Transcriptional Profiling of Melanoma Sentinel Nodes Identify Patients with Poor Outcome and Reveal an Association of CD30+ T Lymphocytes with Progression

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Abstract
Sentinel lymph nodes set the stance of the immune system to a localized tumor and are often the first site to be colonized by neoplastic cells that metastasize. To investigate how the presence of neoplastic cells in sentinel lymph nodes may trigger pathways associated with metastatic progression, we analyzed the transcriptional profiles of archival sentinel node biopsy specimens obtained from melanoma patients. Biopsies from positive nodes were selected for comparable tumor infiltration, presence or absence of further regional node metastases, and relapse at 5-year follow-up. Unsupervised analysis of gene expression profiles revealed immune response to be a major gene ontology represented. Among genes upregulated in patients with progressing disease, the TNF receptor family member CD30/TNFRSF8 was confirmed in biopsy specimens from an independent group of patients. Immunohistochemical analysis revealed higher numbers of CD30+ lymphocytes in nodes from progressing patients compared with nonprogressing patients. Phenotypic profiling demonstrated that CD30+ lymphocytes comprised a broad population of suppressive or exhausted immune cells, such as CD4+Foxp3+ or PD1+ subpopulations and CD4+CD8– T cells. CD30+ T lymphocytes were increased in peripheral blood lymphocytes of melanoma patients at advanced disease stages. Our findings reinforce the concept that sentinel nodes act as pivotal sites for determining progression patterns, revealing that the presence of CD30+ lymphocytes at those sites associate positively with melanoma progression.

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Introduction
Sentinel lymph node (SN) biopsy (SNB) represents the standard of care for the staging and management of clinically localized cutaneous melanomas. SNB detects metastasis in approximately 20% of patients with primary melanoma T1B-T4B. Patients with tumor-positive SNBs are treated with regional lymphadenectomy by completion lymph node dissection (CLND), and approximately 20% show metastatic involvement of additional nodes. Patients with lymph node metastases are classified as stage III, and their estimated 5-year survival rates are 78%, 59%, and 40% for stages IIIA, IIIB, and IIIC, respectively (1). However, this subclassification, which is based on the most predictive independent prognostic factors (i.e., the number of tumor-bearing nodes, the relative tumor burden, and the thickness, ulceration, and mitosis of the primary tumor), is insufficient to provide an individualized patient assessment to determine prognosis and establish treatment plans. A more precise assessment of recurrence risk on a single-patient basis would help to guide adjuvant treatment options, and to reduce the rate of morbidity caused by overtreatment and the associated costs.

The SN is the first lymph node to receive lymphatic drainage from a tumor and represents a relevant immunologic barrier against metastasis. Although providing the optimal environment for the generation of a tumor-specific immune response, in melanoma patients, the SN is usually the first site of metastasis and is the node in the regional basin that is most powerfully influenced by tumor-derived factors and cells (2). In fact, pathologic and functional characterizations of the SN have revealed profound structural alterations, even in the
absence of tumor cells, including an increase in lymphatic vessels and a major reduction in immune functions, facilitating the growth of metastatic tumors and promoting further dissemination of the disease (3). Hence, the SN represents a key component of the tumor microenvironment that potentially promotes immune tolerance. Increasing evidence supports the view that the triggering of immunosuppressive pathways at the microenvironment level, which inactivate tumor-specific T-cell responses, is associated with systemic immune dysfunction (4). These signs of local and systemic immunologic dysfunction, including the accumulation of myeloid-derived suppressor cells (5), tumor-specific CTLs with an anergic phenotype (6), regulatory T cells (Treg) and Th2 cells (7, 8), and dendritic cells (DC) with diminished functionality (9), are definitively detected in those patients with a poor clinical outcome (10, 11).

To obtain insight into the molecular modifications occurring in the SNs that are associated with disease progression, we designed an exploratory study to analyze the genome-wide transcriptional profiles of SNB samples derived from melanoma patients with different outcomes. Here, we report that a broad downmodulation of immune-related genes, together with the upregulated expression of lymphocyte markers defining immunosuppressive and exhausted lymphocytes, identifies patients with progressing melanoma.

Materials and Methods

Clinical samples

SNB samples isolated from 42 patients were studied. A diagram depicting the study design and the analyzed case records is shown in Fig. 1A and is detailed in Supplementary Materials and Methods. The histologic data obtained from the review of the diagnostic sections by a single pathologist and the clinical data for the studied cases are reported in Supplementary Table S1. Lymph node lymphocytes were obtained from patients recurring or not at the 5-year follow-up, whereas blood lymphocytes were obtained from stage IIIC patients recurring or not at the 5-year follow-up. After reviewing the pathology records and assessing the clinical data for the studied cases, independent of the presence or absence of tumor metastases. A three-dimensional scatter plot of the quality of the extracted RNA, 24 SNBs were selected for the analysis (Fig. 1A). After reviewing the pathology records and assessing the quality of the extracted RNA, 24 SNBs were selected for the analysis. We selected two groups of cases with tumor-positive SNBs representing the extremes of the survey (see Materials and Methods): (i) patients with further metastatic nodes at the time of CLND (stages IIIB–C) and disease recurrence within 5 years of follow-up (group PP, positive SNB-positive CLND/progressing) and (ii) patients with tumor-positive SNBs who were melanoma negative at the time of CLND (stages IIA–B) and did not relapse within 5 years (group PN, positive SNB-negative CLND/nonprogressing). In addition, a group of SNB-negative patients who did not have disease recurrence at 5 years (group N, negative SNB/nonprogressing) was also selected to compare the tumor-positive and tumor-negative SNBs (Fig. 1A). After reviewing the pathology records and assessing the quality of the extracted RNA, 24 SNBs were selected for the gene expression profiling study.

Illumina WG-DASL-V3 microarrays were used for sample profiling. The data were normalized using quantile normalization with BeadStudio software and filtered by excluding probes whose detection P values were larger than 0.05 in more than 95% of the samples, thereby resulting in 23,468 probes mapping to 17,811 single genes. Cluster analysis using the affinity propagation method indicated that the SNB samples were clustered into two groups. The first cluster consisted of 16 samples and included eight N and eight tumor-positive SNB samples, seven of which were from the PP progressing group. The second cluster comprised tumor-positive SNB samples, of which seven were PP and one was PN. Therefore, the PP samples from patients with progressing metastatic disease displayed gene expression profiles that were significantly different from those of all other SNB samples from patients with nonprogressing disease, independent of the presence or absence of tumor metastases. A three-dimensional scatter plot

RNA microarrays

Gene expression profiles were generated using Illumina HumanRef-8 WG-DASL v3.0 (Illumina). The Illumina BeadArray Reader was used for scanning the arrays, and Illumina BeadScan software was used for image acquisition and the recovery of primary data. The data were normalized using BeadStudio software and the quantile method. The data were deposited in the Gene Expression Omnibus repository (accession number GSE39945: 12). Data analysis is detailed in Supplementary Materials and Methods.

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed on consecutive sections on a semiautomated platform by standard diagnostic methods and examined in a blinded fashion for semiquantitative estimation (see Supplementary Materials and Methods).

Cytofluorimetric analyses

The fluorescence intensity was measured using a Live/Dead Fixable Dead Cell Stain Kit (Invitrogen) by a Gallios flow cytometer and analyzed with Kaluza software (Beckman Coulter). The monoclonal antibodies (mAb) used are indicated in the Supplementary Materials and Methods.

Statistical analysis

The association between the CD30 IHC staining score and progression was evaluated using the conditional odds ratio and its exact 95% confidence interval (Fisher test R function). Statistical analyses were performed by a Student t test or ANOVA using GraphPad Prism v5.0.

Results

Gene expression profiles distinguish tumor-positive SNBs isolated from patients with or without disease progression

We performed microarrays to obtain gene expression profiles, with the goal of exploring the molecular modifications occurring in SNBs isolated from patients with progressing disease. We selected two groups of cases with tumor-positive SNBs representing the extremes of the survey (see Materials and Methods): (i) patients with further metastatic nodes at the time of CLND (stages IIIB–C) and disease recurrence within 5 years of follow-up (group PP, positive SNB-positive CLND/progressing) and (ii) patients with tumor-positive SNBs who were melanoma negative at the time of CLND (stages IIA–B) and did not relapse within 5 years (group PN, positive SNB-negative CLND/nonprogressing). In addition, a group of SNB-negative patients who did not have disease recurrence at 5 years (group N, negative SNB/nonprogressing) was also selected to compare the tumor-positive and tumor-negative SNBs (Fig. 1A). After reviewing the pathology records and assessing the quality of the extracted RNA, 24 SNBs were selected for the gene expression profiling study.

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Figure 1. Gene expression profiling of the SNB samples. A, study sample design, frequencies, and outcomes at the 5-year follow-up of 752 melanoma patients undergoing SNBs in 2001–2004. The groups selected for the study are indicated in yellow. NED, no evidence of disease; AWD, alive with disease; DOD, dead of disease. B, unsupervised clustering, shown as principal component analysis, displaying the spatial separation of PP from the other samples. C, class comparison analysis displayed as volcano plots. The compared SNB groups are indicated. D, heat map of the genes that were differentially expressed between the PP and PN−N samples. E, heat map of the genes that were differentially expressed between the PP and PN samples. Red, upregulation; blue, downregulation. N, tumor-negative SNBs from cases with nonrecurring disease at the 5-year follow-up (n = 8); PN, tumor-positive SNBs from cases with tumor-negative nodes at the time of CLND and NED at the 5-year follow-up (n = 8); PP, tumor-positive SNBs from cases with tumor-positive nodes at the time of CLND and relapse by the 5-year follow-up (n = 8).
Progression-Related Expression Profiles of Sentinel Nodes

illustrating the characteristics of the samples based on the first three principal components, as generated by principal component analysis, further illustrates the spatial separation of the PP samples from the other samples, resulting in the clustering of the transcript expression (Fig. 1B).

Because the tumor-positive SNB samples were separated into two clusters, the tumor burden of the SNBs was carefully considered. The number, size, and localization of the micro-metastases in the nodes, as identified by IHC staining for the melanoma markers S-100, HMB-45, and Mart-1, were heterogeneous in the two groups. When the tumor burden was quantified on the basis of histopathology by ranking metastatic deposits according to the Rotterdam classification criteria (13), each class was represented in both the PP and PN groups (Supplementary Table S1). To evaluate whether differing tumor loads could explain the variation in expression profiles between the PP and PN samples at the molecular level, the expression levels of the genes that encode the melanoma diagnostic markers S-100, Mart-1, HMB-45/PMEL, MITF, and tyrosinase (SI06B, MLANA, SILV, MITF, and TYR) were evaluated in the array data. These melanoma-associated genes were expressed at similar levels in the PN and PP samples, thereby indicating that the different expression profiles were unlikely to be a reflection of varying tumor burdens. Eleven other genes involved in melanin biosynthesis or highly expressed in melanoma (ASIP, DCT, GP1A/3, MC1R, MLA, PRAME, RAB27A, SLC24A5, SLC45A2, TRPM, and TYRP1) did not significantly differ between the PP and PN samples (Supplementary Fig. S1).

Class comparison analysis of the gene expression data is represented as volcano plots in Fig. 1C, which demonstrates that a large number ($n = 3,078$) of genes were differentially expressed between the PP samples and all other samples (Fig. 1D), that a small number ($n = 58$) of genes were differentially expressed between the PP and all other samples (Fig. 1D), and that a larger number of genes ($n = 18$) were differentially expressed between the PP and PN samples. In contrast, a class comparison analysis of the PP and PN tumor-positive SNB samples yielded a manageable gene list consisting of 337 probe sets identifying 333 genes, which were nearly equally distributed as up- and downregulated genes, as depicted in the heat-map representation (Fig. 1E and Supplementary Table S2). A summary scheme of the number of genes differentially expressed among the different groups is reported in Supplementary Fig. S2.

Taken together, these results indicate that significant variations in gene expression profiles were detected in the tumor-positive SNB samples with a diverse disease course, thereby implying a remarkable influence of tumor aggressiveness or microenvironment-modulating effects once melanoma cells have reached the SN. Therefore, we next focused our study on the transcripts that distinguish the PP samples from the PN samples.

**Differential transcriptional patterns in tumor-positive SNB samples involve immune response genes**

To gain insight into the biologic processes influencing the different gene expression patterns, we used two approaches. First, coordinated expression changes of genes in the PP and PN samples were assessed at the pathway level by Gene Set Enrichment Analysis (GSEA), a statistical functional enrichment analysis. We examined the enrichment of the C2-curated catalog of functional gene sets (minimum gene set size of 18) in the rank-ordered gene list obtained by GSEA using the quantile-normalized data set. The heat map of the top 100 GSEA-ranked, significant individual genes that distinguish the PP and PN samples is shown in Fig. 2A. Interestingly, compared with PN, 556 gene sets were downregulated and none were upregulated in PP [false discovery rate (FDR) < 0.25, $P < 0.05$]. Gene signatures biased toward the regulation of immune responses and gene expression were associated with the most representative gene sets showing downregulation in the PP samples, accounting for 27% and 12%, respectively, of all enriched gene sets (Supplementary Table S3). Among the most significant immune gene sets downregulated in PP, the enrichment plots and heat maps of core enrichment genes for genes associated with antigen processing and presentation (PELLICC-IOTTA_HDAC_IN_ANTIGEN_PRESENTATION_UP), genes associated with chemokines, T- and B-cell receptor signaling (BIOCARTA_CXCR4_PATHWAY), and genes dampened by Foxp3 in Tregs (MARSON_FOXP3_TARGETS_DN) are displayed in Fig. 2B. Genes overlapping in the 16 gene sets associated with immune response regulation were identified using the leading-edge analysis tool and belonged to the KEGG categories "KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY" and "KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY," and mapped to the REACTOME term "REACTOME_SIGNALING_BY_THE_B_CELL_RECEPTOR_BCR" ($P < 0.00005$). GSEA, therefore, revealed a coordinated downregulation of components of adaptive immunity potentially associated with immunosuppression regulated by Foxp3 signaling, in the PP samples compared with the PN SNBs.

In the second approach, the molecular networks involving the 333 genes that were differentially expressed between the two conditions were analyzed using Ingenuity Pathway Analysis (IPA) software. The top IPA network was associated with "inflammatory response, cell death and cell-mediated immune response" functions (Fig. 2C). In accordance with the GSEA results, most of the genes in the immune-response category were downregulated in the PP samples compared with the PN samples, thereby indicating that genes associated with immune modulation principally display reduced expression in SNB samples from patients with progressing disease. Other network functions displaying enrichment were the "cell growth and proliferation," "cell death, post-translational modification, protein folding," and "carbohydrate metabolism, molecular transport, small molecule biochemistry" networks, which all displayed a pattern of reduced gene expression in PP SNBs. Taken together, the results of the combined GSEA and IPA analyses demonstrate that in the presence of comparable tumor infiltration, in PP SNBs, an impaired microenvironment was detected, with dysregulation of the genes that are involved in several aspects of the cellular immune response, including cell survival, proliferation, and metabolism.
Validation studies confirm differential gene expression patterns in additional SNB samples

Validation of the gene-profiling results was performed by testing the expression levels of selected genes in the PP and PN SNB samples by quantitative real-time (qRT) PCR. Genes selected from the ‘Inflammation and Immune Response’ list displayed expression patterns that confirmed the results of the microarray profiling both in the training and in independent samples (Supplementary Table S4 and Fig. S3). Among the immune-response genes, *TNFRSF8* and *BSG* attracted our interest because these genes were upregulated in the PP samples (Fig. 3A) and encode the activation markers CD30 and CD147, which have been associated with regulatory T cells (14, 15), Th2-like response, and chronic T-cell stimulation (16, 17), although these genes are also detected in other immune cell subsets (18). In addition, *TNFRSF8* upregulation was associated with several downregulated genes in the “inflammatory response, cell death, cell-mediated immune response” IPA pathway network shown in Fig. 2C, suggesting a potential role in the major signaling pathway identified by IPA.

To investigate the presence and role of lymphocytes expressing CD30 in SNBs, we first examined CD30 expression by IHC in PP and PN sections. The staining of sparse lymphocytes localized to the paracortical CD3-positive region of the nodes was observed in both samples (Fig. 3B), whereas the metastatic melanoma cells were not stained, in agreement with the published data (19). A total of 26 SNB samples were then analyzed by assigning the CD30⁺ cells a score ranging from 0 to 3. The results demonstrated that most of the PP samples scored 2 to 3, whereas most of the PN samples had a score of 0 to 1, indicating that higher numbers of CD30⁺ cells were present in the PP samples than in the PN SNBs (Supplementary Table S5). To evaluate whether the CD30⁺ lymphocytes coexpressed CD147 and additional immunoregulatory markers, such as Foxp3, a marker of activated Tregs, or PD1, a marker of anergic lymphocytes, the sections were double-stained with specific mAbs. Although these markers were represented in several areas of the nodes, subsets of CD30⁺ cells were shown to be positive, indicating that the CD30⁺ population encompassed different lymphocyte subsets sharing immunoregulatory and dysfunctional traits (Fig. 3C).
CD30+ lymphocytes display features of tolerogenic and exhausted T cells in lymph nodes and increase in peripheral blood of patients with progressing disease

The CD30 receptor is expressed by activated lymphocytes, whether healthy or neoplastic, and is upregulated during chronic inflammation and autoimmunity (14, 20), but a potential function as a tolerogenic marker in cancer immunity has not been reported. A detailed characterization of CD30+ lymphocytes was performed by fluorescence-activated cell sorting (FACS) analysis of cell suspensions obtained from regional nodes at lymphadenectomy. Interestingly, a higher frequency of CD30+ lymphocytes was observed in tumor-invaded nodes compared with tumor-free nodes (Fig. 4A). CD30+ or CD30− lymphocytes were then sorted from tumor-invaded lymph nodes and functionally analyzed ex vivo for proliferative capacity and cytokine secretion upon T-cell receptor triggering by stimulation with anti-CD3 and anti-CD28 antibodies. As reported in Fig. 4B and C, the CD30+ cells displayed a lower proliferative response compared with their CD30− counterparts. Moreover, CD30+ lymphocytes displayed a limited production of Th1 and Th2 cytokines. Thus, these data strongly suggest that the CD30+ T cells in the tumor-invaded nodes include an exhausted or anergic cell population with poor effector function.

To fully explore the nature of cells that express CD30 in the nodes of progressing melanoma patients, lymphocytes obtained from tumor-positive nodes were analyzed by multiparametric flow cytometry. Staining for CD30 was performed in combination with staining for lineage markers specific for T, B, and NK cells and the differentiation/activation markers CD25, CD147, PD1, and Foxp3. The gating strategy and the distribution of the CD30+ cells are illustrated in Fig. 5 A and B, respectively. Approximately 13% of the whole lymph node derived lymphocytes expressed CD30 (panel I). The CD30+
lymphocytes included CD19+ B cells (panels II–III, 35% of the CD30+–gated population) and a population of CD4/CD8 double-negative (DN) CD3+ T cells. The latter population was further characterized as CD56+CD45RA+CD25–PD1–Foxp3– T cells (panel III, 24% of the CD30+ cells), thereby displaying a phenotype compatible with that of tolerogenic DN T cells (21, 22). Approximately one third of CD30+ cells were CD4+ T lymphocytes (panel II), including Foxp3+ PD1–CD25+CD45RA+ Tregs, in line with the IHC data obtained for the SNBs (panel IV, 9.7%). Within the Foxp3+ CD4+ T conventional population, most displayed the phenotype of exhausted/anergic T cells (23), such as the loss of the activation marker CD25 and high expression of PD1 (panel IV, 13.2%), whereas cells negative for PD1 (panel IV, 5.9%) were equally composed of naive and antigen-experienced T cells. Both Tregs and conventional T cells expressed high levels of the activation marker CD147 (data not shown). Only a minority of the CD30+ cells were CD8+ cells (panel II, 5%). Half of these cells were Foxp3+CD25+CD45RA+ and positive for PD1, thus displaying a phenotype compatible with an exhausted/anergic functional state (panel V, 2.6%). The remaining cells, negative for PD1 expression (panel V, 1.9%), were mainly in the naive compartment.

The enhanced expression of CD30 on lymphocytes with suppressive/regulatory functions in tumor invaded lymph nodes of patients with worse prognosis suggests a possible direct role of tumor cells in CD30 upregulation. In vitro experiments have been conducted by coculturing healthy donor peripheral blood mononuclear cells (PBMC) with melanoma cells. A significant CD30 upregulation was detected in the CD3+CD4+CD8– DN cells and in Tregs gated in PBMCs as early as after 48 and 96 hours of coculture with melanoma cells (data not shown). These in vitro data are in agreement with the findings reported in the Figs. 4A and 5. To determine whether CD30+ cells could also be detected in the peripheral blood, PBMCs obtained from patients with regional or systemic disease (stages IIIIC–IV) were analyzed. CD30+ lymphocyte numbers significantly increased in melanoma patients compared with age- and gender-matched healthy donors, thus indicating a systemic accumulation of CD30+ lymphocytes as well (Fig. 6). The increase in CD30+ cells was primarily observed in the CD3+subset and the CD4+ subset and included both activated Tregs and conventional T cells, whereas for CD8–, CD3–CD4–CD8–, CD3–CD4+CD8–, and PD1-expressing subpopulations, the increase did not reach statistical significance.

In sum, the phenotypic characterization depicted here clearly shows that CD30 might represent a common marker for both regulatory and anergic lymphocytes, identifying dysfunctional immune responses both in tumor-neighboring lymph nodes and in the peripheral circulation of melanoma patients.

Discussion

Our study demonstrates that SNBs isolated from patients with progressing melanoma can be distinguished from SNBs derived from patients with good prognoses on the
basis of defined transcriptional profiles. By contrast, few differences were detected between the profiles of tumor-negative and tumor-positive SNBs derived from patients with good outcomes, thereby suggesting that the mere presence of melanoma cell deposits is not sufficient to drive local immune dysfunction, and that defined tumor aggressiveness or immunologic features are required. Genes related to immune response comprised the major ontology of the genes that were differentially expressed between the tumor-positive SNB samples with a good or poor prognosis, again...
indicating that immune changes are not solely driven by local tumor invasion.

The gene expression profiles of melanoma-positive SNBs associated with disease progression were analyzed to identify potential novel markers of poor prognosis in stage III melanoma patients. We identified TNFRSF8/CD30, which displayed upregulated expression in SNBs from patients with disseminated disease and was correlated with the downregulation of a complex network of immune-associated genes. The gene expression data were confirmed at the protein level by IHC, which revealed a higher number of CD30+ T cells in SNBs from progressing patients compared with samples from nonprogressing patients. Interestingly, CD30 expression seemed to encompass a panel of T cells mostly exhibiting tolerogenic or exhausted features, thus representing a potential marker of an immune system that is ‘permissive’ to tumor dissemination in the draining lymph nodes. In fact, CD30 was expressed in SNBs by cells that also stained positive for CD147, an upregulated marker according to mRNA profiles, and either nuclear Foxp3 or PD1. This latter evidence suggests that the CD30+ T cells included both Tregs and exhausted lymphocytes.

Regional nodes from a separate group of stage III melanoma patients were used for in-depth phenotypic and functional characterization, which revealed a higher frequency of CD30+ cells in the presence of tumor metastases compared with tumor-free nodes. Multiparametric flow cytometry confirmed that approximately one third of the CD30+ T cells were CD4 T lymphocytes, most of which coexpressed Foxp3 and CD25 as activated Tregs, or PD1, hence representing exhausted T cells. Another one third of the CD30+ T cells included CD4/CD8 DN lymphocytes. DN T cells were recently shown to exert a regulatory/tolerogenic function (21, 24, 25) and to be enriched in the peripheral blood of melanoma patients (26). Functional studies performed with immunosorted CD30+ lymphocytes from the nodes of stage III melanoma patients have demonstrated that these cells are endowed with low proliferative ability and a limited capacity to produce Th1 and Th2 cytokines. Last, approximately one third of the CD30+ T lymphocyte numbers also increased in the peripheral blood of patients with metastatic melanoma compared with PBMCs from healthy donors, further indicating a potential role of this immune cell subset in melanoma progression.

No information is available about the role of CD30 in cancer-related immune responses. First described in the Reed–Sten-berg cells of Hodgkin lymphoma, CD30 is expressed in normal and neoplastic T lymphocytes, B cells, NK cells, and in several non-lymphoid cells (28). CD30 engagement by its ligand CD30L has been reported to provide costimulatory signals to T cells, leading to the activation of NF-κB, cytokine production, and proliferative responses (16). In addition, CD30 signaling has been found to limit the proliferative potential of autoreactive CD8 effector T cells and protects against autoimmunity in murine models (20). In a human setting, the expression of CD30
marks suppressive Foxp3+ Tregs (14), and the presence of CD30+ T cells in synovial fluid has been correlated with a more favorable course in patients with juvenile arthritis (29). These data support the involvement of CD30 in the negative control of immune responses directed against self-antigens, a setting largely including tumor-specific T cells.

In addition to CD30, other genes were upregulated in SNBs from progressing patients: HMGAI, a transcriptional factor regulating the IFN, IL2, and IL4 genes and recently reported to play a role in transcriptional silencing of the CD4/CD8 loci in the T-cell lineage (30); BSG, which encodes the CD4+Foxp3+ Treg-activation marker CD147 (15), here resulting expressed by nodal CD30+ lymphocytes; and DEFA1, which encodes defensin α, which has been reported to attract immature DCs and sustain chronic inflammation (31). In addition to the upregulation of defined immune-related genes, GSEA demonstrated that SNBs from patients with poor outcomes displayed a prominent pattern of the reduced expression of genes associated with immunologic responses, thus indicating transcriptional down-regulation associated with immune-function impairment.

According to our analysis, the different expression profiles detected in tumor-positive SNBs are unlikely to be a reflection of a difference in tumor burden, as clearly indicated by histopathology and, at the molecular level, by the comparable expression levels of several melanoma-associated genes. Nonetheless, the melanoma genes in the PP and PN samples displayed a different pattern in the array results, for example, PRAME, RAB27A, and TRPM1 showed higher expression in the PP samples than in the N samples. By contrast, DCT, SLC24A5, and MCIR expression were higher in the PN samples (Supplementary Fig. S1), suggesting that the metastatic melanoma cells in the two SNB groups may have different molecular features.

This is the first study to analyze whole-genome gene expression profiling in SNB samples. Broad defects affecting different immunologic components have been extensively documented in SNs with respect to nontumor-draining nodes, including a decreased frequency of CD8+ effector T cells, a reduced presence of stimulatory CD86+ and CD11c+ DCs, and increased Foxp3 density and Th2 cytokine levels (7, 11, 32–37). Our findings partially contradict such observations because we found that immune dysfunction is detected in tumor-positive SNBs in association with disease progression. Interestingly, limited differential expression of genes was detected between tumor-negative and tumor-positive SNBs (n = 58), and even fewer (n = 18) genes were detected when negative SNBs were compared with positive SNBs from patients with a favorable clinical course (Supplementary Fig. S2). By contrast, patients with tumor-positive SNBs and progressing disease displayed a relatively different gene expression profile compared with nonprogressing patients (n = 333) and patients with tumor-negative SNBs (n = 3708).

Although in this study a small number of samples was tested in the discovery and validation experiments (n = 24 and 18, respectively), our results encourage further studies of transcriptional profiles of formalin-fixed paraffin-embedded SNBs as a potential informative tool in the clinical setting, for personalized patient treatment. In addition, the strong correlation that we observed between the presence of exhausted/regulatory CD30+ T cells in SNBs and disease progression suggests a potential role for this marker in the prognostic evaluation and therapeutic targeting of melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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