Effect of Melanoma on Immune Function in the Regional Lymph Node Basin

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Abstract

Purpose: To determine if melanoma within the tumor microenvironment will result in immunosuppression within the draining lymph node as measured by down-regulation of T-cell receptor ζ (TCR ζ) expression.

Experimental Design: Patients with clinical stage I to III melanoma undergoing wide local excision and sentinel lymph node biopsy or therapeutic lymph node dissection were consented to have a portion of their lymph node sampled. Lymph nodes were classified as macroscopically involved (TI), microscopically involved (MI), noninvolved with positive wide excision (NI+), or noninvolved with negative wide excision (NI-). Lymphocytes were stained using antibodies to TCR ζ and other immune cells and analyzed via flow cytometer. Reverse transcription-PCR was used to assess for mediators of immunosuppression.

Results: Fifty patient lymph node samples (15 TI, 7 MI, 9 NI+, and 19 NI-) were evaluated. Increasing involvement of tumor in the lymph node was associated with decreasing TCR ζ chain expression (TI 56%, MI 76%, and NI- 89%). Differences between TI and MI (P = 0.005), TI and NI- (P = 0.0001), and MI and NI- (P = 0.019) were statistically significant. There was also a significant difference between TCR ζ chain expression of NI+ and NI- (73% versus 89%; P = 0.0016). A trend toward increased arginase expression in tumor-involved lymph nodes was detected by reverse transcription-PCR.

Conclusions: Melanoma involvement of regional nodes is associated with loss of TCR ζ expression that is inversely related to tumor burden. Residual melanoma within the wide local excision specimen is associated with TCR ζ loss in noninvolved sentinel lymph nodes, suggesting that immune modulation precedes tumor spread.

Due to the inherent resistance of melanoma to both chemotherapy and radiation, spread of melanoma beyond the primary lesion poses a significant therapeutic challenge. The observed, albeit limited, efficacy of cytokine-based immunotherapy in patients with stage III and IV melanoma (1–4) has suggested that immunotherapeutic strategies may be effective in controlling earlier-stage disease.

Many cancers, including melanoma, have been associated with immunosuppression (5–7). To facilitate spread and immune evasion, many tumors have been shown to down-regulate immune function at distant sites via a variety of cellular or molecular processes. The T-cell receptor ζ subunit (TCR ζ) is a common site of T-cell regulation due to its extreme susceptibility to decreases in amino acid levels (8, 9). A decrease in immune function as evidenced by decreased TCR ζ expression has been seen in many malignancies, including melanoma (10), cervical cancer (11), and pancreatic cancer (12).

Arginase produced from tumor-associated myeloid cells has been mechanistically linked to the down-regulation of TCR ζ in both renal cell carcinoma (9) and murine lung cancer models (13). Indolamine 2,3-dioxygenase (IDO) produced by immunosuppressive dendritic cells in response to interleukin-10 (IL-10) has also been reported to diminish T-cell function through tryptophan depletion (14). Other molecular, cellular, and direct tumor processes may also play a role in immune modulation, including expansion of T regulatory cells (15), and expression of programmed death ligand 1 (16) and transforming growth factor-β (17).

Because melanoma often spreads to regional lymphatics, we postulated that the draining nodal basin, including the sentinel lymph node, would likely serve as the initial site of both immune modulation and tumor spread. The current practice of removal of sentinel lymph nodes as a staging procedure affords a unique window into the progression of immune dysfunction and tumor spread in vivo. We hypothesized that the presence of...
Materials and Methods

Collection of samples. Patients with clinical stage I to III melanoma being evaluated in the Multidisciplinary Cutaneous Oncology Clinic at Beth Israel Deaconess Medical Center for either wide local excision and sentinel lymph node biopsy or therapeutic lymph node dissection were consented to have a portion of their lymph node sampled for special studies of immune function and tumor involvement to be conducted within the Immune Monitoring Core of the Harvard Skin Cancer Specialized Programs of Research Excellence. The Dana-Farber/Harvard Cancer Center Institutional Review Board approved the nodal sampling protocol and all patients provided written informed consent before surgery. Immediately following surgical removal, a small portion of either the sentinel lymph node or a grossly tumor involved lymph node was provided by Beth Israel Deaconess Medical Center pathologists to Core investigators. The nodes were physically disaggregated into a cellular suspension in FCS with 5% DMSO and frozen for later analysis. A wide local excision around the primary melanoma site was done during the same procedure as the sentinel lymph node biopsy and the excision specimen was provided to the pathology department for standard review for presence of residual tumor.

Assessment of TCR ζ chain expression. Immunosuppression in this study was defined by a decrease in the percent of CD3 cells that express the TCR ζ subunit. Samples were thawed and suspended in flow cytometry run buffer (5% fetal bovine serum in 1× PBS with 0.1% sodium azide). The cells were stained externally with antibodies to CD3 (Becton Dickinson) and internally with antibodies to TCR ζ (Santa Cruz Biotechnology). To facilitate the staining of intracellular proteins, the cells were permeabilized using a Fix and Perm Kit (Caltag Laboratories). To properly assess which cells expressed TCR ζ, a mouse isotype (E Bioscience) was used as a control. A Beckman Coulter Cytomics FC 500 was used for all flow cytometry in this experiment. A sample flow cytometry plot is presented in Fig. 1. The vertical gate was set using an isotype control.

Clinical data. Clinical and pathologic data, including timing of surgeries, patient demographics, and pathology results, were captured in a clinical database that was kept separate from the laboratory investigators evaluating the lymph node samples. Samples were classified according to degree of melanoma involvement within the lymph node as determined by the pathologist. Groups included macroscopically involved nodes (tumor detected by surgeon and verified by pathologists), microscopically involved nodes (tumor detected by pathologists), and noninvolved nodes (no cancer cells seen by pathologists). The noninvolved nodes were further separated into two groups—those with and without residual tumor within the wide local excision specimen taken from around the primary melanoma site.

Evaluation of cellular/molecular mediators of immunosuppression. The cellular suspensions were also stained using commercially available antibodies (E Bioscience, CD4 FITC, CD25 APC, Foxp3 PE; Becton Dickinson, CD11b PE), and (Beckman Coulter, CD14 FITC, CD15 PC5) to identify T regulatory cells (CD4+ CD25+ Foxp3+) and tumor-associated myeloid cells (CD11b+ CD14+ CD15+). As above, a Fix and Perm kit (Caltag Laboratories) was used to stain intracellular antibodies. Isotype controls were run alongside the samples to set accurate baselines (E Bioscience, mouse IgG1 + APC for CD25 APC; Beckman Coulter, mouse IgG1 + PC5 for CD15).

Molecular mediators of immunosuppression were analyzed using real-time reverse transcription-PCR (RT-PCR). A portion of each sample had RNA extracted through a TRIzol preparation (Invitrogen). RT-PCR primers and probes for arginase, IDO, transforming growth factor-β, IL-10, and programmed death ligand 1 were commercially obtained (Applied Biosystems). RT-PCR was done within the Beth Israel Deaconess Medical Center PCR Core Facility using a Perkin-Elmer ABI Prism 7700 Sequence Detector per manufacturer’s directions. The presence of mRNA for the gene product in question was defined as achieving linear phase in less than 40 cycles of denaturation. Samples were run in duplicate and only accepted for analysis if both runs were within two cycles.

Molecular studies for melanoma involvement. Specimens were also analyzed by RT-PCR for tyrosinase and MART-1, two genes commonly expressed by melanoma cells. Primers and probes for PCR were commercially obtained (Applied Biosystems) and samples were run within the Beth Israel Deaconess Medical Center PCR Core facility.

Statistical analysis. For flow cytometric data, Tukey’s multiple comparison test was used to determine which lymph node categories significantly differed based on percentage of TCR ζ expression. This test compares each pair of means with appropriate adjustment for the multiple testing. For RT-PCR data, Fisher’s exact tests were used to statistically assess the relationship of node category and gene expression pattern. Throughout the experiment, a P value of 0.05 was considered the limit of statistical significance.
Results

Patient characteristics. Overall, 50 lymph nodes were evaluated from 50 patients. Fifteen nodes had macroscopic tumor involvement (M1) and seven had microscopic tumor involvement (M1). The remaining 28 lymph nodes were completely free of tumor (NI). Of the nodes without tumor involvement, nine had been removed from patients with residual melanoma detected within the primary tumor site wide local excision specimen (NI+), whereas the remaining 19 came from patients with no tumor involvement of either the sentinel lymph node or the wide excision specimen (NI-).

Demographic and clinical features of the patients included in this analysis divided by subgroup are displayed in Table 1. The four subgroups had relatively similar demographic features. The majority of patients were male; however, there were slightly more women in the noninvolved node with negative wide excision (NI-) group. Ages of subjects ranged from 24 to 85 years. Time from initial biopsy to wide local excision and sentinel lymph node biopsy ranged from 3 to 16 weeks (median 6.5 weeks for the NI+ and 7.0 weeks for NI- patients, respectively). As would be anticipated, the macroscopically involved (M1) and microscopically involved (M1) groups had primary tumors with the thickest Breslow depths, median 2.8 and 4.6 mm, respectively. Of patients with noninvolved lymph nodes, those with positive wide excisions (NI+) had similar primary lesion Breslow thickness (median 1.1 mm) compared with those with negative wide excisions (NI-; median 1.1 mm).

Of the 50 lymph nodes analyzed with flow cytometry, 37 had sufficient remaining tissue to conduct PCR. Of these samples, 24 provided adequate data for accurate analysis with respect to tyrosinase and MART-1. Twenty-two of the 24 nodes analyzed from all categories were positive for tyrosinase by RT-PCR. Of those two nodes negative for tyrosinase, one was macroscopically involved and one was noninvolved. Six of 16 tumor-involved nodes (macroscopic and microscopic) were strongly positive for MART-1. Because RNA was extracted only from a small portion of the lymph node, the tumor-involved portion may have been missed in the PCR analysis. All noninvolved nodes (NI- and NI+) were negative for MART-1, supporting the pathologic assessment that these nodes were free of tumor.

Although a relationship was seen between pathologic features in the primary tumor including Breslow thickness, ulceration, and mitotic rate and nodal status, when nodal status was controlled for, none of these factors independently predicted for decreased TCR zeta expression. In addition, tumor lymphocytic infiltrate status within the primary tumor was categorized as either absent, present, or brisk. No significant difference in TCR zeta expression could be detected based on tumor lymphocytic infiltrate category ($P = 0.1037$).

Relation of TCR zeta chain expression tumor burden within the lymph node. The presence of pathologically detectable tumor within the lymph node was associated with a decreased expression of TCR zeta (Fig. 2). Lymph nodes with macroscopic amounts of tumor had the lowest level of TCR zeta expression (56 ± 5%). Those nodes with microscopically detected tumor had a higher percent TCR zeta expression (76 ± 5%). Noninvolved sentinel lymph nodes removed from patients without residual melanoma at the primary site (NI-) showed the highest level of TCR zeta expression (89 ± 4%). The differences between TCR zeta expression in macroscopically involved nodes and that within both microscopically and noninvolved (NI-) nodes were statistically significant with $P$ values of 0.005 and 0.0001, respectively. The difference between TCR zeta chain expression in microscopically involved and noninvolved (NI-) nodes was also statistically significant ($P = 0.019$). Therefore, not only was tumor presence associated with a decrease of TCR zeta expression, but also there seemed to be an inverse relation between tumor burden and TCR zeta chain expression with increased tumor burden being associated with decreased TCR zeta chain expression.

Relation of TCR zeta chain expression to tumor within the wide local excision specimen. Patients with noninvolved sentinel lymph nodes were separated into those with residual tumor within the wide local excision specimen (NI+) and those without residual tumor in either the wide local excision or lymph node (NI-; Fig. 3). Average lymphocyte TCR zeta chain expression within (NI+) group was 73 ± 5% compared with the 89 ± 4% in the NI- group ($P = 0.016$). Thus, residual tumor within the wide local excision specimen was associated with immune modulation within the sentinel lymph node even in the absence of identifiable lymph node tumor involvement.

Cellular and molecular mediators of immunosuppression. T regulatory cells (CD4⁺ CD25⁺ Fox p3⁺) were found in all four groups of lymph nodes; however, there was no statistically significant association between T regulatory cell population and level of tumor involvement of lymph nodes or wide local excision status (data not shown). No significant population of tumor-associated myeloid cells (CD11b⁺ CD14⁺ CD15⁺) was seen in any of the lymph node groups.

![Fig. 2. TCR zeta expression related to tumor involvement. Increasing tumor volume within the lymph node was associated with increased TCR zeta expression (noninvolved 89 ± 5%, microscopically involved 76 ± 5%, and macroscopically involved 56 ± 4%). $P$ values were derived from Tukey’s multiple comparison test.](image-url)
Table 2 displays the PCR results by group. Arginase I was more likely to be detected in the nodes involved with melanoma compared with noninvolved nodes (43% versus 12%; \( P = 0.097 \)). Furthermore, NI+ nodes exhibited a trend toward more arginase I expression than the NI- nodes (20% versus 8%). IDO expression was more likely to be present in involved than noninvolved nodes (47% versus 30%) and NI+ nodes were more likely to express IDO than NI- nodes (40% versus 25%), although all of these trends were not statistically significant. All but two samples were positive by RT-PCR for both transforming growth factor-\( \beta \) and programmed death ligand 1. These two samples, one NI+ and one NI-, were negative for both gene products. IL-10 was shown in 69% of the involved and 63% of noninvolved nodes representing no significant difference.

**Discussion**

A robust relationship has been observed between immunosuppression and the development of malignancy (18). Melanoma, as well as other cancers, are both more prevalent and more aggressive in transplant patients on immunosuppressive drugs (6, 19, 20). Moreover, nonviral-associated cancers also have increased incidence in patients with acquired immune deficiency syndrome (21–23). Recent investigations suggest that tumors can also use a variety of mechanisms to suppress the immune system, perhaps thereby promoting their own survival.

One model of tumor-induced immunosuppression suggests that tumors secrete substances that disable local immune processes. Such cellular messengers could conceivably travel down the regional lymphatics to the sentinel lymph node and thus be capable of suppressing lymphocyte function even in advance of tumor spread. Under this model, immunosuppression could serve to prepare the lymphatic “soil” to enable the subsequent tumor “seed” to take root, grow, and eventually metastasize to distant sites (Fig. 4).

Work by Cochran and Lee supports this model being operant in melanoma. Cochran et al. (5) found that tumor-free sentinel lymph nodes had significant reductions in both the density and maturation of interdigitating dendritic cells related to their tumor-free nonsentinel lymph node counterparts. Thus, the strongest immunomodulatory effects occur in areas that are directly downstream of the primary tumor. In subsequent work, Lee et al. suggested a mechanism for these histologic changes. They compared the cytokine expression in sentinel lymph nodes that were downstream of primary melanomas with positive versus negative margins after initial biopsy. Sentinel lymph nodes downstream of residual primary melanomas showed increased expression of immunosuppressive cytokines IL-10 and IFN-\( \gamma \) (14).

TCR \( \zeta \) chain expression has been shown to be diminished in various acute and chronic inflammatory conditions, including trauma, chronic infection, and a variety of cancers (10–12, 24, 25). Diminished lymphocyte TCR \( \zeta \) expression has been causally related to critical amino acid deprivation and felt to be a measure of immune dysfunction. Rabinowich and colleagues (26) showed decreased TCR \( \zeta \) expression in both lymph node suspensions and peripheral blood lymphocytes in patients with advanced metastatic melanoma compared with normal controls. These lymphocytes regained TCR \( \zeta \) expression in vitro upon being cultured in a medium containing IL-2. Furthermore, the two patients who responded to high-dose IL-2 treatment had a recovery of TCR \( \zeta \) expression in peripheral blood lymphocytes to normal levels. Those who did not recover clinically did not regain TCR \( \zeta \) expression.

Our finding that the extent of melanoma involvement within regional lymph nodes correlates inversely with the percentage of lymphocyte TCR \( \zeta \) expression suggests that the tumor itself may be directly responsible for immune modulation, perhaps facilitating its own survival and progression. Furthermore, we show that even in the absence of lymph node involvement, the presence of residual tumor at the primary site is associated with a significant decrease in TCR \( \zeta \) chain expression in the sentinel lymph node. Our results expand the observations of Lee et al. (14) by implying an effect of residual tumor on the immune function within the sentinel lymph node in addition to the histologic and gene expression abnormalities reported in their

<table>
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<th>Gene</th>
<th>Arginase I</th>
<th>IDO</th>
<th>TGF-( \beta )</th>
<th>IL-10</th>
<th>PD-L1</th>
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<td>Involved nodes* (%)</td>
<td>6/14 (43)</td>
<td>7/15 (47)</td>
<td>14/14 (100)</td>
<td>9/13 (69)</td>
<td>16/16 (100)</td>
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<td>Noninvolved nodes (%)</td>
<td>2/17 (12)*</td>
<td>5/17 (29)</td>
<td>18/20 (90)</td>
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<td>1/5 (20)</td>
<td>2/5 (40)</td>
<td>6/7 (86)</td>
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<tr>
<td>NI- (%)</td>
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<td>3/12 (25)</td>
<td>12/13 (92)</td>
<td>6/9 (67)</td>
<td>12/13 (92)</td>
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**Table 2.** PCR results for molecular mediators of immunosuppression

NOTE: Thirty-seven samples were available for analysis. A sample was considered positive if it achieved linear phase in less than 40 cycles. Not all columns add to 37 because experiment ran in duplicate and data were excluded if results were more than two cycles apart.

Abbreviations: TGF-\( \beta \), transforming growth factor-\( \beta \); PD-L1, programmed death ligand 1.

*Includes macroscopically (TI) and microscopically (MI) involved lymph nodes.

\( P = 0.097 \).
work. Taken together, these data strongly suggest that not only does tumor-induced immunosuppression exist but it also seems to precede tumor spread.

Alternative explanations for these findings are also possible. Of the patients with noninvolved sentinel nodes, those with residual tumor in the wide local excision specimen undoubtedly had larger tumors as indicated by the failure to remove the entire tumor with the initial biopsy procedure. Although the Breslow thicknesses of the primaries within NI+ and NI- groups were similar, it is possible that the larger primary tumor dimensions in the NI+ patients might imply a distinct tumor biology that was capable of producing immunosuppression. Although this is theoretically true, the fact that larger primaries correlated with decreased immune status at a distant, noninvolved site is further proof of both the inverse correlation between tumor volume and immunosuppression and the fact that immunosuppression precedes cancer spread.

Although a decrease in TCR χ expression suggests a decrease in immune function of the affected T cells, this is not invariably the case. Alternatively, diminished TCR χ chain expression could represent T-cell activation and thus this observation might reflect an active immune response rather than immunosuppression. As lymphocytes were not directly assessed for functionality, determination of their immune function must await future experiments. In addition, it is possible that the initial biopsy may have been sufficient to down-modulate TCR χ expression in the sentinel lymph node and that patients with residual tumor at the primary site might have had more expeditious sentinel node procedures. In fact, those with positive wide local excision underwent wide local excision and sentinel lymph node procedure a median of 6.5 weeks postbiopsy compared with those with negative wide excision with a median of 7 weeks between procedures. Restoration of TCR χ expression has been observed to occur within days in vitro and weeks following extensive surgery. Consequently, given that the sentinel node procedures were done well over a month from the biopsy of the primary in most cases, the effect of the initial biopsy is unlikely to persist at the time of surgery and therefore the half-week time discrepancy is unlikely to account for the difference in TCR χ expression between the two groups.

Data from Rodriguez et al. (8) suggest a role for enhanced arginase I expression leading to depletion of local arginine concentrations in tumor-induced immunosuppression. Arginase has been shown to be necessary in the production of the TCR χ subunit, the rate-limiting subunit in the production of the TCR. Tumor-associated myeloid cells (CD11b+, CD14+, CD15+), which produce arginase I, have been implicated in the TCR χ down-modulation seen in both renal cell carcinoma and murine lung cancer (9, 13). Our study showed a trend toward increased arginase I expression in nodes with either visible microscopic tumor involvement or downstream from residual tumor within the wide local excision specimen. This finding potentially supports a role for arginase in the diminished TCR χ expression observed in patients with melanoma. Unlike the studies of Zea et al. (9) and Rodriguez et al. (13), we were not able to find evidence of tumor-associated myeloid cells, suggesting an alternative source for arginase production in patients with melanoma.

Other mechanisms have been suggested to be responsible for diminished TCR χ expression in melanoma containing lymph nodes. Gajewski and Lee et al. have suggested that IDO, the rate-limiting enzyme in tryptophan catabolism, may be a mediator of this immunomodulation. In this theory, IL-10 produced by the tumor leads to proliferation and activation of immunosuppressive dendritic cells, which in turn produce IDO. The IDO production leads to depletion of local tryptophan levels resulting in down-regulation of the TCR χ expression (27–29). Our identification of increased levels of IDO expression with tumor presence either within the lymph node or at the primary site, although lacking statistical significance, supports the existence of this mechanism (14). However, in contrast to the results of Lee, we were unable to detect any association between the level of IL-10 expression and either the degree of tumor involvement or immunosuppression. Perhaps the collection of more samples in various categories would enable a more systematic evaluation of the mediators responsible for diminished TCR χ expression.

Regional immune dysfunction represents a potential target for therapeutic intervention. If immunosuppression of the draining lymph node basin facilitates the metastasis of melanoma, then reversing the local immune dysfunction might theoretically hinder this spread. Lee et al. (14) were able to show an increase in both T-cell area and interdigitating dendritic cell area/density within the sentinel lymph node in patients following injection of recombinant human granulocyte macrophage–colony stimulating factor at the primary site. Theoretically, such a reversal might also be associated with an improvement in TCR χ chain expression. This hypothesis is testable clinically. It remains to be seen whether peritumoral application of agents such as granulocyte macrophage–colony stimulating factor or other immune agonists before wide local excision and sentinel node biopsy would result in increased TCR χ chain expression within the draining lymph node. If so, this could potentially translate into less tumor involvement in the sentinel lymph node and enhanced tumor-specific immunity, which ultimately might lead into improved clinical outcomes for patients with intermediate and high-risk melanomas.
References

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