Long-Term Protective Effect of Mature DC-LAMP+ Dendritic Cell Accumulation in Sentinel Lymph Nodes Containing Micrometastatic Melanoma

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Abstract

Purpose: In a previous immunohistochemical study of dendritic cells (DC) in sentinel lymph nodes (SLN) draining regressing melanomas, we found that the accumulation of mature DC-LAMP+ DCs in SLNs was associated with local expansion of antigen-specific memory effector CTLs and the absence of metastasis in downstream lymph nodes. The aim of this study was to investigate the prognostic importance of the maximal density of mature DCs in SLNs.

Experimental Design: A total of 458 consecutive patients with micrometastatic melanoma within SLNs were eligible for analysis. The maximal density of mature DC-LAMP+ DCs was evaluated by three independent observers and categorized into three classes ( <100, 100 to <200, and ≥200/mm²).

Results: There was excellent interobserver reproducibility for maximum density of mature DC-LAMP+ DC scores (κ score = 0.82). There were differences in the maximal density scores and staining intensity according to the treating melanoma center (P < 0.001). The higher the mature DC density in the SLN is, the longer is the duration of survival [P = 0.047; hazard ratio, 0.70; 95% confidence interval, 0.50-1.00]. Adjusted by thickness and ulceration, the prognostic importance of DC density was lower (P = 0.36).

Conclusion: This study is the first to report the prognostic value of DC-LAMP+ DC counts in SLNs containing metastatic melanoma. Patients with a high density of mature DCs (≥200/mm²) have the lowest risk of death. It also provides evidence that a lack of maturation in the SLNs is important in biological facilitation of melanoma progression.

Dendritic cells (DC) are antigen-presenting cells (APC) whose function, location, and morphology are highly dependent on their degree of maturation (1–3). Mature DCs are the most potent APCs capable of priming tumor-specific T cells. It is generally postulated that following a cutaneous inflammatory reaction, epidermal Langherhans cells that represent immature DCs can migrate to regional lymph nodes where they mature further (4–6). Mature DCs express MHC class I and II, CD80, CD86, CD54, CD83, and CD208/DC-LAMP molecules. The mature DCs contribute to antigen presentation and T-cell costimulation, which results in the induction or restimulation of antigen-specific immune responses, including IFN-γ-producing Th-helper-1 (Th-1) responses that are desirable for antitumor responses (7, 8). Conversely, it has been recognized that Th-2 responses, which result in interleukin-4 (IL-4) and IL-10 production, and regulatory T-cell lymphocyte responses, characterized by CD4-positive/CD25-positive cells and Foxp3-expressing cells, can result in immune suppression (9–11).

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DC-LAMP, the human lysosome-associated membrane, protein-3, was originally described as a molecule specifically expressed by mature dendritic cells (12, 13). Its expression is induced in the later stages of maturation. A definite function of DC-LAMP has not yet been established. DC-LAMP is most homologous to CD68, a lysosomal glycoprotein expressed by macrophages (12). Besides mature dendritic cells, DC-LAMP mRNA has been found in human and murine lungs and recently in pulmonary adenocarcinomas with Clara cell differentiation (13, 14).

The extent of SLN involvement by melanoma metastasis is one of the most powerful independent predictive factors for melanoma survival, and 30% to 40% of patients with SLN micrometastases will eventually die of their disease, compared with 5% to 10% of patients without SLN involvement (15). Melanoma SLNs are privileged sites of T-cell priming and crosstalk between cells of the immune system. In a previous study, we showed that the accumulation of DC-LAMP+ mature DCs (mature DCsDC-LAMP+) was clustered with tumor cells and lymphocytes and was associated with the local expansion of antigen-specific memory effector CTLs and spontaneous regression of melanoma (16). Furthermore, this study also showed that in SLNs containing micrometastatic melanoma, there was a significant correlation between the density of DC-LAMP+ DCs within metastases and the absence of metastasis in downstream nonsentinel lymph nodes (16).

In the current study, we investigated whether the accumulation of mature DCsDC-LAMP+ in melanoma containing SLNs controlled melanoma progression and was an important prognostic variable. A population of 458 micrometastatic melanoma containing SLNs with detailed follow-up information was used to perform the study.

Patients and Methods

Patient population. This retrospective study included all patients with a diagnosis of a cutaneous melanoma who were evaluated and treated between 1992 and 2002 in one of the institutions participating in the European Organization for Research and Treatment of Cancer (EORTC) Melanoma Group Pathology Committee. The inclusion criteria were (a) histologic diagnosis of primary melanoma; (b) a sentinel node procedure was done in the participating institution; (c) the SLN pathologic examination was based, as a minimum standard, on the guidelines published by the EORTC Melanoma Group (protocol 1; ref. 17); (d) at least two unstained slides taken from sequential SLN slides set were available for the study; and (e) the patients either died or were followed up for at least 3 years after SLN excision.

The study was conducted in accordance with local regulations and with the approval of all relevant local institutional review boards. Patients were identified through institutional databases, which provided demographic, clinicopathologic, treatment, and outcome information. In addition, medical records were reviewed to obtain details regarding patient characteristics, surgical treatment, and chemotherapy.

A total of 458 patients were included in the first study phase that aimed to determine the immunostaining intercenter variability. These patients came from the following institutions: the Royal Prince Alfred Hospital (RPAH), Sydney, Australia (center 1, n = 219); Royal Surrey County Hospital, Guildford, United Kingdom (center 2, n = 73); Gustave-Roussy Institute, Villejuif, France (center 3, n = 50); Glasgow University, Glasgow, United Kingdom (center 4, n = 55); Erasmus Hospital, Rotterdam, the Netherlands (center 5, n = 37); and the European Institute for Oncology, Milan, Italy (center 6, n = 24). In total, 1,126 SLN sections corresponding to 537 SLNs were available.

Table 1. Characteristics of included patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (N = 219)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr) at SLN excision</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>53</td>
</tr>
<tr>
<td>Range</td>
<td>5–81</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>74 (33.8)</td>
</tr>
<tr>
<td>Male</td>
<td>145 (67.2)</td>
</tr>
<tr>
<td>Sentinel nodes basin (%)</td>
<td></td>
</tr>
<tr>
<td>Axillary</td>
<td>105 (45.7)</td>
</tr>
<tr>
<td>Groin</td>
<td>89 (38.7)</td>
</tr>
<tr>
<td>Neck</td>
<td>33 (14.3)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (1.3)</td>
</tr>
<tr>
<td>Number of positive/removed sentinel nodes</td>
<td>1/2</td>
</tr>
<tr>
<td>Median</td>
<td>0–4/1–8</td>
</tr>
<tr>
<td>Breslow thickness (%)</td>
<td></td>
</tr>
<tr>
<td>&lt;1.01 mm</td>
<td>7 (3.2)</td>
</tr>
<tr>
<td>1.01–2.00 mm</td>
<td>62 (28.3)</td>
</tr>
<tr>
<td>2.01–4.00 mm</td>
<td>91 (41.6)</td>
</tr>
<tr>
<td>&gt;4.00 mm</td>
<td>59 (26.9)</td>
</tr>
<tr>
<td>Clark’s level (%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>40 (18.4)</td>
</tr>
<tr>
<td>IV</td>
<td>153 (70.1)</td>
</tr>
<tr>
<td>V</td>
<td>25 (11.5)</td>
</tr>
<tr>
<td>Ulceration (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>121 (55.3)</td>
</tr>
<tr>
<td>No</td>
<td>86 (39.3)</td>
</tr>
<tr>
<td>Not available</td>
<td>12 (5.5)</td>
</tr>
</tbody>
</table>
mature DC-LAMP+ DC densities were tested by the Spearman’s rank correlation test.

The overall survival time was defined as time from SLN biopsy until death, whatever the cause; the follow-up of patients still alive has been censored at their latest date of follow-up. The actuarial curves were computed using the Kaplan-Meier technique, and the SEs of the estimates were computed using the Greenwood formula. The prognostic value of mature DC-LAMP+ DC densities (as coded as 0, <100/mm²; 1, 100 to <200/mm²; 2, ≥200/mm²) and Breslow’s thickness (as coded as 0, ≤1, 1–2, 2.1–4; 3, >4 mm), considered as ordered categorical variables, and of ulceration (presence versus absence) were assessed using the Cox’s proportional hazards model. This model provides an estimate and the 95% confidence interval (95% CI) of the hazard ratio (HR) of the death intensity per time unit of a category versus the one of baseline category of the same variable. In the case of an ordered variable (DC-LAMP+, Breslow thickness), a smooth estimate of the HR corresponding to one category versus the previous one was obtained. The same model was used for multivariate analysis to determine whether several variables were of independent prognostic importance. The Wald test was used to determine the prognostic importance of each variable included in the model. All analyses were done according to the intent-to-treat principle. The SAS 9.1 software (SAS Institute Inc.) was used for the survival analysis. All statistical tests were two sided.

**Results**

**Table 2. Distribution of mature dendritic cell maximal density**

<table>
<thead>
<tr>
<th>Center</th>
<th>Maximal density of mature DCs</th>
<th>n</th>
<th>SD</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>157.19</td>
<td>219</td>
<td>60.90</td>
<td>153</td>
</tr>
<tr>
<td>2</td>
<td>184.95</td>
<td>73</td>
<td>77.27</td>
<td>190</td>
</tr>
<tr>
<td>3</td>
<td>214.75</td>
<td>50</td>
<td>66.21</td>
<td>221</td>
</tr>
<tr>
<td>4</td>
<td>169.44</td>
<td>55</td>
<td>66.02</td>
<td>151</td>
</tr>
<tr>
<td>5</td>
<td>134.21</td>
<td>37</td>
<td>31.05</td>
<td>128</td>
</tr>
<tr>
<td>6</td>
<td>170.13</td>
<td>24</td>
<td>48.41</td>
<td>166</td>
</tr>
<tr>
<td>Total</td>
<td>168.19</td>
<td>458</td>
<td>62.98</td>
<td>164</td>
</tr>
</tbody>
</table>

The estimated 5-year survival rates (SE, %) were 45.8% (10.8) in category 1, 56.4% (7.6) in category 2, and 61.5% (7.8) in category 3. Overall, there was a significant association between mature DC-LAMP+ maximal density and survival (P = 0.047, Fig. 1). The higher the mature DC-LAMP+ maximal density is, the lower is the risk of death rate: estimated HR was 0.70, and 95% CI was 0.50-1.00. The protector effect of mature DC accumulation (category 3) in the SLN seems to persist more than 10 years after diagnosis.

**Univariate analysis indicated that Breslow’s thickness (P = 0.029) and ulceration (presence versus absence: P = 0.002) were both of prognostic importance.** Multivariate analysis, done on 207 patients with known ulceration status, including mature DC-LAMP+ density, Breslow’s thickness, and tumor ulceration, identified ulceration as the only independent prognostic factor (presence versus absence: P = 0.02), whereas Breslow’s thickness was only borderline (P = 0.07), and mature DC-LAMP+ density was not significant (P = 0.36). In the entire population (n = 219), when max DC mature DC-LAMP density and tumor thickness were included in the model, they were both of borderline significance (respectively, P = 0.06 and 0.07).

**Discussion**

Exploration of the immunologic status of SLNs, including DC maturation and T-cell responses, is one of the most promising issues in tumor immunology. We reported here the first evidence that high maximum density of mature dendritic cells expressing the DC-LAMP molecule in melanoma-draining SLN is associated with significant overall survival benefit. This benefit is maintained more than 10 years after SLN procedure in the category of highest maximal mature DC density. These results clearly indicate that the preservation of the maturation ability of DCs in SLNs containing melanoma is crucial for the long-term antimitastatic host immune response.

Previous studies have addressed DC status in SLNs in several types of cancers (16, 19–32). However, to the best of our knowledge, this study is the first to have correlated mature DCs in the SLN with overall survival. In melanomas, it was suggested that invasion of lymph nodes by tumor cells results in suppression of lymph node functions with alterations in immune cell ratio and activity (20, 25, 33). Cochran et al. reported that the paracortex of lymph nodes with melanoma metastases contained decreased numbers of DCs (33, 34) that were phenotypically less mature as compared with DCs in nonmetastatic lymph nodes (33). They observed that the SLNs were more affected than the non-SLNs and suggested that there may be a direct effect of tumor or tumor-associated factors on
DC maturation and function. They also showed that a higher frequency of DCs in SLNs was correlated with reduced likelihood of recurrence and death from melanoma (34). The marker they used in that study ($S_{100}$) identifies DCs at different stages of maturation, including mature DCs. More recently, Lee et al. provided molecular evidence of cytokine-mediated SLN immunosuppression associated with the presence of melanoma cells and showed that SLN immunosuppression may be potentially reversed by cytokine therapy using granulocyte macrophage-colony stimulating factor (25).

In breast carcinoma studies, Huang et al. compared $S_{100}$-positive DCs in SLNs and non-SLNs and showed that the SLNs had reduced frequencies of $S_{100}$-positive DCs with a predominance of immature DCs (35). Recently, Matsuura et al. (21) compared SLNs and non-SLNs in patients with breast carcinoma. They observed that metastasis-free SLNs contained significantly less mature DCs and a lower degree of Th-1 response than metastasis-free non-SLNs, suggesting that cellular immune responses are less active in the direct tumor-draining node than in distant nodes. They also observed that the metastasis-positive SLNs showed higher expression of CD83 by DCs than the metastasis-free SLNs, suggesting that once metastasis is established, DC maturation is triggered and is followed by the up-regulation of Th-1 responses. Gannon et al. (36) reported that the metastatic pelvic LN from prostate cancer patients contained decreased numbers of CD20$^+$, CD38$^+$, and CD68$^+$ cells compared with nonmetastatic LN. These studies all point to the development of an immunosuppressive microenvironment in the tumor-draining lymph nodes. However, they have only compared micrometastatic SLNs to either nonmetastatic SLNs or metastatic non-SLNs. In contrast, we have observed that within the micrometastatic SLNs category, the degree of host response is highly variable with 15% of the SLNs containing very few mature DCs and 45% containing a high mature DCs density. Therefore, it is of utmost importance to study the clinical relevance of these variations between individuals.

Visual counting of DC-LAMP$^+$-positive DCs was associated in this study with excellent interobserver reproducibility. However, variability in staining intensity was noticed between participating centers, leading to intercenter variations in the positivity thresholds and to a significant center effect on the DC-LAMP$^+$ maximal density scores. The same center effect was observed when DC-LAMP$^+$ mean densities were studied ($P < 0.001$). This might be explained either by a fixative effect and/or by different protocols used among the six participating pathology departments to process the SLNs. For instance, at least three different fixatives were used to process SLN samples. In a previous pilot study, we observed a strong variation in staining intensity in samples fixed with buffered formalin, AFA (acetic acid, formalin, alcohol), and Bouin liquid (data not shown). This illustrates that multicenter studies for biomarker developments are difficult to carry on due to uncontrolled parameters leading to intercenter variability.

The extent of SLN involvement is one of the most powerful independent predictive factor for melanoma survival, and 30%
to 40% of patients with SLN micrometastasis will eventually die of their disease, compared with 5% to 10% of patients without SLN involvement (15). Moreover, SLNs draining cutaneous melanoma site are privileged sites of both T cell priming and initial metastasis, and therefore, evaluation of SLNs provides a unique opportunity to study the early phases of tumor-lymph node immune interaction. However, the respective roles of the biological characteristics of micrometastatic tumor cells and SLN immune response remain largely unknown. In a genomics study of primary melanomas, we found that primary melanomas with metastatic evolution were characterized by a dysregulation in key pathways driving replication, including the replication origins firing (ROF) system and survivin gene involved in chromatid separations (37, 38). We found no difference in expression signature between primary melanomas and metastases, illustrating once more that the genetic machinery for melanoma metastases evolution is acquired early in melanoma development.

Interestingly, in this study, we observed an inverse correlation between ulceration of the primary melanoma, a factor associated with worse prognosis, and mature DCs^{DC-LAMP^+} density in the draining SLNs. It is still unknown whether tumor ulceration can directly lead to a decreased DC response in the primary melanoma and, therefore, participates to the creation of an immunosuppressive environment, or whether ulceration is an associated phenotype in tumor cells that are poorly immunogenic. In several recent studies, including large population-based studies, it has been shown that ulceration is dependent on mitotic activity (39, 40). Therefore, there might be a relation between melanoma replication and further immune response. The interaction between the micrometastasis biology and immune response is of particular interest for the development of new treatment strategies that combine immunotherapy (e.g., with antiregulatory T cells) and targeted therapy.

In conclusion, this study shows that high maximum density of mature dendritic cells expressing DC-LAMP in melanoma-draining SLNs is associated with significant and prolonged overall survival benefit, reinforcing the notion that the immune system plays an active role in limiting the spread of melanoma. Ulceration of the primary melanoma is associated with low mature DC density in the draining SLNs. These results indicate that the preservation of DC maturation ability in the draining LN, an early event in the development of immune responses, is strongly associated with the establishment of long-term antimetastatic host response. The interactions between mature DCs and other immune cells, as well as the relationship between cellular responses and biological characteristics of micrometastases, are under investigation.

References
IL-12/23p40 


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