

Quantitative Analysis of Melanoma-Induced Cytokine-Mediated Immunosuppression in Melanoma Sentinel Nodes

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ABSTRACT

Purpose: Melanoma sentinel nodes (SN) show evidence of immunosuppression prior to tumor metastasis. Interleukin (IL)-10 and IFN- γ can induce dendritic cells (DC) that express immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). The goals of this study are to evaluate the role of melanoma in SN immunosuppression and to assess reversibility of SN immunosuppression by a cytokine therapy.

Experimental Design: Fifty-seven clinical stage I/II melanoma patients underwent wide local excision and sentinel lymphadenectomy (WLE/SL), with removal of non-SN. In 21 patients, nodal RNA was analyzed by quantitative real-time PCR for expression levels of IL-2, IL-10, IL-12, IFN- γ , and IDO genes. Among the remaining 36 patients, 15 received peritumoral injection of recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) 2 to 5 days prior to WLE/SL. Lymph nodes (LN) from these 36 patients were assessed for T-cell area, DC area, and DC density.

Results: Of 21 patients whose nodal RNA was analyzed, 13 had residual melanoma at the primary site or a tumor-positive SN. In these patients, expression levels of IL-10 ($P = 0.05$), IFN- γ ($P < 0.05$), and IDO ($P = 0.06$) were dramatically higher in SNs than non-SNs. This difference was not evident in the 8 patients without residual melanoma or SN metastasis. Of the 36 patients whose LNs were examined for histologic features, the 15 patients who received rhGM-CSF had significantly higher SN values of T-cell area, DC area, and DC density than those who did not receive rhGM-CSF.

Conclusions: Our data provide molecular evidence of cytokine-mediated SN immunosuppression that is associated

with presence of melanoma. Furthermore, SN immunosuppression can potentially be reversed by a cytokine therapy.

INTRODUCTION

There is no effective treatment for metastatic melanoma. Chemo- and radiation therapies have shown some utility, but are not uniformly effective in melanoma. Immune-based treatment strategies, such as IFN- α -2b (1) and interleukin (IL) 2 (2, 3) have shown encouraging outcomes, resulting in approval of these agents by the U.S. Food and Drug Administration (FDA). In addition, several phase I/II clinical trials have shown survival advantages for advanced-stage melanoma patients who received cancer vaccines for metastatic disease (4–9), further supporting a relationship between melanoma progression and the immune system.

It is believed that a primary melanoma invades the local lymphatic system before spreading to distant sites because most patients have lymph node (LN) metastases prior to development of distant disease. Because the sentinel node (SN) is the first LN draining the primary melanoma and thus the first site of regional lymphatic metastasis, evaluation of SNs provides a unique opportunity to study the early phase of tumor-LN immune interaction. Recent evidence suggests that regional LNs, especially the SNs, are immunosuppressed when compared with neighboring non-SNs in patients with primary melanoma (10–14). Lymphocytes from LNs closest to the primary melanoma show diminished proliferation in response to lectins or IL-2. These LNs also generate migration-inhibitory lymphokines and contain increased numbers of suppressor cells (10–12). Melanoma SNs show a significant decrease in T-cell-rich paracortical areas, as well as profound down-regulation of interdigitating dendritic cells (IDC; ref. 13), which are involved in antigen processing and subsequent T-cell activation. These changes are evident even in the absence of SN metastasis, suggesting that primary melanoma interacts with the SNs to create an immunosuppressive microenvironment that favors melanoma metastasis and growth. The significance of peritumoral infiltration of activated dendritic cells (DC) in antitumor activity and clinical prognosis has been shown in various cancers, including melanoma (15–18).

IL-10 is a potent regulator of immunosuppression (19–25) and may play a significant role in melanoma progression (20, 21). IL-10 can interfere with T-cell activation by inducing immunosuppressive DCs (22–25). The recent discovery of indoleamine 2,3-dioxygenase (IDO)-expressing DCs in the SNs of melanoma patients provides insight into the relationship between SN immunosuppression and local cytokine microenvironment. DCs are professional antigen-presenting cells that play a crucial role in T-cell activation. There exists a subset of CD123+/CCR6+ DCs that express IDO (25), which is a rate-limiting enzyme in tryptophan catabolism (26). IDO expression by these CD123+/CCR6+ DCs have shown to be immunosuppressive, as shown by inhibition of T-cell proliferation *in vitro* (27) and reduction of

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T-cell response *in vivo* (28). Enhanced IDO activity has been shown after stimulation with Th-1 type cytokines (29), and induction of immunosuppressive IDO+ DCs can be regulated by IL-10 (25).

It has been well documented that a local cytokine milieu can influence monocyte differentiation as well as DC maturation (30, 31). These studies suggest that the local cytokine milieu can have a significant impact on maturation and subsequent function of DCs, and that the ultimate fate of a DC (i.e., immune stimulatory versus suppressive) is not determined until the terminal stage of its differentiation (30). Given these findings, we hypothesize that primary melanoma interacts with the locoregional immune system, especially the SN, inducing local immunosuppression and thereby creating a microenvironment conducive for lymphatic metastasis and tumor growth. Here, we provide evidence that SN immunosuppression is induced by melanoma and is mediated by local cytokines. The resulting SN cytokine milieu favors immunosuppressive DCs and SN immune dysfunction. In addition, we show that histologic evidence of SN immune dysfunction can be reversed by cytokine-mediated locoregional therapy.

MATERIALS AND METHODS

Study and Experimental Design. Both parts of the study, which involved a total of 57 patients (21 and 36) with informed consents, were approved by the John Wayne Cancer Institute/Saint John's Health Center Joint Institutional Review Board.

Comparison of SN and Non-SN Cytokine Microenvironment. This is a prospective study comparing immune-regulatory gene expression levels in melanoma SNs and neighboring non-SNs as a function of the presence or absence of residual melanoma either in the SN or primary site. This portion of the study involved 21 patients with biopsy-proven American Joint Committee on Cancer (AJCC) clinical stage I/II (no palpable LNs) melanoma. All patients underwent wide local excision and sentinel lymphadenectomy (WLE/SL) along with concomitant removal of adjacent non-SNs, as described in a later section. A patient was considered positive for postbiopsy residual melanoma [PRM(+)] if any of the following conditions applied: (a) pathologically identifiable positive margin in the initial diagnostic biopsy specimen of the primary melanoma, (b) pathologically identifiable residual melanoma in the WLE specimen, or (c) tumor-positive SN. Using quantitative real-time reverse transcription-PCR (RT-PCR), relative levels of immune-regulatory genes expressed in the SNs were analyzed as a function of presence or absence of PRM. The cytokines studied were IL-2, IL-10, IL-12, and IFN- γ . The immunosuppressive enzyme IDO was used as a molecular surrogate for LN immunosuppression (25–29). Non-SNs from the same lymphatic drainage basin were used as surrogates for normal LNs (13). Statistical analyses were done by using Fisher's exact test, χ^2 test, Wilcoxon rank sum test, and Wilcoxon matched-pair signed rank test.

Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor Study. We previously reported that melanoma SNs show histologic evidence of immune dysfunction (13). A pilot study was done to determine potential reversibility of SN immune dysfunction by a cytokine-mediated therapy. This portion of the study included additional 36 AJCC

clinical stages I/II patients with biopsy-proven melanoma. Fifteen patients received peritumoral i.d. injection of recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) (Sargramostim, Berlex Corp., Seattle, WA; gift of Mark Gilbert, MD, Berlex Corp., Seattle, WA), 2 to 5 days prior to WLE/SL in a consecutive dose-escalation protocol (5 patients received 100 $\mu\text{g}/\text{m}^2$, 5 patients received 150 $\mu\text{g}/\text{m}^2$, and 5 patients received 200 $\mu\text{g}/\text{m}^2$); 21 patients did not receive rhGM-CSF. WLE/SL was done with concomitant removal of adjacent non-SNs, as described in a later section. SNs and non-SNs were stained by immunohistochemistry on 4- μm formalin-fixed and paraffin-embedded tissue sections to detect S-100 protein (for IDCs) and CD43 (for T cells; Dako Corp., Carpinteria, CA). The relative T-cell area, IDC area, and IDC density were used as the histologic surrogates of LN immune function (11, 13, 14). Morphometric analyses of the LNs by measuring these histologic parameters were done by using an IBM PC-assisted image analysis system (Planar Morphometry, version 2.1, Southern Micro Instruments, Atlanta, GA) by an observer blinded to patients' clinical status and samples analyzed. T-cell and IDC areas were measured as the percent of LN cross-sectional surface occupied by T cells and IDCs, respectively. IDC density was measured by averaging the number of IDCs present in a cross-sectional area of the LN (number of IDCs per square millimeter). Statistical analyses used Wilcoxon rank sum test.

WLE/SL and RNA Extraction. All patients had previously undergone a skin biopsy for diagnosis of their primary melanoma. After an informed consent (for both routine surgery and our research protocol) was obtained, WLE/SL was done as previously described (32, 33). In brief, preoperative $^{99\text{m}}\text{Tc}$ -labeled sulfur colloid lymphoscintigraphy to identify the nodal basin at risk for metastasis was followed by intraoperative peritumoral i.d. injection of isosulfan blue dye (Lymphazurin, Tyco International, Norwalk, CT). In our review of the literature, we did not find any data to suggest that DCs are sensitive to either sulfur colloid or Lymphazurin. The SN was localized intraoperatively by using a hand-held γ probe and by visual inspection of the blue dye, which was used as the gold standard for identifying the SNs (34). An additional random neighboring non-SN (nonblue and nonradioactive) was harvested within 2 cm of the SN at the same time. A portion of each SN and non-SN was collected and placed immediately in either RNAlater (Qiagen, Valencia, CA) or a -20°C container (for earlier samples) for RNA preservation and then processed at 4°C . RNA extraction and purification was done by using either Tri-Reagent (Molecular Research Center, Cincinnati, OH; ref. 35) or RNeasy kit (Qiagen), according to the manufacturer's instructions (RNeasy Mini Handbook). The initial RNA concentration and quality were assessed by optical densitometry at 260 and 280 nm. The final concentration, quality, and purity of total RNA were determined by using the RNA 6000 Nano Assay kit on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) per manufacturer's instructions (Agilent Bioanalyzer Handbook).

Quantitative Real-Time PCR to Measure Gene Expression. Commercially available forward/reverse primer sets and probes for IL-2, IL-10, IL-12, and IFN- γ were used (Applied Biosystems, Foster City, CA). A forward/reverse primer set and probe for IDO were custom designed by using published mRNA

sequence (36) and ABI Prism Primer Express Software v2.0, purchased from Applied Biosystems. The mRNA segments were amplified on a panel of cDNA derived from phytohemagglutinin-stimulated lymphocytes by using RT-PCR. Subsequently, the amplicons were separated by using 2% agarose gel electrophoresis and then recovered by using the QIAquick gel extraction method (Qiagen) according to the manufacturer's instructions. The amplicons were ligated into pCR II-TOPO cloning vector (Invitrogen, San Diego, CA) and transformed into DH5- α *E. coli* cell line for plasmid amplification (TOPO TA Cloning, Invitrogen, Carlsbad, CA). The transfected cell colonies were identified and cell cultures were expanded as described elsewhere (35). The amplicons were subsequently isolated and purified. To confirm the correct size of the inserted PCR product, plasmids were digested with specific restriction enzymes, and the cDNA clone PCR products were evaluated by using 2% agarose gel electrophoresis. After the concentration of the plasmid was determined by optical densitometry at 280 nm, the plasmid solution was serially diluted and amplified via quantitative RT-PCR to generate the standard curves.

RNA from the LN samples was reverse-transcribed into cDNA by using random hexamers and M-MLV kit (Promega, Madison, WI), and then evaluated by quantitative RT-PCR using the ABI Prism 7000 sequence detection system and the Taqman assay (Applied Biosystems). Briefly, 5 μ L of cDNA (reverse-transcription product) was combined with 1.25 μ L of 20 \times target primer and 2 \times TaqMAN universal PCR Master Mix (Applied Biosystems) for a total volume of 25 μ L. The thermocycler parameters were 95 $^{\circ}$ C for 10 minutes (for initial denaturation) followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 seconds and annealing and extension at 60 $^{\circ}$ C for 1 minute. The resultant fluorescence, which correlates with the amount of cDNA, was measured and plotted against the cloned plasmid standard curve to determine mRNA copy number for the gene of interest. The samples were evaluated in duplicate.

RESULTS

Effects of Postbiopsy Residual Melanoma on SN Cytokine Gene Expression. To evaluate the effects of PRM on the cytokine microenvironment of locoregional LNs, we measured cytokine gene expression levels in the regional LNs from early-stage melanoma patients. Twenty-one patients with biopsy-proven AJCC clinical stage I/II melanoma underwent WLE/SL, during which SNs and neighboring non-SNs were collected. Of 21 patients, 13 (62%) had a PRM present at the time of WLE/SL, whereas 8 (38%) showed no evidence of PRM. Univariate analysis shows that the two patient groups were significantly different with regard to Breslow thickness of their primary tumor (median, 2.00 mm for PRM(+) versus 1.05 mm for PRM(-); $P = 0.03$) but not in age, gender, ulceration of primary tumor, Clark level, or postbiopsy days (number of days from initial skin biopsy to WLE/SL; Table 1). The time interval between skin biopsy and WLE/SL did not contribute to differential cytokine gene expression (correlation coefficient <0.20 for each of the genes tested). Within the PRM(+) group, comparison of 9 SN(-) patients versus 4 SN(+) patients failed to show any significant difference in cytokine expression patterns (data not shown).

Table 1 Clinicopathologic features of 21 patients in the cytokine expression study

	PRM(+), n = 13	PRM(-), n = 8	P
Age (y)			
Mean \pm SD	55.38 \pm 13.33	48.88 \pm 16.31	
Median	58.00	44.50	0.27*
Range	30-76	32-74	
Gender			
Male	9	4	0.65†
Female	4	3	
Breslow (mm)			
Mean \pm SD	2.67 \pm 1.93	1.18 \pm 0.39	
Median	2.00	1.05	0.03*
Range	>0.3-5.5	0.93-2.0	
Clark level			
II	1	1	0.12‡
III	4	4	
IV	8	3	
Ulceration			
Positive	4	1	0.61†
Negative	9	7	
Postbiopsy days			
Mean \pm SD	29.15 \pm 8.85	24.37 \pm 12.01	
Median	29	24	0.31*
Range	14-45	7-43	

NOTE: Univariate analysis of difference between the two groups is shown. Differential Breslow thickness is not associated with differential cytokine expression between the two groups (data not shown).

*Wilcoxon rank sum test.

†Fisher's exact test.

‡ χ^2 test.

We used quantitative RT-PCR to compare gene expression levels (absolute mRNA copy numbers per microgram RNA) in pairs of SNs and non-SNs from the same lymphatic drainage basin. Differences between the SN and non-SN of each pair were calculated and expressed as percent increase or decrease from the non-SN. The relative levels of genes expressed in the SNs were analyzed as a function of presence or absence of PRM (Table 2). The presence of PRM was significantly associated with increased expression of IL-10 (median increase of 48%, $P = 0.05$) and IFN- γ (median increase of 40%, $P = 0.04$) genes in SNs compared with matched non-SNs. No significant differences were noted for gene expression of IL-2 and IL-12, the

Table 2 The relative levels of genes expressed in the SNs versus non-SNs analyzed as a function of the presence or absence of PRM

	Median % Δ	Range % Δ	P*
PRM(+), n = 13			
IL-2	+25.97	-8.56 to 672.11	0.20
IL-10	+48.04	-50.35 to 1056.60	0.05
IL-12	+37.04	-53.50 to 1271.51	0.19
IFN- γ	+40.03	-16.86 to 2213.20	0.04
IDO	+83.26	-50.50 to 941.80	0.06
PRM(-), n = 8			
IL-2	-28.02	-63.11 to 83.94	1.00
IL-10	-1.67	-53.41 to 2009.72	0.94
IL-12	-28.07	-69.02 to 9786.63	0.84
IFN- γ	-56.73	-99.49 to 185.29	0.46
IDO	-11.13	-58.93 to 58.62	0.57

NOTE: % $\Delta = [(SN - non-SN)/non-SN] \times 100$.

*Wilcoxon matched-pair signed rank test.

prototypical Th-1 type cytokines. Gene expression of immunosuppressive enzyme IDO showed a trend toward increase in PRM(+) SNs (median increase of 83%, $P = 0.06$). When PRM was not present, expression of immune-regulatory genes was not significantly different between SNs and non-SNs (Table 2). To evaluate the thickness of the primary tumor as a possible confounding factor, we analyzed the data for differences in immune-regulatory gene expression at 1-mm intervals of Breslow thickness (from 0.5 to 4.5 mm). Breslow thickness was not a significant factor in determining immune-regulatory gene expression in the SNs (data not shown).

rhGM-CSF Reverses Melanoma-Associated SN Immune Dysfunction. A pilot study was done with additional 36 AJCC clinical stage I/II melanoma patients to determine whether histologic evidence of SN immune dysfunction could be reversed by using a cytokine therapy. rhGM-CSF was chosen for the following reasons: (a) GM-CSF has been shown to facilitate monocyte differentiation *in vitro*, (b) rhGM-CSF is effective against melanoma *in vivo* when injected intralesionally (37–39), (c) rhGM-CSF-based treatments have been shown to prolong survival of stage III/IV melanoma patients (9, 40), and (d) rhGM-CSF is FDA approved for human usage. Although we had initially considered using anti-IL-10 antibody to show a cause-and-effect relationship, no FDA-approved anti-IL-10 antibody exists currently for human usage.

Thirty-four SNs and nine non-SNs were obtained from 15 patients who received preoperative peritumoral injection of rhGM-CSF, and 21 pairs of SNs and non-SNs were obtained from 21 patients who did not receive rhGM-CSF. By using antibodies against S-100 protein and CD43, immunohistochemistry was done on the LNs to identify IDCs and T cells, respectively. The relative T-cell area, IDC area, and IDC density were all significantly higher ($P < 0.001$) in SNs from patients who had received preoperative peritumoral rhGM-CSF when compared with SNs from patients who did not receive rhGM-CSF (Table 3). Furthermore, the relative T-cell area, IDC area, and IDC density were not different between SNs and non-SNs of the rhGM-CSF-treated group. These findings show that morphologic differences between SNs and non-SNs can be reversed with a cytokine (rhGM-CSF) therapy.

DISCUSSION

Local cytokine milieu can influence monocyte differentiation as well as DC maturation. For example, a monocyte population of peripheral blood mononuclear cells can be differentiated into DCs by incubation in GM-CSF/IL-4-enriched media. A study by Palucka et al. (30) indicates that monocyte-derived CD1a+/CD14- DCs can revert to CD1a-/CD14+ macrophage phenotype and vice versa, depending on the locally dominant cytokine environment. This plasticity is maintained until the terminal stage of DC differentiation. Geissman et al. (31) show that GM-CSF/IL-4-derived CD1a+ DCs grown in transforming growth factor β 1 and tumor necrosis factor α show a persistent immature phenotype but become functionally mature once exposed to CD40L. These studies suggest that local cytokine milieu and immunostimulatory signals can have a significant impact on maturation and subsequent function of DCs and that the

Table 3 Nodal histologic features according to rhGM-CSF exposure

Node and treatment type	<i>n</i>	T-cell area (%)	IDC area (%)	IDC density (/mm ²)
(1) SN, no rhGM-CSF	21	8.48 ± 8.25	1.24 ± 2.48	15.59 ± 19.84
(2) Non-SN, no rhGM-CSF	21	38.49 ± 20.1	21.29 ± 12.58	119.61 ± 56.33
(3) SN, rhGM-CSF	34	28.72 ± 19.50	23.43 ± 20.23	122.16 ± 84.74
(4) Non-SN, rhGM-CSF	9	39.33 ± 20.89	20.72 ± 19.39	108.10 ± 64.06
Wilcoxon rank sum test, <i>P</i>				
(1) vs. (3)	<0.001	<0.001	<0.001	
(3) vs. (4)	0.43	0.27	0.81	
(2) vs. (4)	0.65	0.57	0.37	

NOTE: T-cell area, IDC area, and IDC density were significantly lower in the SNs from patients who did not receive peritumoral rhGM-CSF. The SNs from patients who received peritumoral rhGM-CSF had these values restored to the “normal” level. Values are mean ± SD.

ultimate fate of a DC (i.e., immune stimulatory versus suppressive) is not determined until the terminal stage of its differentiation (30). Given these findings, it is plausible to hypothesize that melanoma SN immunosuppression is mediated in part by cytokines.

IL-10 has been identified as a potent regulator of immunosuppression (19–25) and plays a role in melanoma disease progression (20, 21). Animal studies involving xenotransplantation of B16 melanoma cells show enhanced tumor growth after injection of intralesional IL-10 (20). In addition, several melanoma cell lines are known to secrete IL-10 (21). Detailed studies suggest that IL-10 can interfere with T-cell activation by induction of immunosuppressive DCs (22–25). Our recent studies show that increased expression of IL-10 in peripheral blood mononuclear cells is associated with poor survival of patients who received polyvalent irradiated whole-cell vaccine therapy for late-stage melanoma (41). Additional studies using magnetic bead reverse-isolation method show that the CD14+ population, not CD8+ or CD4+ T cells, within peripheral blood mononuclear cells is responsible for differential IL-10 expression (42). These findings also suggest a significant interaction between IL-10 and immune-regulatory cells (i.e., monocytes, macrophages, and DCs) during melanoma progression.

The significance of IL-10 and local immunosuppression is further documented by discovery of IDO+ DCs. DCs are professional antigen-presenting cells that play a crucial role in T-cell activation. DC-activated T cells were initially thought to be only immune stimulatory and cytotoxic. However, a subset of CD123+/CCR6+ DCs that express IDO, which is a rate-limiting enzyme in tryptophan catabolism, has recently been identified (25, 26). Catabolism of tryptophan into its metabolites by these IDO-expressing DCs can inhibit T-cell proliferation *in vitro* (27) and reduce T-cell response *in vivo* (28). Enhanced IDO activity

has been shown after stimulation with Th-1 type cytokines (29), and it can also be induced by stimulation of immature CD123+/CCR6+ DCs with IFN- γ (a classic proinflammatory cytokine), with subsequent reduction in T-cell proliferation (25). For these reasons, IDO expression was used as a molecular surrogate for LN immunosuppression in this study. Furthermore, induction of immunosuppressive IDO+ DCs may be regulated by IL-10. Whereas stimulation of mature DCs with IFN- γ resulted in down-regulation of IDO activity, IFN- γ stimulation of DCs matured in the presence of IL-10 resulted in enhancement of IDO expression (25). The relevance of IDO in local down-regulation of immune function in patients with early-stage melanoma has been suggested by the presence of IDO+ DCs in melanoma SNs (25).

To test the hypothesis that SN immunosuppression is induced by melanoma and mediated in part by cytokines, we used quantitative RT-PCR to investigate the SN and non-SN cytokine milieu. Direct measurement of cytokine levels seems simple and attractive but is very difficult due to relative instability of the cytokines and small amount of cytokines available in the LN fragments. Leong et al. (43) attempted direct measurement of several cytokines from SNs of melanoma patients by using ELISPOT assay but were unsuccessful except when LN-derived lymphocytes were stimulated for 40 hours with staphylococcal enterotoxin A. Unfortunately, this type of stimulation assay does not accurately represent what occurs *in vivo*. To ensure *in vivo* fidelity, we used freshly preserved tissue taken uniformly from the tissue collected for routine pathologic analysis. In addition, RNA from each sample was assessed for purity, quality, and integrity by using the Agilent 2100 BioAnalyzer. Our data indicate that if PRM was present at the time of WLE/SL, the SNs have significantly different cytokine microenvironment when compared with neighboring non-SNs (Table 2). More specifically, expression levels of IL-10 and IFN- γ genes were significantly higher in the SNs (compared with matching non-SNs) when PRM was present, suggesting that melanoma interacts with the SNs to increase expression of these genes. However, SN expression of IL-2 and IL-12 genes was not changed by the presence of PRM, suggesting that SNs have a rather specific cytokine response to neighboring melanoma. A median increase of 83% in IDO gene expression in PRM(+) but not PRM(-) SNs further supports the theory that SN immunosuppression is associated with, if not contributed by, neighboring melanoma. Our data are also in agreement with the finding that IL-10 and IFN- γ may have a combined coregulatory role in expression of IDO by DCs (25). In other words, the presence of IL-10 can turn a proinflammatory signal of IFN- γ into an immunosuppressive signal (25).

Our data also show that SNs from PRM(-) patients do not show significant difference in immune-regulatory gene expression when compared with its matched neighboring non-SNs, suggesting that immunosuppressive SN cytokine microenvironment is potentially reversible once melanoma is completely removed from the locoregional environment. Therefore, to determine potential reversibility of SN immune dysfunction by altering SN cytokine milieu, we did a pilot study by peritumorally injecting rhGM-CSF 2 to 5 days prior to WLE/SL. Histologic examinations of SNs and non-SNs by

using immunohistochemistry show that preoperative peritumor administration of rhGM-CSF significantly increases T-cell area, IDC area, and IDC density in the SNs. This finding, which suggests that we can potentially restore SN immune function by altering SN cytokine microenvironment, further supports the hypothesis that SN immunosuppression is cytokine mediated. Previous studies have already shown the importance of activated DCs in antitumor activity (15–18) and the therapeutic response of intralesional rhGM-CSF in melanoma patients (37–39), proving a support for using T-cell area, IDC area, and IDC density as a measure of antitumor immune response in this setting.

Functional and histologic evidence of immunosuppression in melanoma regional LNs, especially the SNs, has already been shown (10–14). In this study, we used fresh nodal tissue to examine melanoma SN cytokine profiles as a function of PRM. Our data provide molecular evidence that SNs have different cytokine profiles than neighboring non-SNs, depending on the presence or absence of locoregional melanoma. These findings suggest that SN immunosuppression is induced by melanoma and is mediated in part by cytokines such as IL-10 and IFN- γ . SN cytokine microenvironment is similar to that of non-SN in the absence of PRM, suggesting that immunosuppressive SN cytokine microenvironment can be reversed. Findings from the pilot study show that manipulation of local cytokine microenvironment by using a cytokine, such as rhGM-CSF, can reverse the histologic evidence of immune dysfunction, suggesting a restoration of SN immune function. The data offer a strong argument for complete surgical resection of locoregional tumor in patients with early-stage disease in an attempt to reverse tumor-induced locoregional immunosuppression. Applied systemically, our data provide a possible mechanism of tumor immune evasion and a good rationale for immune-based systemic treatment strategy in a postsurgical adjuvant setting. Finally, our data also suggest that a single cytokine, such as IFN- γ , can neither be classified solely as “proinflammatory” in its function nor be reliably used as a measure of desired enhanced immune response following an immunotherapy. Different temporal and combined cytokine exposures can significantly alter its effects on a given group of target cells.

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