjusting on prognostic factors previously identified (tumor size, grade, estrogen receptor status, HER2 expression). The adjusted hazard ratios of DFS and OS rates for patients with DC-LAMP<sub>low</sub> tumors versus DC-LAMP<sub>high</sub> tumors were 3.56 (95% CI, 1.42–8.94, p = 0.007) and 4.14 (95% CI, 1.28–13.32, p = 0.017), respectively (Table I). We concluded that a high density of DC-LAMP+ DCs correlates with the presence of HEV blood vessels within breast tumors and constitutes an independent factor of good clinical outcome for breast cancer patients.

**Progressive loss of DC-LAMP+ DCs and tumor HEVs during breast cancer progression**

The tumorigenesis and progression of breast cancer constitute a multistep process that is influenced by many factors including genetic composition, age, hormonal status, and immune environment. IDC is thought to derive from a series of intermediate hyperplastic and neoplastic stages including DCIS. To better understand the role of tumor HEVs during breast tumor development, we performed MECA-79 immunohistochemistry on 15 nonmalignant breast tissues, 29 pure DCISs, and 173 IDCs (Fig. 5A). HEVs were present in breast tumor tissues, but not in normal breast tissue samples (Fig. 5A), and their density was significantly higher in DCIS (7.467 ± 1.324) than in IDC (0.6363 ± 0.1132; Fig. 5B). To evaluate the role of tumor HEVs in the progression of DCIS to invasive carcinomas, we compared the density of MECA-79+ HEVs in matched IDC and DCIS components of 31 cases of invasive carcinomas with a DCIS component (Fig. 5C). We found a preferential localization of HEVs around in situ tumor components (Fig. 5C). A strong reduction in the density of tumor HEVs between DCIS components and IDC components was observed for all tumor samples analyzed (Fig. 5D). Given the clear association between DCs and HEVs in peripheral lymph nodes (15) and breast tumor tissues (this study), we then analyzed the density of DC-LAMP+ DCs in invasive carcinomas containing matched invasive and DCIS components (Fig. 5E). Similar to the density of tumor HEVs, we found that the density of DC-LAMP+ DCs was significantly reduced between DCIS and IDC components (Fig. 5F). Finally, we analyzed the density of CD3+ T cells in matched DCIS and IDC tumor areas (Fig. 5G), and we found that it was significantly reduced in IDC compared with DCIS areas (Fig. 5H). Together, these results indicate that breast cancer invasiveness is associated with a progressive loss of HEV blood vessels, DC-LAMP+ DCs, and CD3+ T cells around DCIS structures.

**Human breast tumor HEVs develop in the presence of FOXP3+ Tregs**

Tumor HEVs have recently been found to be induced in mouse tumor models upon depletion of Tregs (14). To better define the link between Tregs and HEV blood vessels within breast tumor stroma, we analyzed MECA-79, FOXP3, and CD3 markers by immunohistochemistry for 40 invasive breast cancer patients (Fig. 6A). We found a significant increase in the density of tumor-infiltrating FOXP3+ Tregs (Fig. 6B) and CD3+ T cells (Fig. 6C) in tumors containing a high density of HEV blood vessels (p < 0.01), and a significant correlation between the density of FOXP3+ Tregs and tumor HEVs (Spearman r = 0.54, p < 0.003). However, we also observed a significant reduction in the FOXP3+ Tregs/CD3+ T cell ratio in tumors with a high density of tumor HEVs (p < 0.01; Fig. 6D). These findings demonstrate that HEV blood vessels can fully develop in the presence of Tregs within breast tumor stroma, but that their density is influenced by the Tregs/ T cells ratio.

**Discussion**

The current dogma in the field of tumor angiogenesis is that blood vessels contribute to tumor growth, and they are thus generally
associated with poor prognosis. Recently, we proposed the novel concept that "tumor blood vessels are not all the same and that some types of blood vessels found in the tumor microenvironment (i.e., tumor HEVs) can be associated with favorable clinical outcome" (3). Tumor HEVs were frequently observed in the stroma of human solid tumors and appeared to contribute to tumor suppression by allowing high levels of lymphocyte infiltration (including CD8+ cytotoxic T cells infiltration) into tumors (3,7). A better understanding of tumor HEVs and their regulation is important because it could allow, in the future, the manipulation of tumor blood vessel phenotype to transform regular tumor blood vessels into tumor HEVs.

The lymphotoxin pathway has been shown to regulate HEV blood vessels in both lymphoid organs and chronically inflamed tissues (16, 18–20, 22, 23). We demonstrated previously that DCs and DC-derived lymphotoxin are critical for maintenance of HEV blood vessel phenotype in mouse lymph nodes (4,15). In this study, we asked whether similar mechanisms regulate tumor HEVs in human breast tumors. We found that LTα and LTβ were both overexpressed in HEVhigh tumors, and DCs were the major producers of membrane-associated LTβ (LTα1β2) within breast tumors. Whereas most T and B cells expressed LTβ after polyclonal activation of PBMCs, we observed only a few T and B lymphocytes expressing LTβ in breast tumors. In contrast, a considerable fraction (~30%) of CD11c+ DCs from HEVhigh breast tumors expressed membrane-bound LTβ. In addition, DC-LAMP expression was strongly correlated to LTβ expression in breast tumor samples. These results suggested that DCs may contribute to the formation of HEVs in human breast tumors through LTβ production. In support of this possibility, tumor HEVs were often surrounded by clusters of mature Fascin+ and DC-LAMP+ DCs, and high densities of DC-LAMP+ DCs clusters were observed in HEVhigh breast tumors. The density of DC-LAMP+ DCs clusters was strongly correlated with the density of tumor HEVs, T and B cell infiltration, and favorable clinical outcome (longer DFS and OS) in a retrospective cohort of 146 primary invasive breast cancer patients. Together, these results indicated that, similar to mouse lymph nodes (15), DCs and lymphotoxin pathway may be critical regulators of HEV blood vessels in human breast tumors.

We observed that densities of DC-LAMP+ DCs and tumor HEVs were significantly higher in DCIS than in IDC. Interestingly, in a series of 31 invasive breast carcinomas containing matched DCIS and IDC components, the density of tumor HEVs and DC-LAMP+ DCs was strongly reduced between DCIS and IDC components. These observations suggest that loss of tumor HEVs and DC-LAMP+ DCs during breast cancer progression may represent a critical step in the transition from in situ carcinoma to invasive carcinoma.

Although our results strongly suggest that DCs foster HEV development and/or maintenance within human breast tumors, we cannot exclude the alternative possibilities that DCs arriving via tissue lymphatics may preferentially be attracted to pre-existing HEVs or that DCs may enter the stroma via tumor HEVs. Functional analyses in mouse tumor models will be required to demonstrate the direct role of DCs in the induction and/or maintenance of tumor HEVs.

Surprisingly, increased densities of Tregs were observed in breast tumors containing high densities of tumor HEVs. In contrast, it was previously reported that in mouse methylcholanthrene-induced fibrosarcomas, formation of tumor HEVs only occurs after depletion of Tregs (14). Our results indicate that the absence of Tregs is not an essential prerequisite for development of HEVs in human breast tumors. However, the relative proportion of Tregs compared with other T cell populations appears to be important because HEVhigh breast tumors were associated with low FOXP3+ Tregs/CD3+ T cell ratios. Therefore, Tregs may limit HEV neogenesis in human breast tumors, similar to mouse tumor models (14). LTα and LTβ were upregulated in mouse fibrosarcomas upon depletion of Tregs (14), suggesting that Tregs may inhibit HEV development through reduction of LTα1β2 levels.

Interestingly, targeting of LTα to mouse melanomas tumors has been shown to result in formation of tumor HEVs, T cell infiltration, and eradication of the tumors (12, 13). Overexpression of LIGHT, the second ligand for LTβR, in mouse tumors has also been shown to induce antitumor immunity and eradication of the
tumors (28, 29). However, it remains unclear whether tumor HEVs were generated upon overexpression of LIGHT. Although DCs in lymph nodes express both LTα/LTβ and LIGHT (15), it is unknown yet whether LIGHT plays a role in the regulation of HEVs. In this study, we observed that LIGHT was not overexpressed in HEV-positive breast tumors, indicating that LTαβ rather than LIGHT, is likely to be the major LTβR ligand involved in the generation of human breast tumor HEVs.

Further characterization of the cellular and molecular mechanisms regulating the formation of tumor HEVs (including the roles of DCs and lymphotixin) will be important because it could lead to the development of improved therapeutic strategies for human solid tumors. For instance, it could provide means to induce the HEV endothelial cell differentiation program in tumor blood vessels. This would increase the density of tumor HEVs without increasing tumor angiogenesis (total number of tumor blood vessels) and would suppress tumor growth through enhanced recruitment of cytotoxic lymphocytes. Novel therapeutic strategies based on the modulation of tumor HEVs could thus have a major impact on tumor growth and clinical outcome of cancer patients.

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Disclosures
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