

Regional immunity in melanoma: immunosuppressive changes precede nodal metastasis

Aaron S Mansfield^{1,2}, Shernan G Holtan^{1,2}, Travis E Grotz³, Jake B Allred⁴, James W Jakub³, Lori A Erickson⁵ and Svetomir N Markovic^{1,2}

¹Division of Hematology, Department of Internal Medicine, Mayo Clinic, Rochester, MN, USA; ²Department of Oncology, Mayo Clinic, Rochester, MN, USA; ³Department of Surgery, Mayo Clinic, Rochester, MN, USA; ⁴Cancer Center Statistics, Mayo Clinic, Rochester, MN, USA and ⁵Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

In order to characterize the degree of immunosuppression in regional immunity in patients with melanoma, we used immunohistochemistry to analyze markers of T-cell subtype and polarity, costimulation, dendritic cell maturation, monocytes, lymphatic vasculature, and angiogenesis. Specifically, we analyzed expression of CD4, CD8, CD14, CD40, CD86, CD123, HLA-DR, IL-10, LYVE, VEGFR3, and VEGF-C in lymph nodes. We compared sentinel lymph nodes with and without metastasis from patients with melanoma with both infection inflamed (reactive) and dormant human lymph nodes. There were no differences demonstrated between sentinel lymph nodes with or without metastasis from patients with melanoma in any of the markers that were tested. Both groups of sentinel lymph nodes had fewer CD8⁺ T cells than either set of control nodes. Whereas the infection inflamed lymph nodes demonstrated Th2 polarity, the dormant lymph nodes demonstrated Th1 polarity. In conclusion, changes in regional immunity appeared to precede metastasis in melanoma. Whether there was tumor present in sentinel lymph nodes or not, these nodes demonstrated a marked decrease in cytotoxic T cells compared with both sets of controls. Furthermore, the control lymph nodes used for comparison can significantly impact interpretation, as the dormant and reactive lymph nodes markedly varied in their immune profiles. These immunologic changes may explain the successful metastasis of melanoma in the midst of the immune environment of the sentinel lymph node, and lend insights into the mechanisms of lymphatic metastases in other solid malignancies.

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Lymph node metastasis is one of the most important prognostic factors in melanoma.¹ Whereas patients without nodal involvement generally have excellent outcomes, those with nodal involvement more commonly experience recurrence after resection and diminished survival. Previous investigations suggest that melanoma-draining lymph nodes demonstrate immunologic alterations and are likely immunosuppressed.² Sentinel lymph nodes are the first lymph nodes to drain a tumor bed, and represent the first expected site of metastasis of

melanoma.^{3,4} Sentinel lymph nodes are a site of contact between tumor-associated antigens and the adaptive immune system and represent a regional unit of metastasis and immune response. Accordingly, sentinel lymph nodes are an ideal setting for the investigations of the early immunologic (endocrine and paracrine) events that lead up to melanoma progression from a primary tumor to metastatic cancer. In the past year, we have described that patients with metastatic melanoma exist in a state of systemic chronic inflammation driven by tumor-derived vascular endothelial growth factor (VEGF) overproduction leading to systemic immune polarization to a state of CD4 cell Th2 bias.⁵ Consequently, we sought to determine if similar differences in regional immune profiles in sentinel lymph nodes of patients with melanoma existed.

Correspondence: Dr SN Markovic, MD, PhD, Mayo Clinic 200 First Street SW, Rochester, MN 55905, USA.

E-mail: markovic.svetomir@mayo.edu

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Presented is our immunohistochemical analysis of sentinel lymph nodes with and without evidence of melanoma metastases compared with infection inflamed and dormant human lymph nodes.

Materials and methods

Patients

Archival formalin-fixed paraffin-embedded sentinel lymph nodes from patients with melanoma with (SLN+, $n=8$), or without (SLN-, $n=12$) detectable sentinel lymph nodes metastases were used for the analysis (Table 1). Control infection inflamed 'reactive' lymph nodes ($n=10$) and dormant lymph nodes ($n=10$) were also collected and analyzed. Reactive lymph nodes were from patients with no evidence of malignancy who presented with lymphadenopathy and underwent lymph node excision for diagnosis, or underwent lymph node excision in conjunction with another primary operation. Dormant lymph nodes were collected from patients with breast cancer who underwent prophylactic contralateral mastectomy in conjunction with sentinel lymph node biopsy. These patients had no known active cancer or inflammatory process at the time of lymph node excision. This retrospective study was reviewed and approved by the Mayo Clinic Institutional Review Board.

Immunohistochemistry

Formalin-fixed paraffin-embedded nodes were sectioned into 3 to 4 μm slices and affixed on glass slides. Samples were heated for half an hour at 56 °C, and then deparaffinized in xylene, rehydrated in a graded alcohol series, and washed in water. Heat-induced epitope retrieval was used for antigen retrieval. Endogenous peroxidase activity was quenched in a bath of methanol and hydrogen peroxide. Only one stain was used per slide. We stained samples for CD4 (no. ab864; Abcam Cambridge, MA, USA) at 1:100, CD8 (no. ab4055; Abcam) at 1:100, CD14 (no. ab8676; Abcam) at 1:50, CD40 (no. ab27224; Abcam) neat, CD86 (no. AF-141-MA; R&D Systems, Minneapolis, MN, USA) at 1:20, CD123 (no. 14-1234; eBiosciences, San Diego, CA, USA) at 1:100, HLA-DR (no. 14-990-82; eBiosciences) at 1:100, LYVE (no. DP3500; Acris, Herford, Germany) at 1:25, IL-10 (no. ab33471; Abcam) at 1:400, VEGFR3 (cat no. ab27278; Abcam) at 1:100, VEGF-C (no. sc1881; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100, Foxp3 (ab22510; Abcam) at 1:100, T-bet (no. MAB5385; R&D) at 1:50, GATA-3 (BAF2605; R&D) at 1:5, Galectin 3 (no. ab58086; Abcam) at 1:200, placental growth factor (no. ab9542; Abcam) at 1:1000, osteopontin (MAB14331; R&D) at 1:10, and HLA-G (no. ab7759; Abcam) at 1:100 (the reported function of each marker is described in Table 2). Samples

Table 1 Patient characteristics

	<i>Sentinel lymph nodes with metastasis</i>	<i>Sentinel lymph nodes without metastasis</i>	<i>Dormant lymph nodes</i>	<i>Reactive lymph nodes</i>
Number	8	12	10	10
Age (years) ^a	63 (55–74)	43 (36–67)	52 (49–67)	50 (42–59)
Sex				
Male	5	4	0	2
Female	3	8	10	8
Tumor thickness (mm) ^a	3.5 (1.9–5.2)	1.8 (1.1–2.2)		
Tumor stage ^b				
Ia		2		
Ib		5		
IIa		1		
IIb		4		
IIIa	4			
IIIb	2			
IIIc	2			
Location				
Axillary	4 (50%)	6 (50%)	10 (100%)	1 (10%)
Inguinal	4 (50%)	6 (50%)		
Cervical				2 (20%)
Mediastinal				2 (20%)
Mesenteric				1 (10%)
Pericystic (gall bladder)				2 (20%)
Perinephric				1 (10%)
Periparotid				1 (10%)

^aMedian, 25–75% quartile.

^bAs defined by the American Joint Committee of Melanoma Staging Cancer System.⁴⁸

Table 2 Summary of markers used and their putative function or expression

Marker	Reported function, expression
CD4	Helper T cells
CD8	Cytotoxic T cells
CD14	Surface protein expressed by monocytes and macrophages ⁴⁹
CD40	Tumor necrosis receptor family, costimulatory molecule ¹⁶
CD86	Costimulatory molecule of antigen-presenting cells ¹⁸
CD123	IL-3 receptor α chain, primarily expressed on plasmacytoid dendritic cells ⁵⁰
HLA-DR	Major histocompatibility class II antigen ⁵¹
LYVE	Hyaluronan uptake, present on luminal and abluminal surfaces of lymphatic vessels ⁵²
IL-10	Immunoregulatory cytokine produced by Th2 cells ⁵³
VEGFC	Endothelial cell proliferation ⁵⁴ , angio- and lymph- angiogenesis ⁵⁵
VEGFR3 (also known as FLT4)	VEGF-C and -D receptors on lymphatic endothelium ³⁰
Foxp3	Immunologic self-tolerance, immune response suppression ⁵⁶
T-bet	Transcription factor that promotes Th1 lineage commitment ⁵⁷
GATA-3	Transcription factor that promotes Th2 lineage commitment ⁵⁷
Galectin 3	Melanoma cell plasticity and vasculogenic mimicry ⁴⁵
Placental growth factor	Neoangiogenesis and lymphangiogenesis ⁵⁸
Osteopontin	Chemotaxis (macrophages, T cells, DCs), increased metastatic potential ⁴³
HLA-G	Suppression of natural killer cell and cytotoxic T-cell cytolytic killing ⁵⁹

were incubated overnight with antibody. Peroxidase activity was localized for all samples with 3,3'-diaminobenzidine and were counterstained with hematoxylin and mounted. Immunohistochemistry was performed by Histoserv (Germantown, MD, USA).

Image Analysis

We analyzed one section for each stain per patient. Owing to exhaustion of the tissue block, there were two fewer samples of sentinel lymph nodes with metastasis available for analysis of the exploratory stains (osteopontin, HLA-G, PGF, and galectin 3). Each slide was automatically scanned at $\times 20$ using 4096×64 pixel charged-couple device sensor continuous scan technology (NanoZoomer 2.0; Olympus America, Center Valley, PA, USA) to produce a virtual slide of each section. Digitized images of control tissues were examined to standardize thresholds of each stain. Standardized thresholds were then applied to determine the positively stained area of each section for each stain using IHCscore (Bacus Laboratories, Lombard, IL, USA, now Olympus America). For sentinel lymph nodes with metastasis, the areas of metastasis were not included in the analysis to exclude incorporation of expression of markers by tumor.

Statistics

Basic statistics were used to determine the median and lower and upper quartiles of each stain per group. The expression of each marker was compared between the groups using the Kruskal–Wallis test, and multiple pairwise comparisons were made using Tukey's multiple comparison test if the Kruskal–Wallis test was significant. SAS 9.1 (SAS

Institute, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA) were used for statistical analyses and data figure preparation, respectively. A Bonferroni correction was applied because of the multiple comparisons, and a P -value of ≤ 0.004 was considered significant.

Results

T-Cell Polarity

The biomarkers of T-cell polarity were primarily distributed in the T cell-rich paracortex of lymph nodes (Figure 1). There were significant differences in the areas of expression of the Th1 marker T-bet ($P=0.0004$), the Th2 marker GATA-3 ($P<0.0001$), and cytotoxic T-cell marker CD8 ($P<0.0001$) between groups of lymph nodes. No differences were noted in the areas of expression of Foxp3 or CD4 between the groups. There were no differences between sentinel lymph nodes with or without metastasis in areas of expression of any of the T-cell markers. There was greater expression of T-bet in dormant lymph nodes (median 22.1%, lower and upper quartiles 19.7–33.9%) than reactive lymph nodes (6.7%, 4.5–13.4%), sentinel lymph nodes without metastasis (6.7%, 6.0–9.6%), and sentinel lymph nodes with metastasis (4.0%, 3.3–12.6%; all comparisons $P<0.0001$). There was greater expression of CD8⁺ cells in dormant lymph nodes (35.7%, 26.0–48.2%) than reactive lymph nodes (21.0, 15.5–30.2%; $P=0.0091$), sentinel lymph nodes without metastasis (1.8%, 1.0–2.1%; $P<0.0001$) and sentinel lymph nodes with metastasis (1.1%, 0.3–2.0%; $P<0.0001$) and between reactive lymph nodes, sentinel lymph nodes without metastasis ($P<0.0001$), and sentinel lymph nodes with metastasis ($P<0.0001$). There was greater GATA-3 expression in reactive lymph nodes (6.4%, 4.5–10.6%)

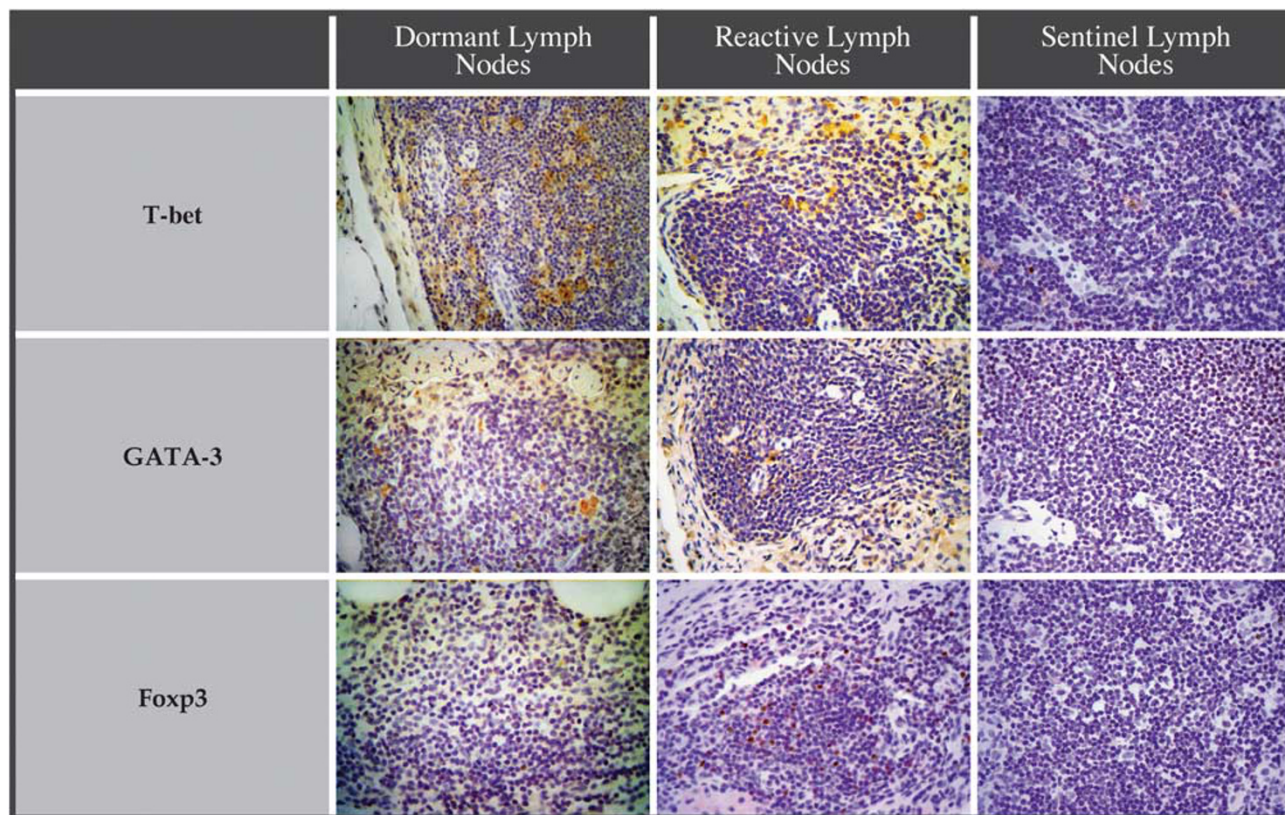


Figure 1 T-cell polarity images. Representative images at high power are shown for dormant, reactive, and sentinel lymph nodes stained for the markers of T-cell polarity, T-bet, GATA-3, and Foxp3.

than dormant lymph nodes (0.4%, 0.3–0.6%), sentinel lymph nodes without metastasis (1.9%, 1.0–4.1%), and sentinel lymph nodes with metastasis (1.7%, 0.5–2.9%; all comparisons $P < 0.0001$).

Antigen-Presenting Cell and Costimulatory Markers

There were significant differences in the areas of expression of CD14 ($P = 0.0001$), CD40 ($P < 0.0001$), and CD86 ($P < 0.0017$) between groups of lymph nodes. There were no differences in the areas of expression of CD123 or HLA-DR between the groups. There were no differences between sentinel lymph nodes with or without metastasis in areas of expression of any of the antigen-presenting cell and costimulatory markers. There was greater expression of CD14 in sentinel lymph nodes without metastasis (0.94%, 0.49–2.27%) compared with reactive lymph nodes (0.05%, 0.04–0.20%; $P = 0.0038$). There was greater expression of CD40 in sentinel lymph nodes without metastasis (59.2%, 50.9–62.0%) than dormant lymph nodes (32.3%, 20.5–49.7%; $P = 0.0002$) and reactive lymph nodes (15.5%, 13.1–26.6%; $P < 0.0001$). There was greater expression of CD40 in sentinel lymph nodes with metastasis (61.3%, 42.6–67.9%) than dormant lymph nodes (32.3%, 20.5–49.7%; $P < 0.0001$) and reactive lymph nodes

(15.5%, 13.1–26.6%; $P < 0.0001$). There was greater expression of CD86 in dormant lymph nodes (23.8%, 18.3–34.7%) than reactive lymph nodes (8.6%, 4.3–16.1%, $P = 0.0007$), sentinel lymph nodes without metastasis (5.2%, 3.6–10.5%; $P < 0.0001$), and sentinel lymph nodes with metastasis (5.0%, 1.5–11.2%; $P < 0.0001$).

Vascular Markers

There were significant differences in the areas of expression of VEGFC ($P = 0.0001$), VEGFR3 ($P = 0.0025$), but not LYVE ($P = 0.32$), between groups of lymph nodes. There were no differences between sentinel lymph nodes with or without metastasis in areas of expression of any of the vascular markers. The greater area of expression of VEGFC in dormant lymph nodes compared with sentinel lymph nodes without metastasis was not statistically significant by pairwise comparison because of the correction factor. There was greater expression of VEGFR3 in dormant lymph nodes (36.7%, 20.9–50.5%) compared with reactive lymph nodes (7.5%, 6.0–12.9%; $P < 0.0001$), sentinel lymph nodes without metastasis (12.2%, 8.1–18.4%; $P = 0.0003$), and sentinel lymph nodes with metastasis (10.3%, 4.7–19.5%; $P = 0.0005$).

Exploratory Biomarkers of Inflammation

There were significant differences in the areas of expression of galectin 3 ($P=0.0011$), osteopontin ($P=0.0004$), IL-10 ($P=0.0013$), HLA-G ($P=0.0038$), and PGF ($P=0.0014$) between groups of lymph nodes. There were no differences between sentinel lymph nodes with or without metastasis in areas of expression of any of the exploratory biomarkers of inflammation. The differences in expression of IL-10, HLA-G, and PGF were not significant by pairwise comparison. There was greater expression of galectin 3 in dormant lymph nodes (12.5%, 8.5–22.4%) compared with sentinel lymph nodes without metastasis (4.0%, 3.4–6.3%; $P=0.0010$) and sentinel lymph nodes with metastasis (2.1%, 1.8–5.2%; $P=0.0011$). There was greater expression of osteopontin in dormant lymph nodes (35.1%, 27.2–39.9%) compared with reactive lymph nodes (5.9%, 3.3–22.2%; $P<0.0001$), sentinel lymph nodes without metastasis (13.2%, 6.7–15.2%; $P<0.0001$), and sentinel lymph nodes with metastasis (6.3%, 1.6–11.0%; $P<0.0001$).

Discussion

We evaluated T-cell markers in sentinel lymph nodes and found that: (1) there were no differences between sentinel lymph nodes with or without metastasis in any of the T-cell markers we investigated, (2) dormant lymph nodes showed greater Th1 polarization than sentinel lymph nodes, and (3) there were fewer CD8 cells in sentinel lymph nodes compared with dormant and reactive lymph nodes. These results suggest that evidence of immune suppression in sentinel lymph nodes of melanoma patients is present even before metastasis. We are the first, to our knowledge, to report these findings in T-cell polarity in sentinel lymph nodes of melanoma patients. An earlier investigation, before the advent of sentinel lymph node biopsy, reported that in patients with clinical stage II disease there was no difference in T-cell markers between lymph nodes with and without metastasis.⁶ Although we did not look at T-cell receptors, one group found decreased expression of T-cell receptor ζ among CD3⁺ T cells in melanoma lymph nodes with metastasis compared with those without metastasis, which was thought to suggest that metastatic lymph nodes are immunosuppressed.⁷ We did not see any differences in CD4⁺ cells between any of the lymph nodes; however, in breast cancer patients, sentinel lymph nodes without metastasis were found to have higher populations of CD4⁺ cells than sentinel lymph nodes with metastasis.⁸ Similar to another group,⁹ we also did not detect any differences in Foxp3⁺ cells between sentinel lymph nodes groups. Unlike our findings, two other groups have reported increases in Foxp3⁺ cells in melanoma-involved lymph nodes compared with those without metastasis,^{10,11} which is similar to results reported in

breast cancer.^{12–14} As we did not include metastatic areas of lymph nodes in our quantification of positively stained cells in order to exclude the inclusion of positively stained melanoma cells, we may not have detected the difference noted by the other groups. Also, the lymph nodes from patients with melanoma evaluated in the other studies were from patients with palpable lymph node disease (per personal communication with authors^{10,11}). Accordingly, their patient populations may represent those with more advanced disease than our patients who did not have clinically detectable lymph node disease before sentinel lymph node biopsy. As circulating melanoma antigen-specific regulatory T cells have been reported in patients with metastatic disease,¹⁵ regulatory T cells may have a role in later stage disease in melanoma.

We also looked at markers of antigen-presenting cells and costimulation and found that: (1) there were no differences between sentinel lymph nodes with or without metastasis in any of the antigen-presenting cell and costimulatory markers we investigated, (2) sentinel lymph nodes demonstrated greater expression of the costimulatory marker CD40 than dormant and reactive lymph nodes, and (3) sentinel lymph nodes had diminished expression of CD86 compared with dormant and reactive lymph nodes. Although there was increased expression of costimulatory CD40 in sentinel lymph nodes, the significance of this finding is uncertain. CD40 is expressed on B cells and antigen-presenting cells, and when activated by CD40 ligand, CD40 stimulates multiple signaling pathways via tumor necrosis factor receptor-associated factors. These downstream effects are critical to the development of cellular and humoral immune responses,¹⁶ however, sole CD40 stimulation in murine melanoma vaccine studies has impaired development of durable tumor responses.¹⁷ Our findings with CD86 are similar to others showing diminished expression of this marker in peripheral blood monocytes of patients with melanoma.¹⁸ Diminished expression of CD40 and CD86 has been found in sentinel lymph nodes without metastasis when compared with non-sentinel lymph nodes without metastasis in melanoma¹⁹ and breast cancer.¹⁴ In breast cancer, no difference in another costimulatory marker, CD83, was found between sentinel lymph nodes with or without metastasis,²⁰ but diminished expression of CD83 has been described in sentinel lymph nodes without metastasis compared with non-sentinel lymph nodes without metastasis.¹⁴ Other groups have looked specifically at dendritic cells. Fewer interdigitating (morphologically mature) dendritic cells have been found in sentinel lymph nodes than non-sentinel lymph nodes in melanoma,²¹ and greater numbers of plasmacytoid dendritic cells have been found in sentinel lymph nodes with metastasis than sentinel lymph nodes without metastasis.²² Many dendritic cells in lymph nodes of melanoma patients have been found to express

indolamine 2,3-dioxygenase.²³ Indolamine 2,3-dioxygenase has been shown to mediate immunosuppression by blocking T-cell proliferation.²⁴ Mature dendritic cells (often measured by DC-Lamp expression) have been associated with expansion of tumor antigen-specific memory effector cytotoxic T cells. Furthermore, the presence of mature dendritic cells in sentinel lymph nodes in melanoma patients is associated with absence of downstream lymph nodes metastases,²⁵ which is similar to what was found in breast cancer.²⁶

We looked at markers of angio- and lymphangiogenesis and found that: (1) there were no differences between sentinel lymph nodes with or without metastasis in any of the markers we investigated, and (2) there was greater expression of VEGFR-3 in dormant lymph nodes compared with sentinel lymph nodes. Angiogenesis is critical for new blood vessel formation and tumor growth.²⁷ Lymphangiogenesis is important for the metastasis of tumors.²⁸ Both angiogenesis and lymphangiogenesis are partially controlled by VEGFs and their receptors.^{28,29} We decided to look at VEGF-C and VEGFR-3 expression in sentinel lymph nodes of melanoma patients. Despite many associations of VEGFR3-mediated lymphangiogenesis with metastasis,³⁰ we found greater expression of VEGFR-3 in dormant lymph nodes compared with sentinel lymph nodes. The presence of a receptor however does not imply its activation. VEGF-A and -C have been shown to promote lymphangiogenesis and metastasis in murine models of skin cancer,^{31,32} and in human breast cancer.³³ Similarly, VEGF-D in the presence of VEGFR-3 has been associated with peritumoral lymphatic vessels and lymph node metastasis in breast cancer.³⁴ Tumor lymphangiogenesis is predictive of metastasis in melanoma^{35–37} and breast cancer.³⁸ VEGF may also have a role in immune suppression. VEGF-C has been shown not only to induce angio- and lymphangiogenesis, but it also attracts macrophages,³⁹ which are also associated with sentinel lymph node metastasis.⁴⁰ Tumor-associated macrophages demonstrate M2 polarization and promote tumor progression.⁴¹ In a previous study, our group showed that patients with stage 4 melanoma are in a systemic state of chronic inflammation (Th2 helper T-cell bias) that is likely mediated by VEGF-A.⁵

We looked at a number of exploratory markers and found: (1) no differences between sentinel lymph nodes with or without metastasis in any of the markers we investigated, (2) greater expression of osteopontin in dormant lymph nodes than sentinel lymph nodes and reactive lymph nodes, and (3) greater expression of galectin 3 in dormant lymph nodes than sentinel lymph nodes and reactive lymph nodes. There were no differences in the expression of IL-10, HLA-G, or PGF. Our group has taken interest in how a placenta invades the uterus and a semiallogeneic fetus escapes rejection from the maternal immune system. Accordingly, we

selected a number of exploratory markers found to be important for proliferation, invasion, and immune tolerance at the fetomaternal interface.⁴² One of these markers, osteopontin, has chemotactic properties for macrophages, T cells, and dendritic cells, and is associated with increased metastatic potential.⁴³ Regardless, we found greater expression of osteopontin in dormant lymph nodes than sentinel lymph nodes. Similar to our results, no differences in the expression of osteopontin was demonstrated between sentinel lymph nodes with or without metastasis in breast cancer.⁴⁴ As osteopontin has chemotactic properties, the diminished expression of osteopontin in sentinel lymph nodes is consistent with the diminished areas of CD8⁺ T cells and CD86⁺ cells that we observed in sentinel lymph nodes. Although galectin 3 is associated with vasculogenic mimicry and metastatic potential,⁴⁵ we found diminished galectin 3 in sentinel lymph nodes without metastasis compared with dormant lymph nodes. Elevated levels of galectin 3 in serum have prognostic value in patients with regionally advanced and metastatic melanoma.⁴⁶ We found a significant difference in IL-10 expression between the lymph node groups; however, there were no significant differences on pairwise comparisons, likely because of the wide variability in our data. Similar to our findings, other groups have not found any difference in IL-10 expression between sentinel lymph nodes with or without metastasis in melanoma,^{9,47} and disparate findings have been reported in breast cancer.^{14,20}

This study raises the issue as to what represents an appropriate control lymph node in studies of regional lymph nodal immunity. We used both reactive and dormant lymph nodes. Whereas the dormant lymph nodes showed marked Th1 polarity, the reactive lymph nodes showed Th2 polarity. As the dormant lymph nodes were removed from patients without any known ipsilateral inflammatory process, they likely represent a resting, Th1-dominant state. On the other hand, reactive lymph nodes were excised from patients with lymphadenopathy or a concurrent inflammatory process, and likely represent the resolution of an immune response with Th2 dominance. The comparison of lymph nodes with just one of these control groups could lead to inappropriate conclusions on immune status.

In aggregate, these findings suggest that changes in regional immunity in sentinel lymph nodes precede metastasis, and may be secondary to the elaboration of soluble mediators by the primary tumor. The change in T-cell polarity, diminished CD8 cell population, and decrease in a costimulatory marker suggest that these mediators are immunosuppressive. These immunologic changes may explain the successful metastasis of melanoma in the midst of the immune environment of the sentinel lymph nodes, and lend insights into the mechanisms of lymphatic metastases in other solid malignancies.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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