A specific immune and lymphatic profile characterizes the pre-metastatic state of the sentinel lymph node in patients with early cervical cancer

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INTRODUCTION

The “pre-metastatic niche” is a supportive microenvironment generated by the primary tumor in a secondary site prior to tumor cell dissemination. First described by Kaplan et al. for the hematogenous dissemination, it is now increasingly accepted that the pre-metastatic niche can support the metastatic process. A wide range of tumor-derived factors and bone marrow-derived hematopoietic progenitors have been demonstrated to participate to the formation of a pre-metastatic microenvironment in secondary hematogenous sites (i.e., lung and bones). Less is known regarding pre-metastatic niche in lymph nodes (LNs), which are the initial site of metastatic cell dissemination in most carcinomas. Previous experimental studies in animal models have highlighted a lymphovascular remodeling (lymphangiogenesis) in the first draining LN (sentinel LN or SLN) that occurs prior to metastasis. So far, only a few clinical studies have documented pre-metastatic lymphovascular process in the SLN of patients with lung, breast and oral squamous carcinomas. Moreover, the mechanisms underlying the pre-metastatic regulation remain unknown and its relationship with inflammation and immune responses is not documented.

In pre-clinical models, the formation of new lymphatic vasculature is thought to enhance tumor cell dissemination since it is associated with a higher incidence of nodal metastases. In this context, Hirakawa et al. have proposed the concept of a...
“lymphvascular niche” that can facilitate tumor cell transport to LNs and promote the retention and growth of cancer cells with metastatic potential. In addition, a specific immunoreactive profile has also been described within SLN. In murine models, an increased density of T regulatory lymphocytes (Foxp3) associated with a reduced dendritic cell number has been noted and led to a suppression of T cytotoxic (CD8^+) reactivity. Furthermore, B lymphocyte accumulation has been reported. A link between the lymphatic vasculature and the elaboration of a specific immune microenvironment is emerging. For instance, the formation of a SLN immunosuppressive microenvironment could be regulated by lymphatic endothelial cells through the secretion of chemokines and cytokines as well as through the expression of molecules such as PD-L1 involved in T-cell regulation at their cell surface. The increase of lymphatic flow as a consequence of nodal lymphangiogenesis may also enhance the drainage of tumor-derived factors (i.e., growth factors, cytokines, soluble antigens and antigen-presenting cells) to SLN, influencing thereby the immune microenvironment. All together, these experimental data highlight the importance to evaluate both the lymphovascular and the immune microenvironment.

In this study, we investigated modifications of the lymphatic vasculature, as well as T and B cell compartments in LNs of patients with early cervical cancer FIGO stage IB1. Due to its specific dissemination throughout a complex bilateral pelvic lymphatic system, such early cervical cancer is considered as a good candidate for studying the early nodal metastatic process. We provide a detailed whole slide computer-assisted characterization of both the lymphovascular pattern and the immune climate in metastatic cell-free SLN (SLN−), metastatic cell-free distant pelvic LNs (DLN−) and metastatic cell positive lymph nodes (MLN+). In addition to characterizing the lymphangiogenic profile in pre-metastatic SLN, we establish for the first time a link between the lymphatic vasculature remodeling and the humoral immune profile.

**Results**

**Lymphatic vascular network**

A digital image analysis approach was applied on whole scanned tissue sections in order to first characterize the lymphatic vascular network immunostained with an anti-D2/40 antibody (Fig. 1). Lymphatic vessels were primarily detected close to the tissue edge, in the capsule and subcapsular sinus. Only a few lymphatic vessels were sparsely distributed throughout the paracortex and the medulla (Fig. 1A–F). In DLN−, some large lymphatic vessels were found within the capsule (Fig. 1A and B). In sharp contrast, SLN− displayed numerous large and tortuous vessels in their capsule and subcapsular sinus (Fig. 1C and D). Interestingly, in MLN+, tumor cells were found around and within enlarged capsular and subcapsular lymphatic vessels that invade the paracortex (Fig. 1E and F).

For quantitative analyses, two computer-assisted methods applied on whole tissue sections led to the measurement of (1) the global lymphatic vessel density (LVD) and (2) the spatial distribution of lymphatic vessels according to a procedure previously described. LVD was twice higher in SLN− than in DLN− (p < 0.01), highlighting an early remodeling of lymphatic vasculature occurring before the arrival of metastatic cells (Fig. 1G). A double immunostaining for D2-40 and Ki67 revealed the proliferative status of lymphatic endothelial cells, reflecting thereby an active lymphangiogenesis in SLN− (Fig. 1H). On the contrary, the nuclei of lymphatic endothelial cells were Ki67-negative in DLN− supporting their quiescence. In LNs colonized by tumor cells (MLN+), the LVD was again 2-fold higher than in DLN− (p < 0.01), but remained similar to that measured in SLN− (p > 0.05) (Fig. 1G). In line with the global immunohistological analysis, the tight proximity between lymphatic vessels and the tissue edge was further supported by the analysis of lymphatic vessel spatial distribution (Fig. 1I–K). Indeed, the LVD peaked at a distance of around 0.19–0.24 mm from the LN border (0.24 ± 0.54 mm in DLN−; 0.19 ± 0.24 mm in SLN− and 0.22 ± 0.22 mm in MLN+). Furthermore, 90% of lymphatic vessels were found at a distance ranging from 0.8 to 1.3 mm to the tissue edge (0.88 ± 0.74 mm in DLN−, 0.92 ± 0.55 mm in SLN− and 1.29 ± 0.97 mm in MLN+). Lymphatic vessel distribution was almost similar in DLN− and SLN−, with a slight settlement of the peak in SLN− (Fig. 1I and J). In MLN+, the enlarged peak of the distribution curve reflects the infiltration of lymphatic vessels deeper in the paracortex (Fig. 1K). Accordingly, half of the lymphatic vessels (50th percentile) were found at a twice longer distance from the border (0.46 ± 0.09 mm in MLN+ versus 0.22 ± 0.04 mm in SLN−; p < 0.05). Altogether, our data point out an early capsular and subcapsular modification of the lymphatic vasculature in SLN prior to tumor cell arrival. After metastatic cell colonization, although the global LVD remains unchanged, dilated lymphatic vessels appeared deeper inside LNs.

**Immune microenvironment**

Accumulating experimental evidence has pointed out a link between the lymphatic vasculature and the establishment of a specific immune response within LNs. Accordingly, we analyzed the immune profile by investigating T lymphocytes (CD8^+ or Foxp3^+) and B lymphocytes (CD20^+). We also tested PD-1, a transmembrane protein that acts as an immune checkpoint playing a regulatory role in the immune system. Given that our main goal was to characterize pre-metastatic changes, we next compared the immune environment in DLN− and SLN− (Fig. 2A–D).

A paracortical hyperplasia with increased density of CD8^+ and Foxp3^+ T lymphocytes was observed in SLN− compared with DLN− (Fig. 2A–C). The spatial distribution of CD8^+ and Foxp3^+ T lymphocytes was similar in DLN− and SLN− (Fig. 2D). The distribution curves peaked at around 0.5 mm and 0.3 mm for CD8^+ and Foxp3^+ T lymphocytes, respectively (Table S1), confirming the main paracortical localization of these cells. CD20^+ B cells were mainly found within germinal centers located in the superficial cortex, as well as in the medullary cords (Fig. 2A and B). Although no difference was found in CD20^+ B cell global density between SLN− and DLN−, a higher amount of CD20^+ B cells was detected in the
Figure 1. Lymphatic vascular network characterization. (A–F) Illustration of lymphatic endothelial cell immunostaining (D2-40, brown, first row) and corresponding binary image of whole tissue (second row) in DLN (A, B), SLN (C, D) and MLN+ (E, F). The capsule (Ca), subcapsular sinus (arrows), tumor cell emboli within a lymphatic vessel section (TE, panel E) and invading tumor cells (IT, panel E) are highlighted. In binary images (B, D, F), lymphatic vessels (red) are detected on whole tissue (gray). Clusters of tumor cells in MLN+ are represented in white. (G) The graph corresponds to lymphatic vessel density measured on whole DLN−, SLN− and MLN+ sections. It is presented as a scatter plot of individual data points. Means are presented by horizontal bars together with the standard deviation. **p < 0.01. (H) Double immunostaining of lymphatic endothelial cells (D2-40, pink) and proliferating cells (Ki67, brown). The inset shows a magnification of a proliferating lymphatic vessel with a Ki67-positive endothelial cell (arrow head). Scale bars on immunostained tissues represent 100 μm. (I–K) Spatial lymphatic vessel distribution analysis from tissue edge to tissue center measured on whole tissues of DLN− (I), SLN− (J) and MLN+ (K). The gray area represents the distance from the border at which 90% of lymphatic vessels are located. Curves were normalized such that sum of heights equals one.
The subcapsular area of SLN− compared with DLN− (p < 0.01) (Fig. 2C and D). Indeed, the maximal frequency of CD20+ B cells found at a similar distance (0.18 ± 0.02 mm in DLN− versus 0.15 ± 0.03 mm in SLN−, p > 0.05) (Table S1) was 1.7-fold higher in SLN− than in DLN− (Fig. 2D, p < 0.01). Moreover, 90% of CD20+ B lymphocytes were detected at a lower distance from tissue edge in SLN− than in DLN− (p < 0.01) (Table S1).

Interestingly, PD-1 immunostaining was strongly positive on the surface of cells located in germinal centers, which were both positive for Ki67 and CD20 (Figs. 2A and B and S1A). Within those germinal centers, some CD20+ and CD4+ cells were found to be positive for PD-1 as illustrated in the image obtained after a confocal acquisition (Fig. S1B). CD8+ cells were also found to express PD-1 at their cell surface within those areas. However, this event appears anecdotic since among CD8+ cells detected in germinal center (ranging from 0 to 5 cells) only a few of them were found to be positive for PD-1 (ranging from 0 to 2 CD8+ cells). These data suggest that in addition to T cells some follicular B cells also express PD-1. The number of PD-1+ germinal centers was 4.9-fold higher in

Figure 2. Analysis of the immune response profile in DLN− and SLN−. (A, B) Illustration of the immunostaining of CD8+ T lymphocytes, Foxp3+ T lymphocytes, CD20+ B lymphocytes and PD-1+ germinal centers (brown). Insets show the immunostaining of single cells at a higher magnification. Scale bars represent 100 µm. (C) Computerized quantification of whole slide immunostaining densities. Graphs are presented as scatter plots of individual data points. Means are presented by horizontal bars together with standard deviations. (D) Spatial distribution analysis from tissue edge to tissue center. Curves were normalized such that sum of heights equals one. *p <0.05, **p <0.01, ***p <0.001.
In line with the distribution of CD20\(^+\) B cells, the mode of the distribution curve (the highest frequency) was higher in SLN— than in DLN— (Fig. 2D, \(p < 0.01\)). Moreover, the 90th percentile of PD-1 distribution corresponded to a lower distance from tissue edge in SLN— than in DLN— (\(p < 0.01\)) (Table S1). Together, our data highlighted important modifications of the immune landscape in SLN—, which are characterized by a paracortical CD8\(^+\) and Foxp3\(^+\) T lymphocyte hyperplasia, and increased positivity for CD20 and PD-1 in germinal centers.

**Association between lymphatic vascular network and immune microenvironment**

In order to determine whether lymphatic vasculature remodeling could be or not linked to modifications observed in the immune profile, scatter plots of LVD in a function of immune cell densities were generated and linear regression curves were interpolated (Fig. 3). No association was seen between LVD and CD8\(^+\) or Foxp3\(^+\) T lymphocyte density. However, in DLN—, an inverse correlation was found between LVD and CD20\(^+\) cell density (\(p < 0.01\), \(r^2 = 0.2480\)). On the opposite, in SLN—, the LVD was positively associated with both CD20 and PD-1 positivities (\(p < 0.05\), \(r^2 = 0.1523\) for CD20 and \(r^2 = 0.1756\) for PD-1).

Given the association found between lymphatic vessels and CD20\(^+\) and PD-1\(^+\) germinal centers, we finally examined the spatial distribution of lymphatic vessels around germinal centers. To address this issue, distances between the center of each PD-1\(^+\) germinal center and the closest region of each lymphatic vessel were calculated (Fig. 4). Interestingly, 90% of lymphatic vessels were detected at a distance of 1.4 mm from the follicular tissue center (1.41 ± 0.22 mm in DLN— and 1.44 ± 0.27 mm in SLN—). Maximal LVD was found at 0.2 ± 0.016 mm and 0.291 ± 0.021 mm for SLN— and DLN—, respectively (\(p < 0.004\)). These findings point out that lymphatic vessels are at the vicinity of PD-1\(^+\) germinal centers.

**Discussion**

During the last few years, experimental studies highlighted that lymphatic vessels could play multifaceted roles on regulating immune function inside the LN. They may have a more complex role than to simply transport tumor cells. Indeed, lymphatic cells could be involved in the formation of a pre-metastatic niche.\(^{19-25}\) However, evidence supporting this emerging concept in patients are sorely lacking. Early cervical cancer is a good candidate for studying the early nodal metastatic process due to its well described dissemination throughout a complex bilateral pelvic lymphatic system. As schematically presented in Fig. S2, we demonstrate a subcapsular extension of the lymphatic vascular network in pre-metastatic sentinel LNs, which is associated with a paracortical hyperplasia. We also establish an unappreciated link between LVD, CD20 and PD-1 immunostainings. Our findings are supported by robust computerized quantifications applied on whole tissue sections.

We first demonstrate the presence of a lymphangiogenic response in metastatic free SLN, confirming the results previously reported in experimental models and patients with other cancer types.\(^{3-14}\) The original spatial distribution analysis used in the present study additionally highlighted that main lymphatic vascular modulations occur in the subcapsular sinus, the
canal through which tumor cells reach the LN parenchyma. Such a modified lymphatic landscape is conserved in metastatic LNs. In this case, we noted a slight shift of the lymphatic vessel distribution toward the tissue center, which is likely due to a dilatation of the subcapsular sinus induced by the presence of tumor cell clusters. Taken together, our data support the concept of "lymphvascular niche" initially proposed by Hirakawa et al.\(^16\) in an experimental model. Therefore, a pre-metastatic lymphangiogenesis occurs in metastatic free SLN of patients with early cervical cancer and could play an important part in preparing the soil for metastatic dissemination.

In order to test whether SLN lymphangiogenesis could have an impact on the establishment of a specific immune profile, we have next characterized the cellular and humoral immune response in SLN— and DLN— using the most common markers used to detect T cytotoxic lymphocytes (CD8\(^{+}\)), T regulatory lymphocytes (Foxp3\(^{+}\)) and B lymphocytes (CD20\(^{+}\)). Regarding the cellular immune response, a paracortical hyperplasia was detected in SLN— due to an accumulation of cytotoxic CD8\(^{+}\) and regulatory Foxp3\(^{+}\) T lymphocytes within the paracortex. One explanation of the concomitant presence of these two immune profiles could be the latency between the increased number of Foxp3\(^{+}\) T cells and their apoptotic activity on CD8\(^{+}\) T cells. In line with this hypothesis, we report a higher modulation of Foxp3\(^{+}\) T lymphocyte density in SLN— compared with CD8\(^{+}\) one, which could reflect the establishment of an immunosuppressive microenvironment.

In parallel to cellular immune changes, we provide evidence for a modulation of humoral immune response in the SLN—. On the one hand, a spatial distribution analysis revealed a prominent density of CD20\(^{+}\) B lymphocytes in the superficial cortex of SLN— compared with DLN—. Therein, these cells are mainly found within germinal centers, structures in which naive B lymphocytes mature into antibody secreting memory B and plasma cells in response to T cell-dependent antigen.\(^{29}\) CD20 is a protein expressed as of the pro-B phase and has a progressively increasing expression until maturity. Analysis of other B cell markers should be used to further characterize B cell subtypes present within germinal centers.\(^{30}\) On the other hand, a strong expression of the immune regulator PD-1 is detected within germinal centers with strong Ki67 and CD20\(^{+}\) B cell positivity. A drastic increase of the density of germinal centers positive for PD-1 also occurs in SLN— compared with DLN—. So far, pre-clinical works pointed out PD-1 as a negative regulator of B cell proliferation and differentiation. This may be due to its direct expression on B cells or secondary to its effect on T cells.\(^{31-33}\) In the present work, we demonstrated that some B lymphocytes can express PD-1 at their cell surface in germinal centers. Today, although the inhibitory role of PD-1 in activated T cells has been widely studied, a few information is available on its triggering on human B cell surface.\(^{28}\) Recently, Thibult et al.\(^{34}\) were first to report PD-1 expression by human B lymphocytes that could negatively regulate their activation, proliferation and IL-6 production. To date, the putative roles of B lymphocytes in generating a specific pre-metastatic microenvironment in the SLN— is also not well documented. To the best of our knowledge, one study in line with our data reported an influx of B cells within the SLN— of patients with melanoma.\(^{35}\) Authors proposed that B cells could contribute to the establishment of a Th2 immune response, which may be permissive for chronic inflammation. In addition, experimental studies revealed that B cells can both positively and negatively regulate T cell mediated antitumor immune response, by acting as antigen presenting cells or via chemokine secretion.\(^{36-39}\) Altogether our data shed light on a subcapsular B cell rich microenvironment whose function in generating a specific pre-metastatic niche for metastase hosting remains to be determined. Of utmost interest, we establish for the first time a link between lymphatic vessels, CD20\(^{+}\) lymphocytes and PD-1-positive germinal centers in SLN of cancerous patients. Notably, correlations between those parameters were noted both for their density and their spatial distribution. Several explanations could be proposed for this original finding. First, B lymphocytes may themselves enhance LN lymphangiogenesis by producing vascular growth factors.\(^{6,19-21,40}\) Second, the formation of a prominent subcapsular lymphatic vascular network in SLN— may promote the delivery of tumor antigens, leading to immune reaction.\(^{26,41}\) Finally, lymphatic endothelial
cells may also express a wide range of stimulating chemokines.\textsuperscript{22,42} Although future investigations remain mandatory to clarify these issues, our work sheds light on underconsidered modulations that could play an important role in generating a favorable microenvironment for tumor cell metastasis.

In conclusion, we provide evidence that the SLN of patients with early cervical cancer could be early prepared to host metastasis. Of great interest, we have demonstrated for the first time a link between the lymphangiogenic response and the presence of a prominent PD-1 regulated humoral immune response. This support the concept that lymphatic vessels do not act as simple channels transporting tumor but also contribute to the elaboration of a specific immune microenvironment in cancerous patients. In this context, we argue that modulations of lymphatic vessel proliferation combined with current antitumor immunotherapy hold promise for preventing the formation of LN metastasis.

Materials and methods

Tissue samples

A total of 70 pelvic LNs surgically collected from 48 patients suffering early cervical cancer (FIGO stage IB1) were obtained from the biobanks of the University of Liège (CHU, Liège, Belgium) and the Centre Hospitalier Universitaire of Montreal (CHUM, Hôpital Notre-Dame, Montreal, Canada) after study approval by local ethic committees. All tissues were reviewed by pathologists blinded to the study purpose. Specimens were selected after the examination of hematoxylin eosin stained tissues. Only those showing at least the presence of the superficial cortex together with the paracortex, all surrounded by a fibrous capsule, were included.

Two groups of patients were studied. In the first group, patients had no LN metastasis (30/48). In 22 of them, one sentinel lymph node (SLN–) and one distant pelvic lymph node (DLN–) were selected. In the 8 other patients, either 1 SLN (4/30) or 1 DLN (4/30) was chosen due to the absence of includable corresponding SLN/DLN specimens (see above). The absence of HPV-positive tumor cells in those tissues was confirmed by (1) immunohistochemistry targeting CDKN2A/p16\textsuperscript{INK4a} (Abcam, ab7962) well known to be overexpressed in case of carcinogenic HPV infection, and (2) a conventional GP5+/GP6+ PCR amplifying the conserved region within the L1 HPV gene (Forward primer: 5'-TTTGTACTGTGTA-GATACTAC-3' and reverse primer: 5'-GAAAATAAAGT- TAAATCATATTCC- 3') (Fig. S3). The second group consists of patients diagnosed with at least one metastatic pelvic lymph node (MLN+) (18/48). When these patients were considered, one metastatic LN permitting optimal tumor and peri-neoplastic evaluation was selected in each case regardless the LN status (SLN or DLN). SLN were studied according to the international pathologic recommendations. Four serial sectioning of 4-µm thick were performed each 150 µm. Hematoxylin eosin staining and immunohistochemical analyses involving anti-P40 antibody in case of squamous carcinoma and pan-cytokeratin in case of adenocarcinoma for each SLN were done with the aim to rule out the presence of micrometastasis or ITC (isolated tumor cells) before the SLN was declared negative/positive. Tissue sections were oriented according to standard histological guidelines. Clinical and pathological features are summarized in Table 1.

Immunohistochemistry

Lymphatic vessels (D2-40), proliferating lymphatic vessels (D2-40 and Ki67), T cytotoxic lymphocytes (CD8\textsuperscript{+}), T regulatory lymphocytes (Foxp3), B lymphocytes (CD20) and Programmed cell Death 1 (PD-1), were studied using peroxydase and/or phosphatase immunostaining methods. Double fluorescent immunolabelings of CD20\textsuperscript{+} PD-1 and CD4\textsuperscript{+} PD-1 were also performed. In agreement with our referring pathologists, one representative 5-µm thick section per LN has been used for each marker. Positive and negative controls of immunohistochemical staining are presented in Fig. S4. All information related to antibody references, dilutions and immunohistochemical procedures are summarized in Table S2.

Virtual image acquisition and processing

Virtual images of tissues immunostained using peroxydase and/or phosphatase methods (CD8\textsuperscript{+}, Foxp3, CD20, PD1, D2-40 and D2-40 + Ki67) were acquired at high magnification with a fully automated digital microscope system (Olympus, BX51TF, Aartselaar, Belgium). Images of tissues immunolabeled with Alexafluor dyes (CD20\textsuperscript{+} PD-1 and CD4\textsuperscript{+} PD-1) were obtained by a confocal microscope (Nikon, A1R, Brussels, Belgium). Virtual images acquired using the fully automated digital microscope system were converted into a standard TIF format. The immunostaining of CD8\textsuperscript{+}, Foxp3\textsuperscript{+} and CD20\textsuperscript{+} lymphocytes was thereafter automatically segmented on a whole slide at high resolution thanks to a Maximum Entropy Thresholding performed on the excess blue (twice blue value minus green and red values) component. Due to the absence of a clear delimitation of PD-1-positive (PD-1\textsuperscript{+}) germinal centers

Table 1. Patient’s features.

| Age (years) | Patients N– (n = 30) | Patients N+ (n = 18) |
|------------|---------------------|---------------------|
| 41.63 ± 11.13 | 42.94 ± 9.967 |
| Tumor Type | | |
| Squamous cell carcinoma | 23 | 13 |
| Adenocarcinoma | 6 | 5 |
| Both | 1 | 0 |
| Grade | | |
| I | 6 | 2 |
| II | 15 | 10 |
| III | 9 | 6 |
| Lymphvascular space invasion | | |
| Yes | 12 | 11 |
| No | 18 | 4 |
| Unknown | 0 | 3 |
| Lymph node | | |
| Sentinel lymph node (SLN) | 26 | – |
| Non-sentinel lymph node (DLN) | 26 | – |
| Not mentioned | – | 18 |
| Metastasis | | |
| Lymph node metastasis | | |
| No | 30 | 0 |
| Yes | 0 | 18 |
| Distant metastasis | | |
| No | 29/30 | 17/18 |
| Yes | 1/30 | 1/18 |
and the presence of thin discontinuous lymphatic endothelia that do not allow an optimal computerized detection, these structures were drawn manually using the Aperio ImageScope v10.2.1.2314 software. Tissues and tumor nodules were also delineated manually. Finally, all binary images of the detected structures were generated and decimated as previously described.\textsuperscript{27} Image processing is detailed in Fig. 5.

**Image measurements**

Image measurements were performed on whole tissue surface using image analysis library Pandore (GREYC, Caen, France), Aphelion 3.2 (ADCIS, Saint-Contest France) and image toolbox of MATLAB 9.2 (MathWorks, Natick, US-MA) software. In metastatic LNs, quantifications were performed in the non-tumor area. Densities of lymphatic vessels (LVD), CD8\textsuperscript{+}, Foxp3\textsuperscript{+} and CD20\textsuperscript{+} lymphocytes are represented as the ratio between the immunostained area and the total tissue surface. The density of the PD-1\textsuperscript{+} germinal centers is defined as the number of germinal centers positive for PD-1 per mm\textsuperscript{2} of tissue. Finally, the Euclidean distance separating the centroid of each detected structure from the tissue border was measured. From these values, histograms were constructed in order to determine the spatial distributions of structures. For comparison, distribution curves display relative frequencies (sum of the heights equaling 1). We calculated the distance up to which

![Figure 5. Illustration of image processing. (A–E) Virtual images of a non-metastatic lymph node tissue. Cytotoxic (CD8\textsuperscript{+}) (A) and regulatory (Foxp3) (B) T lymphocytes as well as B lymphocytes (CD20) (C), programmed cell death-1 expressing cells (PD-1) (D) and lymphatic endothelial cells (D2-40) (E) were detected by immunostaining. These structures are visualized in an intense and highly contrasted brown color (zoom in the inset). (F–J) CD8\textsuperscript{+} (F), Foxp3\textsuperscript{+} (G) and CD20\textsuperscript{+} (H) lymphocytes were automatically segmented on virtual images, whereas PD-1-positive germinal centers (I) and lymphatic vessel sections (J) were manually drawn. Automatic and manual detections of immunostained structures were superimposed on virtual images (red). (k-o) Binary images of CD8\textsuperscript{+} (K), Foxp3\textsuperscript{+} (L) and CD20\textsuperscript{+} (M) lymphocytes as well as PD-1-positive germinal centers (N) and lymphatic vessel sections (O) on whole tissues. Segmented structures and the tissue are represented in red and gray, respectively. Scale bars in insets represent 100 \(\mu\)m.](image-url)
50% and/or 90% of the lymphatic vessel distribution was detected (50th and/or 90th percentile, respectively) and the depths at which the maximal density is found. This latter parameter represents the mode of the distribution curve, defined as the distance at which the probability to localize the highest density is maximal.

**Statistical analysis and graphs**

Densities and spatial distributions were analyzed using the GraphPad Prism 5.0 (San Diego, CA) and the MATLAB 9.2 (Natick, MA) software, respectively. Wilcoxon non-parametric paired test was used to determine whether quantified values in SLN- and DLN- could be considered as significant. When comparing values of SLN- and DLN- to that of MLN+, statistical analyses were performed using the Mann–Whitney test. The level of statistical significance was set at \( p < 0.05 \) for all comparisons.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We address a particular acknowledgment to Paulette Herlin and Benoit Plancoûlaine from PathImage/BioTICLA, Inserm (UMR 1199), University Caen Normandy, Cancer Center F. Baclesse, Caen, France for their technical support for image decimation.

**Funding**

This work was supported by grants from the Fonds de la Recherche Scientifique – FNRS (F.R.S.-FNRS, Belgium), the Fondation contre le Cancer (foundation of public interest, Belgium), the Fonds spéciﬁque de la Recherche from the Service Public de Wallonie (SPW, Belgium), the Inter-University Attraction Poles Programme, (4) GIGA-Bioinformatics platform, and (5) GIGA-Mouse facility and Transgenics platform. CB is recipient of funds provided by the Centre Hospitalier Universitaire de Liége.

**References**

1. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005; 438:820-7; PMID:16341007; http://dx.doi.org/10.1038/nature04186
2. Sceney J, Smyth MJ, Moller A. The pre-metastatic niche: finding common ground. Cancer Metast Rev 2013; 32:449-64; PMID:23636348; http://dx.doi.org/10.1007/s10555-013-9420-1
3. Hirakawa S, Kodama S, Kunstfeld R, Kajiyama K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. J Exp Med 2005; 201:1089-99; PMID:15809353; http://dx.doi.org/10.1084/jem.20041896
4. Qian CN, Berghuis B, Tsarfaty G, Bruch M, Kort El, Ditej L, Tsarfaty I, Hudson E, Jackson DG, Petillo D et al. Preparing the “soil”: the primary tumor induces vasculature reorganization in the sentinel lymph node before the arrival of metastatic cancer cells. Cancer Res 2006; 66:10365-76; PMID:17062557; http://dx.doi.org/10.1158/0008-5472.CAN-06-2977
5. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. Blood 2007; 109:1010-7; PMID:17032920; http://dx.doi.org/10.1182/blood-2006-05-1758
6. Harrell MI, Iritani BM, Ruddle A. Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. Am J Pathol 2007; 170:774-86; PMID:17255343; http://dx.doi.org/10.2353/apjpath.2007.060761
7. Ozasa R, Ohno J, Iwahashi T, Taniguchi K. Tumor-induced lymphangiogenesis in cervical lymph nodes in oral melanoma-bearing mice. J Exp Clin Cancer Res 2012; 31:83; PMID:23031500; http://dx.doi.org/10.1186/1756-9966-31-83
8. Liersch R, Hirakawa S, Berdel WE, Mesters RM, Detmar M. Induced lymphatic sinus hyperplasia in sentinel lymph nodes by VEGF-C as the earliest premetastatic indicator. Int J Oncol 2012; 41:2073-8; PMID:23076721; http://dx.doi.org/10.3892/ijo.2012.1665
9. Ogawa F, Amano H, Eshima K, Ito Y, Matsui Y, Hosono K, Kitasato H, Iyoda A, Iwabuchi K, Kumagai Y et al. Prostanoid induced premetastatic niche in regional lymph nodes. J Clin Investig 2014; 124:4882-94; PMID:25271626; http://dx.doi.org/10.1172/JCI73530
10. Kawai H, Minamiya Y, Ito M, Saito H, Ogawa J. VEGF121 promotes lymphangiogenesis in the sentinel lymph nodes of non-small cell lung carcinoma patients. Lung Cancer 2008; 59:41-7; PMID:17868952; http://dx.doi.org/10.1016/j.lungcan.2007.08.001
11. Zhao YC, Ni XJ, Wang MH, Zha XM, Zhao Y, Wang S. Tumor-derived VEGF-C, but not VEGF-D, promotes sentinel lymph node lymphangiogenesis prior to metastasis in breast cancer patients. Med Oncol 2012; 29:2594-600; PMID:22562153; http://dx.doi.org/10.1007/s12032-012-0205-0
12. Ishii H, Chikamatsu K, Sakakura K, Miyata M, Furuya N, Masuyama K. Primary tumor induces sentinel lymph node lymphangiogenesis in oral squamous cell carcinoma. Oral Oncol 2010; 46:373-8; PMID:20308006; http://dx.doi.org/10.1016/j.joraloncology.2010.02.014
13. Chung MK, Do IG, Jung E, Son YI, Jeong HS, Baek CH. Lymphatic vessels and high endothelial venules are increased in the sentinel lymph nodes of patients with oral squamous cell carcinoma before the arrival of tumor cells. Ann Surg Oncol 2012; 19:1595-601; PMID:22124758; http://dx.doi.org/10.1245/s10434-011-2154-9
14. Wakisaka N, Hasegawa Y, Yoshimoto S, Miura K, Shiotani A, Yokoyama J, Sugasawa M, Moriyama-Kita M, Endo K, Yoshizaki T. Primary tumor-secreted lymphangiogenic factors induce pre-metastatic lymphvascular niche formation at sentinel lymph nodes in oral squamous cell carcinoma. PloS One 2015; 10:e0144056; PMID:26630663; http://dx.doi.org/10.1371/journal.pone.0144056
15. Garny-Susini B, Avraamides CJ, Desgrosellier JS, Schmid MC, Fouquet P, Ellies LG, Lowy AM, Blair SL, Vandenberg SR, Datnow B et al. P13Kalpha activates integrin alpha4beta1 to establish a metastatic niche in lymph nodes. Proc Natl Acad Sci USA 2013; 110:9402-7; PMID:23671668; http://dx.doi.org/10.1073/pnas.1219603110
16. Hirakawa S. From tumor lymphangiogenesis to lymphvascular niche. Cancer Sci 2009; 100:983-9; PMID:19385973; http://dx.doi.org/10.1111/j.1349-7006.2009.01142.x
17. Cochran AJ, Huang RR, Lee J, Itakura E, Leong SP, Essner R. Tumour-induced immune modulation of sentinel lymph nodes. Nat Rev Immunol 2006; 6:659-70; PMID:16932751; http://dx.doi.org/10.1182/blood-2006-05-021758
18. Nakamura S, Yaguchi T, Kawamura N, Kobayashi A, Sakurai T, Higuchi H, Takaiishi H, Hibi T, Kawakami Y. TGF-beta1 in tumor microenvironment induces immunosuppression in the tumors and sentinel lymph nodes and promotes tumor progression. J Immunother 2014; 37:63-72; PMID:24509168; http://dx.doi.org/10.1097/CJI.0000000000000011
19. Ruddle A, Harrell MI, Furuya M, Kirschbaum SB, Iritani BM. B lymphocytes promote lymphogenous metastasis of lymphoma and melanoma. Neoplasia 2011; 13:748-57; PMID:21847366; http://dx.doi.org/10.1593/neo.11756
20. Ruddle A, Kelly-Spratt KS, Furuya M, Parghi SS, Kemp CJ. p19/Arf and p53 suppress sentinel lymph node lymphangiogenesis and
carcinoma metastasis. Oncogene 2008; 27:3145-55; PMID:18059331; http://dx.doi.org/10.1038/sj.onc.1210973

21. Shrestha B, Hashiguchi T, Ito T, Miura N, Takenouchi K, Oyama Y, Kawahara K, Tanchamoun S, KI YI, Arimura N et al. B cell-derived vascular endothelial growth factor A promotes lymphangiogenesis and high endothelial venule expansion in lymph nodes. J Immunol 2010; 184:4819-26; PMID:20308631; http://dx.doi.org/10.4049/jimmunol.0903063

22. Card CM, Yu SS, Swartz MA. Emerging roles of lymphatic endothelium in regulating adaptive immunity. J Clin Investigat 2014; 124:943-52; PMID:24590280; http://dx.doi.org/10.1172/JCI73316

23. Cohen JN, Guidi CJ, Tewalt EF, Qiao H, Rouhani S, Ruddell A, Farr AG, Tung KS, Engelhard VH. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. J Exp Med 2010; 207:681-8; PMID:20308365; http://dx.doi.org/10.1084/jem.20092465

24. Lund AW, Duraes FV, Hirosus S, Raghaban VR, Nembrini C, Thomas SN, Issa A, Hugues S, Swartz MA. VEGF-C promotes immune tolerance in B16 melanomas and cross-presentation of tumor antigen by lymph node lymphatics. Cell Rep 2012; 1:191-9; PMID:22832193; http://dx.doi.org/10.1016/j.celrep.2012.01.005

25. Tewalt EF, Cohen JN, Rouhani S, Guidi CJ, Qiao H, Fahl SP, Conaway MR, Bender TP, Tung KS, Vella AT et al. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. Blood 2012; 120:4772-82; PMID:22993390; http://dx.doi.org/10.1182/blood-2012-04-427013

26. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nat Rev Cancer 2012; 12:210-9; PMID:22362216; http://dx.doi.org/10.1038/nrc3186

27. Balsat C, Signolle N, Goffin F, Delbecque K, Plancoulaine B, Sauthier F, Cowen J, Delbet A, Munaut C, Foidart JM et al. Improved computer-assisted analysis of the global lymphatic network in human cervical tissues. Mod Pathol 2014; 27:887-98; PMID:24309324; http://dx.doi.org/10.1038/modpathol.2013.195

28. Riela LV, Paterson AM, Sharpe AH, Chandraker A. Role of the PD-1 pathway in the immune response. Am J Transplantat 2012; 12:2575-87; PMID:22900886; http://dx.doi.org/10.1111/j.1600-6143.2012.04224.x

29. De Silva NS, Klein U. Dynamics of B cells in germinal centres. Nat Rev Immunol 2015; 15:137-48; PMID:25656706; http://dx.doi.org/10.1038/nri3804

30. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. J Allergy Clin Immunol 2013; 131:959-71; PMID:23465663; http://dx.doi.org/10.1016/j.jaci.2013.01.046

31. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 1996; 8:765-72; PMID:8671665; http://dx.doi.org/10.1038/intimm.8.5.765

32. Nishimura H, Minato N, Nakano T, Honjo T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. Int Immunol 1998; 10:1563-72; PMID:9796923; http://dx.doi.org/10.1093/intimm/10.10.1563

33. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nat Immunol 2010; 11:535-42; PMID:20453843; http://dx.doi.org/10.1038/ni.1877

34. Shalapour S, Font-Burgada J, Di Caro G, Zhong Z, Sanchez-Lopez E, Thibault ML, Barret-Dardenne J, Pastor S, Just-Landi S, Xerri L, Chetaille B, Olive D. PD-1 is a novel regulator of human B-cell activation. Int Immunol 2013; 25:129-37; PMID:23087177; http://dx.doi.org/10.1038/intimm.dxd098

35. Grotz TE, Jakub JW, Mansfield AS, Goldenstein R, Enninga EA, Nevala WK, Leonovich AA, Markovic SN. Evidence of Th2 polarization of the sentinel lymph node (SLN) in melanoma. Oncoimmunology 2015; 4:e1026504; PMID:26405583; http://dx.doi.org/10.1080/2162402X.2015.1026504

36. Freed C, Schuetz F, Sohn C, Beckhove P, Domschke C. B cell-regulated immune responses in tumor models and cancer patients. Oncoimmunology 2013; 2:e25443; PMID:24073382; http://dx.doi.org/10.4161/onci.25443

37. Carmi Y, Spitzer MH, Linde IL, Burt BM, Prestwood TR, Perlman N, Davidson MG, Kenkel JA, Segal E, Pusapati GV et al. Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity. Nature 2015; 521:99-104; PMID:25924063; http://dx.doi.org/10.1038/nature14424

38. Zirakzadeh AA, Marits P, Sherif A, Winqvist O. Multiplex B cell characterization in blood, lymph nodes, and tumors from patients with malignancies. J Immunol 2013; 190:5847-55; PMID:23630345; http://dx.doi.org/10.4049/jimmunol.1203279

39. Shalapour S, Font-Burgada J, Di Caro G, Zhong Z, Sanchez-Lopez E, Dhar D, Willimsky G, Ammirante M, Strasser A, Hansel DE et al. Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy. Nature 2015; 521:94-8; PMID:25924065; http://dx.doi.org/10.1038/nature14395

40. Ruddell A, Mezquita P, Brandvold KA, Farr A, Iritani BM. B lymphocyte-specific c-Myc expression stimulates early and functional expansion of the vasculature and lymphatics during lymphomagenesis. Am J Pathol 2003; 163:2233-45; PMID:14633517; http://dx.doi.org/10.1038/ajpath.2004.89

41. Roozendaal R, Mempel TR, Pitcher LA, Gonzalez SF, Verschoor A, Mebius RE, von Andrian UH, Carroll MC. Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. Immunity 2009; 30:264-76; PMID:19185517; http://dx.doi.org/10.1016/j.immuni.2008.12.014

42. Christiansen AJ, Dieterich LC, Ohs I, Bachmann SB, Bianchi R, Proulx ST, Holleen M, Aebischer D, Detmar M. Lymphatic endothelial cells attenuate inflammation via suppression of dendritic cell maturation. Oncotarget 2016; 7:39421-39435; PMID:27270646; http://dx.doi.org/10.18632/oncotarget.9820