Prognostic Significance of CD169⁺ Lymph Node Sinus Macrophages in Patients with Malignant Melanoma

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Abstract

CD169 (sialoadhesin) is a sialic acid receptor that is specifically expressed on macrophages, including lymph node sinus macrophages. Animal studies suggest that CD169⁺ macrophages in lymph nodes have properties in preventing cancers. In order to determine the significance of CD169⁺ macrophages in patients with malignant melanoma, we evaluated tissue samples from 93 patients to investigate CD169 expression in regional lymph nodes (RLN) and determine the relationship of this expression with overall survival and various clinicopathologic factors. Higher densities of CD169⁺ cells were significantly associated with longer overall survival (P = 0.001). A multivariate analysis showed that the density of CD169⁺ cells was an independent prognostic factor, with higher densities correlating with higher density of CD8⁺ cytotoxic T cells within tumor sites. High CD169 expression in macrophages could be induced by IFNα, IFNγ, and IFNα-producing macrophages and CD303⁺ plasmacytoid dendritic cells were identified surrounding CD169⁺ macrophages. These data suggest that IFNα-stimulated CD169⁺ macrophages in RLNs are closely involved in T-cell–mediated antitumor immunity and may be a useful marker for assessing the clinical prognosis and monitoring antitumor immunity in patients with malignant melanoma. Cancer Immunol Res. 3(12); 1356–63. ©2015 AACR.

Introduction

Malignant melanoma is an aggressive tumor characterized by the malignant proliferation of melanocytes. Worldwide, approximately 41,000 patients die of the disease per year (1). However, in rare cases, complete regression can be achieved by activating antitumor immunity leading to the elimination of tumor cells by various clinicopathologic factors.

Research Article

Cancer Immunology Research


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doi: 10.1158/2326-6066.CIR-14-0180
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Cancer Immunol Res. 3(12); 1356–63. ©2015 AACR.

Published OnlineFirst August 21, 2015; DOI: 10.1158/2326-6066.CIR-14-0180
primary lesions and between these RLN macrophages and various clinicopathologic factors.

**Materials and Methods**

**Patients**

The present study utilized paraffin-embedded specimens of primary lesions and RLNs resected from 93 patients with malignant melanoma who had undergone surgery at Kumamoto University Hospital in the period from 2002 to 2008 (Table 1). No patients were treated with IFN therapy. Each RLN was identified using sentinel node mapping or based on the anatomic location, and both types of lesions were included in this study. All patients provided their written informed consent for participation in the study, in compliance with protocols approved by the Kumamoto University Hospital Review Board.

**Immunostaining and double immunostaining**

All 3-μm serial sections were stored in a deep freezer until immunostaining. Before immunostaining, to decolorize the melanin in the sections (Supplementary Fig. S1), warm 3% hydrogen peroxide in phosphate buffer (0.05 mol/L, pH 7.4) was applied at 55°C for 2 hours (14). For antigen retrieval, the sections were immersed in EDTA solution (pH 8.0) and heated in a microwave (for CD169 and IFNα) or pressure cooker (for CD8, CD68, CD163, and CD303). The primary antibodies were anti-CD8 antibodies (clone C8/144B; Nichirei), anti-CD68 antibodies (clone MOPC-21; Wako) used as a negative control. Following the reaction of the primary antibodies, the samples were incubated with horseradish peroxidase in phosphate buffer (0.05 mol/L, pH 7.4) was applied at 24 hours of incubation to induce the CD169 expression.

In order to calculate the number of positive cells per unit area, we measured the areas (mm²) using the ImageJ software program. To estimate the degree of CD8⁺ T-cell infiltration in the primary lesions, we used the scoring system based on a modification of a previous method (16). The degree of CD8⁺ T-cell infiltration was evaluated in three tumor compartments: the tumor nest, defined as the area within solid melanoma nests; the tumor stroma, defined as the connective tissue between the melanoma nests; and the invasive front, defined as the interface between the host stroma and the invading tumor edge. The extent of CD8⁺ cell infiltration in each area was graded from 1 (weak or absent) to 3 (massive), and the sum of these scores was defined as the total CD8 score (from 3 to 9). Cases in which all compartments could not be identified were excluded from the statistical analysis, and the total scores were divided into weak (<5) and strong (≥5) categories. We were able to evaluate RLN macrophages in 84 patients, and the scores for CD8⁺ cell infiltration in 57 of the 93 patients were studied. All samples were evaluated by two pathologists (Y. Saito and K. Ohnishi) via microscopic observation, and the data were averaged for the statistical analysis.

**Cell culture**

RPMI 1640 (Wako) supplemented with 1% penicillin/streptomycin (Wako) and 10% FBS was used as the complete culture medium. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood obtained from healthy volunteer donors, who provided their written informed consent, via density gradient centrifugation with Lymphoprep (Axis-Shield). Monocytes were isolated from the PBMCs using a magnetic bead–based isolation procedure (MACS CD14 microbeads, MACS column and separator; Miltenyi Biotec). The monocytes were cultured in polystyrene dishes (Becton Dickinson) in complete medium with granulocyte macrophage colony-stimulating factor (GM-CSF; 5 ng/mL, Wako) for 3 to 7 days to induce the production of macrophages. IFNα (100 U/mL, MSD) was added during the last 24 hours of incubation to induce the CD169 expression.

**Flow cytometry (FACS)**

Anti-CD169 monoclonal antibodies (clone 7–239, mouse IgG1, κ) labeled with a fluorochrome (phycoerythrin) were purchased from BioLegend. Mouse IgG1, κ (clone MOPC-21) was used as an isotype-matched control. The cell samples were treated with FcR-blocking reagent (Miltenyi Biotec) for 10

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**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (total = 93)</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>53</td>
<td>57.0</td>
</tr>
<tr>
<td>Male</td>
<td>40</td>
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</tr>
<tr>
<td><strong>Age, years</strong></td>
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<tr>
<td>Median</td>
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<td></td>
</tr>
<tr>
<td>Range</td>
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<td></td>
</tr>
<tr>
<td><strong>Histologic subtype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acral lentiginous melanoma</td>
<td>47</td>
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<tr>
<td>Lentigo maligna melanoma</td>
<td>15</td>
<td>16.1</td>
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<tr>
<td>Superficial spreading melanoma</td>
<td>14</td>
<td>15.1</td>
</tr>
<tr>
<td>Nodular melanoma</td>
<td>11</td>
<td>11.8</td>
</tr>
<tr>
<td>Mucosal melanoma</td>
<td>6</td>
<td>6.5</td>
</tr>
<tr>
<td><strong>Tumor thickness, mm</strong></td>
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<td></td>
</tr>
<tr>
<td>Median</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0–24</td>
<td></td>
</tr>
<tr>
<td>≤1.0</td>
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<td>25.8</td>
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<td>3</td>
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<td>1</td>
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<td>28.0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1.1</td>
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minutes and stained with the fluorochrome-conjugated monoclonal antibodies for 20 minutes. The stained cell samples were analyzed with a FACSVerse flow cytometer (Becton Dickinson) and the FACSuite (Becton Dickinson) software package.

**Western blotting**

Cells were solubilized with 1% Triton X-100, and the protein (20 µg) was run on an 8% SDS-polyacrylamide gel. After the proteins were transferred to polyvinylidene fluoride transfer membranes (Millipore), the membranes were cut into pieces and each membrane was incubated with anti-CD169 antibodies (clone HSn 7D2; Abcam) or anti-β-actin antibodies (clone C4; Santa Cruz Biotechnology). The membranes were then visualized using HRP-conjugated anti-mouse IgG antibodies with Pierce Western Blotting Substrate Plus (Thermo Scientific). Signals were detected with an LAS-4000 image analyzer (Fujifilm).

**Statistical analysis**

The statistical analyses were performed with the JMP 10 software program (SAS Institute). All in vitro data represent the results of three independent experiments. The data are expressed as the mean ± SD. Two-group comparisons were made using the Mann–Whitney U test, and the associations between different categorical variables were assessed using ANOVA. The cumulative survival rates for the two groups were compared via the log-rank test and generalized Wilcoxon test, and the simultaneous relationships between multiple prognostic factors for survival were assessed with the Cox proportional hazards model with stepwise backward reduction. A P value of less than 0.05 was considered to be statistically significant.

**Results**

**Number of CD169⁺ sinus macrophages in RLNs was lower in patients with recurrences**

We first performed immunostaining to investigate the expression of CD68, CD163, and CD169 in the RLNs obtained from the melanoma patients. Because melanin deposition was occasionally found in the tissues, melanin was completely cleared by bleaching before immunostaining, as described in Materials and Methods (Supplementary Fig. S1). The RLN sinus was clearly identified as a demarcated area filled with macrophages on both H&E staining and immunostaining (Fig. 1A and Supplementary Fig. S2). As shown in Fig. 1B, all sinus macrophages were positive for CD169, CD68, and CD163, indicating their typical histologic features. The number of CD169⁺ macrophages was lower in patients with recurrences (Fig. 1C). The overall Kaplan–Meier survival curves for the 84 melanoma patients as related to the number of CD169⁺ macrophages, ratio of CD169⁺ macrophages to CD68⁺ macrophages, number of CD163⁺ macrophages, and number of CD68⁺ macrophages in the RLNs.

Figure 1: Immunostaining of sinus macrophages in the RLNs and the overall survival curves of the melanoma patients. A and B, H&E staining of the sinus areas in the RLNs and the immunohistochemical analysis of CD169⁺, CD68⁺, and CD163⁺ macrophages in the RLNs. Typical results are shown for CD169⁺ high- and low-cell-number cases. B, higher-magnification image of the squared area in A. C, overall Kaplan–Meier survival curves for the 84 melanoma patients as related to the number of CD169⁺ macrophages, ratio of CD169⁺ macrophages to CD68⁺ macrophages, number of CD163⁺ macrophages, and number of CD68⁺ macrophages in the RLNs.
for the pan-macrophage marker CD68, and the number of CD169⁺ sinus macrophages varied in each patient, although the density of sinus macrophages was consistent in each case. In all cases, sinus macrophages expressed CD163, and the distribution of CD163⁺ cells was similar to that of CD68⁺ cells (Fig. 1B and Supplementary Fig. S3). We also checked the distribution of CD14⁺ cells that seemed to overlap that of CD68⁺ macrophages (Supplementary Fig. S4).

We then analyzed the correlations between the clinicopathologic features and number of sinus macrophages in the patients with malignant melanoma. CD68⁺, CD163⁺, and CD169⁺ cells in the sinus areas were counted as described in Materials and Methods (Supplementary Fig. S2), and the patients were subsequently classified into two groups—those with a low or high cell number—based on the median value. The results of the statistical analysis showed that a lower number of CD169⁺ cells and lower ratio of CD169⁺ cells to CD68⁺ macrophages were significantly correlated with recurrence; however, the number of CD169⁺ cells per mm² and ratio of CD169⁺ cells to CD68⁺ macrophages were not associated with age, sex, or the clinical stage (i.e., tumor thickness and lymph node metastasis; Table 2). CD163⁺ and CD68⁺ cells were not significantly associated with any of these factors (Table 2).

Higher density of CD169⁺ sinus macrophages associated with better clinical prognosis

We next evaluated the relationship between the CD169 or CD163 expression of sinus macrophages in the RLNs and overall survival. The results of the univariate analysis showed that the

### Table 2. Associations between the number of macrophages in RLNs and clinicopathologic features

<table>
<thead>
<tr>
<th>Clinicopathologic feature</th>
<th>n</th>
<th>CD169⁺ cells/mm² in RLNs</th>
<th>P value</th>
<th>CD169⁺ cells/CD68⁺ cells in RLNs</th>
<th>P value</th>
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<tr>
<td></td>
<td></td>
<td>&lt;350</td>
<td>&gt;350</td>
<td>&lt;0.9</td>
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<td>36</td>
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<td>51</td>
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<td>Recurrence</td>
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<td>Positive</td>
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<td>8</td>
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<tr>
<td>CD8⁺ cells score in tumor</td>
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<td>20</td>
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<td>11</td>
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<td>Stroma (1-3)</td>
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<td>17</td>
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<td>Invasive front (1-3)</td>
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<td>24</td>
<td>17</td>
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<tr>
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<td>6</td>
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<td>≥5</td>
<td>58</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

NOTE: Underlining indicates statistically significant results.
Abbreviation: NS, not statistically significant.

CD169⁺ Lymph Node Macrophages in Malignant Melanoma

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number of CD169<sup>+</sup> cells and ratio of CD169<sup>+</sup> cells to CD68<sup>+</sup> macrophages were significantly correlated with favorable overall survival (P = 0.001 and P = 0.0003, respectively, log-rank test; Fig. 1C and Table 3). However, the number of CD163<sup>+</sup> cells and ratio of CD163<sup>+</sup> to CD68<sup>+</sup> macrophages were not associated with the clinical prognosis (Fig. 1C, Table 3, and Supplementary Table S1A). A lower clinical stage (< stage 2), reduced tumor thickness (<2 mm), and absence of both lymph node metastasis and recurrence were also associated with a longer overall survival in recurrence-free melanoma cases, and these results were significant (Fig. 1C, Table 3, and Supplementary Table S1A).

According to our previous findings, CD169<sup>+</sup> sinus macrophages directly contact CD8<sup>+</sup> T cells expressing CD43, a major ligand of CD169, in the sinus area of RLNs in patients with colorectal cancer (17). The present immunostaining analysis showed that considerable CD43<sup>+</sup> cells were also detected surrounding the sinus macrophages in the RLNs of the patients with malignant melanoma (Supplementary Fig. S8).

**IFN<sub>a</sub>-producing cells were located close to sinus macrophages in RLNs**

We previously reported that macrophages stimulated with type 1 IFN (IFN<sub>a</sub> and IFN<sub>b</sub>) show a strong CD169 expression (17) and confirmed this finding in the present study (Fig. 3A and B). We therefore hypothesized the existence of an intrinsic environment containing IFN<sub>a</sub> around sinus macrophages in RLNs. Double immunostaining with antibodies against CD169 and IFN<sub>a</sub> showed that IFN<sub>a</sub>-secreting cells were close to CD169<sup>+</sup> sinus macrophages in the RLNs and that CD169<sup>+</sup> sinus macrophages were negative for IFN<sub>a</sub> (Fig. 3C). Furthermore, almost all the IFN<sub>a</sub><sup>+</sup> cells were larger than lymphocytes and were morphologically suspected to be histiocytes. On double immunostaining, we found that the majority of IFN<sub>a</sub><sup>+</sup> cells expressed CD68 (Fig. 3D). On the other hand, scant small-sized IFN<sub>a</sub><sup>+</sup> cells were partially positive for CD303, a specific marker of plasmacytoid dendritic cells (pDC; Fig. 3E).

**Discussion**

In the present study, we observed higher numbers of CD169<sup>+</sup> cells and ratios of CD169<sup>+</sup> to CD68<sup>+</sup> macrophages in the recurrence-free melanoma cases, and these results were significantly
Figure 2. Interaction between CD169+ macrophages in the RLNs and CD8+ T cells in the melanomas. A, an evaluation of infiltrating CD8+ T cells in the melanomas. Three distinct areas (tumor nest, tumor stroma, and invasive front) formed the basis of the semiquantitative CD8 scoring: 1 to 3 for each area and 3 to 9 for the total score. B, examples of scores are shown for a case with a high total score (9) and a case with a low total score (4). C, overall survival curves for the patients according to the CD8 total score.

Figure 3. IFN-induced CD169 expression in the cultured macrophages and distribution of IFNα-producing cells, including pDCs, in the RLNs. A, flow cytometry analysis of CD169 in cultured human macrophages stimulated with IFNs or IL10 (red) in the last 24 hours of culture compared with untreated control (blue) macrophages. One representative result (of three) is shown. The black dashed lines in the histograms represent isotype controls. B, Western blot analysis of CD169 on day 3 in macrophages cultured with or without IFN stimulation during the last 24 hours of culture. C, double immunostaining of CD169 and IFNα in the RLNs. D, double immunostaining of CD68 and IFNα in the RLNs. E, double immunostaining of IFNα and CD303, a specific marker of plasmacytoid dendritic cells (pDC), in the RLNs. Arrowheads indicate double-positive (CD68+/IFNα+ and CD303+/IFNα+) cells.
correlated ($P = 0.001$ and $0.0003$) with favorable overall survival of the patients with malignant melanoma. In contrast, CD163 expression on sinus macrophages was not associated with recurrence or survival rate. The significant correlation between the number of CD169$^+$ sinus macrophages and density of infiltrating CD8$^+$ T cells in the primary lesions suggests that CD169$^+$ RLN macrophages have a close association with the activation of CD8$^+$ T-cell–mediated antitumor immunity.

Our previous study of colorectal cancer cases also showed that a high number of CD169$^+$ sinus macrophages correlates with a better prognosis and higher density of infiltrating CD8$^+$ T cells in tumors (17). CD43 is a major ligand of CD169 (18), and CD169$^+$ sinus macrophages were in direct contact with CD8$^+$/CD43$^+$ T cells in the RLNs of colorectal cancer (17) and malignant melanoma (Supplementary Fig. S8). These findings suggest that CD169$^+$/CD43$^+$ ligation in RLNs mediates cell–cell interactions between sinus macrophages and CD8$^+$ T cells. Asano and colleagues found that CD169$^+$ lymph node sinus macrophages engulf necrotic tumor cells and induce the stimulation of antigen-specific CTLs in a murine tumor model (19). Müerköster and colleagues also demonstrated, using an adoptive immunotherapy model with tumor-bearing mice, that CD169$^+$ liver macrophages promote the activation of CD4$^+$ and CD8$^+$ T cells (20). These findings strongly suggest that CD169$^+$ macrophages act as activators of CD8$^+$ T cells. The activation of CD8$^+$ T cells by CD169$^+$ macrophages is thought to occur by two mechanisms: (i) direct antigen presentation to CD8$^+$ T cells by CD169$^+$ macrophages; and (ii) antigen transfer presentation to dendritic cells (9). Our study using flow cytometry showed that the expression of costimulatory factors, especially CD86, is elevated in monocyte-derived IFN$\alpha$-induced CD169$^+$ macrophages (Supplementary Fig. S9). Human monocyte-derived macrophages are generally used for in vitro experiments because preparations of these cells are readily available. However, recent findings demonstrate that some tissue-resident macrophages and monocyte-derived macrophages are derived from different lineages (21, 22). Sinus macrophages are believed to be resident macrophages (9, 23); however, it is ethnically difficult to obtain these cells from healthy donors. It is necessary to assess the stimulatory ability of CD169$^+$ resident macrophages in the future.

Because our previous and present studies demonstrated that IFN$\alpha$ strongly induces the CD169 expression in human macrophages (17), we postulated that CD169$^+$ macrophages are constantly exposed to IFN$\alpha$ in lymph node sinus areas. Our studies revealed that IFN$\alpha$-producing cells are present around CD169$^+$ macrophages in lymph node sinus areas, suggesting IFN$\alpha$ as a key regulator of CD169 expression in sinus macrophages. IFN$\alpha$ is used clinically as an adjuvant treatment for melanoma patients and produces good outcomes (24, 25). In addition, the perilesional injection of IFN$\beta$ in malignant melanoma patients with multiple metastases results in an increased number of CD8$^+$ CTLs (26). The subcutaneous injection of IFNs into perilesional sites induces the drainage of IFNs into RLNs similar to that observed for tumor antigens as a result of the lymph flow and has the potential to induce CD169 expression in sinus macrophages. The induction of CD169$^+$ macrophages in RLNs may thus have some involvement in the efficacy of IFN therapy. IFN$\alpha$-producing cells were morphologically divided into two cell types in the present study: many large-sized histiocyte-like cells and scant small-sized round cells (Fig. 3). The large-sized IFN$\alpha^+$ cells were positive for CD68 (Fig. 3D) and therefore considered to be macrophages. In contrast, we expected that the CD68-negative small-sized IFN$\alpha^+$ cells might be pDCs because it is generally accepted that pDCs produce high amounts of IFN$\alpha$ (27). Double immunostaining demonstrated that these small-sized IFN$\alpha^+$ cells were partially positive for CD303, a marker of pDCs (28). The effect of IFN$\alpha$ may be to influence sinus macrophages through paracrine signaling, because the CD169$^+$ sinus macrophases were themselves negative for IFN$\alpha$. These results suggest that IFN$\alpha$-producing macrophages and pDCs may be key cells for maintaining CD169$^+$ sinus macrophages in lymph nodes.

In conclusion, we herein demonstrated the significance of CD169$^+$ sinus macrophages in the RLNs of patients with malignant melanoma. In addition, we identified the density of CD169$^+$ macrophages to be an independent risk factor for overall survival. CD169$^+$ sinus macrophages present in RLNs may thus be involved in CTL-mediated antitumor immunity (as summarized in Fig. 4). Evaluating the existence of CD169 antigens in RLNs may aid in assessing the clinical prognosis and monitoring the antitumor immune response in patients with malignant melanoma. Additional studies of CD169$^+$ sinus macrophages will provide new insights into tumor progression and cancer immunotherapy in the setting of malignant melanoma and other malignancies.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Saito, K. Ohnishi, Y. Fujiwara, Y. Komohara
Development of methodology: K. Ohnishi, Y. Komohara
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Saito, A. Miyashita, S. Nakahara, T. Motohashi, S. Fukushima, H. Ihn
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Saito, K. Ohnishi, Y. Fujiwara, H. Ihn, Y. Komohara
Writing, review, and/or revision of the manuscript: Y. Saito, K. Ohnishi, Y. Fujiwara, Y. Takeya, Y. Komohara

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Horlad, S. Fukushima, H. Ihn
Study supervision: M. Jinmin, M. Takeya, Y. Komohara

Acknowledgments
The authors thank Yui Hayashida, Osamu Nakamura, and Takenobu Nakagawa for their valuable technical assistance.

Grant Support
This study was supported in part by Grants-in-Aid for Scientific Research (B24793533 and B25293089) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was also supported in part by a scholarship from the Graduate School of Medical Sciences, Kumamoto University, Japan.

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Received September 24, 2014, revised June 30, 2015, accepted July 6, 2015, published OnlineFirst August 21, 2015.

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Prognostic Significance of CD169+ Lymph Node Sinus Macrophages in Patients with Malignant Melanoma

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doi:10.1158/2326-6066.CIR-14-0180

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