Indoleamine 2,3-Dioxygenase Expression in Primary Cutaneous Melanoma Correlates with Breslow Thickness and Is of Significant Prognostic Value for Progression-Free Survival

Felicia Rubel, Johannes S. Kern, Kristin Technau-Haft, Sibylle Uhrich, Kaethe Thoma, Georg Häcker, Nikolas von Bubnoff, Frank Meiss and Dagmar von Bubnoff

The enzyme indoleamine 2,3-dioxygenase (IDO) is emerging as a facilitator of cancer development through its effects on cancer-associated inflammation. Recent studies report a significant improvement of the response rates in melanoma patients to PD-1 antibodies when IDO inhibitors were added to the regimen. Data on IDO expression in primary human melanomas are, however, incomplete and conflicting. Here, we show that the level of IDO expression in primary human melanoma cells significantly correlates with Breslow thickness (\( P = 0.003 \)), the presence of tumor-infiltrating lymphocytes (\( P = 0.029 \)), and the intensity of the peritumoral inflammatory infiltrate (\( P = 0.001 \)). The expression of IDO in melanoma cells predicted independently of Breslow thickness and tumor stage (\( P = 0.04 \)). We further show that CD11c\(^+\) dendritic cells and CD68\(^+\) macrophages in the microenvironment of melanomas express IDO. The level of IDO expression in antigen-presenting cells correlated positively to peritumoral inflammation (\( P = 0.001 \)) but not to tumor-infiltrating lymphocytes. Significant negative correlation with progression-free survival was found for patients for whom antigen-presenting cells were very strongly IDO positive. These results suggest that IDO induction within melanoma cells may directly reflect tumor progression, whereas IDO in antigen-presenting cells may determine immune surveillance with impact on local and systemic tolerance.


INTRODUCTION

The interaction between melanoma cells and infiltrating immune cells or dermal cells is an important determinant in the development of malignant melanoma (Das et al., 2017; Ziani et al., 2017). Chronic inflammation drives the development of many cancers, but an exact definition of what these specific factors are has been difficult to determine (Holzel and Tuting, 2016). The two situations, progression of treatment-naïve melanoma and relapse of treated melanoma (e.g., after checkpoint inhibitor therapy) are both under the influence of the immune system. In both cases, the selection of tumor cell clones with high antigen plasticity and the induction of T-cell tolerance may, in part, account for the progression of the disease (Landsberg et al., 2012). Regulatory signals from the tumor, in turn, are important for stimulating or inhibiting T cells.

One such regulatory signal is the expression of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) (Prendergast et al., 2010). IDO is an intracellular heme-containing enzyme that initiates the first and rate-limiting step of tryptophan degradation along the kynurenine pathway (Routy et al., 2016). The expression of IDO by mesenchymal stromal cells, fibroblasts, and various myeloid-derived antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages has been shown to block T-cell responses in a number of situations such as in immune tolerance toward allogeneic fetuses and the inhibition of graft rejection (Chen et al., 2012; Li et al., 2006; Munn et al., 1998; von Bubnoff et al., 2004). On the contrary, IDO expression by cancer cells can substantially add to immune evasion by tumors (Godin-Ethier et al., 2011; Prendergast et al., 2014). IDO expression primarily affects T-cell activity but also APC function. Tryptophan deficiency specifically activates the GCN2 kinase in T cells and prevents T-cell activation (Munn et al., 2005). DCs, when generated in low tryptophan conditions in vitro, show low T-cell stimulatory capacity because of the induction of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T regulatory cells (Brenk et al., 2009). In vitro, after activation with CD40L, IDO-positive APCs express increased levels of B7...
family molecules such as CD40 and CD80, as well as PD-L1 (B7-H1) and PD-L2 (B7-DC), compared with IDO-negative DC subset (Von Bubnoff et al., 2011a). It is therefore conceivable that in the tumor microenvironment, a complex process of immune editing takes place where IDO⁺CD80⁺CD86⁺PD-L1/PD-L2⁺ APCs and tumor cells contribute to the induction of tolerance in CTLA-4⁺PD1/2⁺ T cells.

In melanoma but also in other cancers, it has been shown that these inhibitory pathways are strongly up-regulated and affect the development of an immune response in vivo. For instance, injection of stimulatory CTLA-4 Ig into mice induced IDO expression in specific DC subsets of the spleen by reverse signaling via CD80/86 into DCs. The induction of IDO in this setting prevented the development of a specific CD8 T-cell response (Mellor et al., 2003). When the CTLA-4-blocking antibody ipilimumab was injected into B16 melanoma-bearing mice, IDO-deficient animals showed a striking delay in B16 melanoma tumor growth (Holmgaard et al., 2013). The delay was associated with an increased number of tumor-infiltrating CD8⁺ and CD4⁺ effector T cells. Blockade of CTLA-4 in melanoma thus not only releases the checkpoint blockade on T cells but may also block induction of IDO in APCs.

In human melanoma patients and in mouse models, IDO has been shown to be expressed by APCs in tumor draining lymph nodes (Gerlini et al., 2010; Sharma et al., 2009). An independent negative prognostic effect of IDO expression in the sentinel lymph node on progression-free survival (PFS) and overall survival (OS) in patients with stage I and II melanoma has been reported (Speeckaert et al., 2012). IDO expression could be a very early determinant of immune instruction in melanoma development, because IDO expression in tumor negative sentinel lymph nodes confers a negative prognostic value for patients (Chevolet et al., 2014).

We provide a detailed semiquantitative analysis of IDO expression in primary human melanoma cells and in cells of the peritumoral microenvironment from skin biopsy samples of melanoma patients. Findings were correlated to melanoma Breslow index, histopathologic parameters, peritumoral inflammation, PFS, and OS. Because IDO inhibitors have been shown to enhance tumor immunity in vivo (Brochez et al., 2017), these data are of potential value should immunotherapy be established at earlier stages in melanoma.

RESULTS

Patient characteristics according to tumor thickness (groups 1–3)

A total of 99 patients with primary melanoma were divided into three groups according to tumor thickness as outlined in the Materials and Methods section; characteristics are shown in Table 1. The follow-up time was 15 years (mean = 8 years). Patient numbers, sex, and mean age did not differ significantly, although female patients tended to have earlier melanoma development than male patients. As expected, ulceration, microsatellitosis, lymphangioinvasion and tumor-related death were most often seen in group 3.

The level of IDO expression in primary human melanoma significantly correlates to Breslow tumor thickness

The expression of IDO was analyzed in all samples from groups 1, 2, and 3 (Figure 1a). In melanoma cells, very strong IDO expression (IDO⁺⁺⁺) was seen in 25% of group 3 specimens and in 13% of group 2 and 6% of group 1 samples. Strong IDO expression (IDO⁺⁺) was seen most frequently in samples of group 3 (36%); 39% of samples in group 3 were IDO negative (IDO⁻⁻) or of low IDO expression (IDO⁺⁺), whereas this number was 64% in group 2 and 66% in group 1. The correlation of the level of IDO expression in primary melanoma cells to Breslow thickness was highly significant (P = 0.003). IDO expression in APCs did not correlate with Breslow thickness of primary melanoma (P = 0.35, data not shown). Thin melanomas with tumor thickness of less than 0.75 mm expressed IDO right at the site of cancer development (Figure 1b).

Patient characteristics, clinical and histopathological data, and their correlation with the level of IDO expression in melanoma cells and APCs

Patients were stratified according to the level of IDO expression in melanoma cells to assess its association with...
patient characteristics and histopathological and clinical parameters (Table 2). Parameters that were positively and significantly correlated with the level of melanoma IDO expression were ulceration \((P = 0.0023)\), lymphangioinvasion \((P = 0.0068)\), and microsatellitosis \((P = 0.01)\). The presence of tumor-infiltrating lymphocytes (TILs) was highly positively correlated with the level of IDO expression in tumor cells \((P = 0.029)\) but not with the level of IDO APC expression \((P = 0.298)\). The level of IDO APC expression correlated only positively with ulceration \((P = 0.015)\).

Table 2. Patient characteristics and clinical and histological data according to the level of IDO expression in melanoma cells

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>IDO-</th>
<th>IDO+</th>
<th>IDO++</th>
<th>IDO+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n (%)</td>
<td>35 (100)</td>
<td>20 (100)</td>
<td>29 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td>Male 13 (37)</td>
<td>11 (55)</td>
<td>15 (52)</td>
<td>7 (47)</td>
</tr>
<tr>
<td>Female 22 (63)</td>
<td>9 (45)</td>
<td>14 (48)</td>
<td>8 (53)</td>
<td></td>
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<tr>
<td>Breslow thickness (1,2), in mm</td>
<td>Range 0.2–12.01</td>
<td>0.25–10.0</td>
<td>0.2–17.0</td>
<td>0.45–15.1</td>
</tr>
<tr>
<td>Mean 2.01</td>
<td>2.20</td>
<td>3.58</td>
<td>5.38</td>
<td></td>
</tr>
<tr>
<td>Mitotic rate (1,2), per mm(^2)</td>
<td>Range 0–24</td>
<td>0–7</td>
<td>0–16</td>
<td>0–20</td>
</tr>
<tr>
<td>Mean 6.9</td>
<td>4.7</td>
<td>5.8</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>Ulceration (1,2), n (%)</td>
<td>3 (9)</td>
<td>1 (5)</td>
<td>8 (28)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Regression (3), n (%)</td>
<td>5 (14)</td>
<td>3 (15)</td>
<td>4 (14)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Lymphangioinvasion (1,2), n (%)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>4 (14)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Microsatellitosis (1,2,3), n (%)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>6 (21)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Tumor-infiltrating lymphocytes (4), n (%)</td>
<td>Absent 12 (34)</td>
<td>5 (25)</td>
<td>3 (10)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Non-brisk 13 (37)</td>
<td>8 (40)</td>
<td>12 (41)</td>
<td>6 (40)</td>
<td></td>
</tr>
<tr>
<td>Brisk 10 (29)</td>
<td>7 (35)</td>
<td>14 (48)</td>
<td>7 (47)</td>
<td></td>
</tr>
<tr>
<td>IDO expression in APZs (1), n (%)</td>
<td>IDO- 19 (54)</td>
<td>2 (10)</td>
<td>2 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>IDO+ 5 (14)</td>
<td>11 (55)</td>
<td>4 (14)</td>
<td>1 (7)</td>
<td></td>
</tr>
<tr>
<td>IDO++ 9 (26)</td>
<td>7 (35)</td>
<td>18 (62)</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td>IDO+++ 2 (6)</td>
<td>0 (0)</td>
<td>5 (17)</td>
<td>11 (73)</td>
<td></td>
</tr>
<tr>
<td>T stage (1,2), n (%)</td>
<td>T1 26 (74)</td>
<td>15 (75)</td>
<td>16 (55)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>T2 0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>T3 1 (3)</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>T4 8 (23)</td>
<td>5 (25)</td>
<td>12 (42)</td>
<td>9 (60)</td>
<td></td>
</tr>
<tr>
<td>N stage (1,2), n (%)</td>
<td>N0 34 (97)</td>
<td>20 (100)</td>
<td>22 (76)</td>
<td>10 (67)</td>
</tr>
<tr>
<td>N1 1 (3)</td>
<td>0 (0)</td>
<td>4 (14)</td>
<td>2 (13)</td>
<td></td>
</tr>
<tr>
<td>N2 0 (0)</td>
<td>0 (0)</td>
<td>3 (10)</td>
<td>3 (20)</td>
<td></td>
</tr>
<tr>
<td>M stage (1), n (%)</td>
<td>M0 35 (100)</td>
<td>20 (100)</td>
<td>29 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>AJCC (1,2), n (%)</td>
<td>I 26 (74)</td>
<td>15 (75)</td>
<td>16 (55)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>II 8 (23)</td>
<td>5 (25)</td>
<td>6 (21)</td>
<td>4 (27)</td>
<td></td>
</tr>
<tr>
<td>III 1 (3)</td>
<td>0 (0)</td>
<td>7 (24)</td>
<td>5 (33)</td>
<td></td>
</tr>
<tr>
<td>Clinical outcome (4), n (%)</td>
<td>Alive 26 (74)</td>
<td>17 (85)</td>
<td>24 (83)</td>
<td>7 (47)</td>
</tr>
<tr>
<td>Disease progression 8 (23)</td>
<td>3 (15)</td>
<td>6 (21)</td>
<td>9 (60)</td>
<td></td>
</tr>
<tr>
<td>Dead 9 (26)</td>
<td>3 (15)</td>
<td>5 (17)</td>
<td>8 (53)</td>
<td></td>
</tr>
<tr>
<td>Tumor-related death</td>
<td>Yes 2 (6)</td>
<td>2 (10)</td>
<td>2 (7)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>No 7 (20)</td>
<td>1 (5)</td>
<td>3 (10)</td>
<td>4 (27)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AJCC, American Joint Committee on Cancer; APC, antigen-presenting cell; IDO, indoleamine 2,3-dioxygenase.

\(1\)At time of primary diagnosis.

\(2\)\(P < 0.05\).

\(3\)Eight patients were not specified.

\(4\)At the end of follow-up time.

Committee on Cancer 2009 staging classification, and tumor-related death was highest in IDO+++ patients. The level of IDO expression in primary melanoma cells appears to correlate with the established TNM classification for melanomas for the investigated stages.
IDO in Melanoma Correlates to Breslow Thickness and Is Prognostic for Progression-Free Survival

The intensity of IDO expression in primary melanoma cells and in APCs significantly correlates with the strength of the peritumoral inflammatory infiltrate.

Because IDO is mainly induced by proinflammatory stimuli, we correlated the intensity of IDO expression in melanoma cells and APCs to the strength of the peritumoral inflammatory infiltrate (Figures 2a and b). First, most samples with moderate and intense infiltrates also showed substantial IDO expression (IDO++ and IDO+++ in melanoma cells (moderate, 62%; intense, 70%) and APCs (moderate, 76%; intense, 60%). Accordingly, 78% of samples with scarce infiltrate showed no IDO expression in melanoma cells and in APCs, respectively. The remaining 22% of samples with scarce infiltrate were associated with only mild (IDO+) melanoma cell IDO expression or with mild (11%) or moderate (11%) IDO expression in APCs. The correlation between the IDO expression in melanoma cells and in APCs with the density of the peritumoral inflammatory infiltrate was highly significant ($P = 0.001$).

CD68+ macrophages and CD11c+ DCs express IDO in the melanoma microenvironment

The cells in the peritumoral inflammatory infiltrate that expressed IDO were aligned beneath the invasive front of the tumor cells (Figures 3a and g) and were peritumoral in foci of inflammation (Figures 3a and h). CD68+ macrophages and CD11c+ myeloid APCs most often encircled the abundant CD3+ T-cell inflammatory infiltrate (Figures 3c–e). Melanoma cells, epidermal Langerhans cells, and some myeloid cells in the infiltrate stained S100 positive (Figure 3f).

Double-immunofluorescence staining showed that CD68+ macrophages and CD11c+ myeloid DCs strongly expressed IDO (Figure 4).

The level of IDO expression in primary melanoma cells and in peritumoral APCs shows significant negative correlation with PFS

Clinical progression of melanoma within the first 4 years was especially pronounced in the patient group with very high IDO expression (IDO+++ in melanoma cells (Figure 5a). In line with this, the median PFS was reached by this patient group at 4 years of follow-up, whereas median PFS was not reached by the other groups. The between-group differences in PFS related to the level of IDO expression in melanoma cells was highly significant ($P = 0.02$). Because IDO expression positively correlates to tumor thickness and tumor thickness is known to predict PFS, we determined if IDO expression independently correlates with PFS. Covariate Cox regression analyses showed an independent correlation of IDO expression in melanoma cells with PFS ($P = 0.04$).

With regard to the level of IDO expression in APCs, neither patient curve reached median PFS (Figure 5b). The patient curves with the strongest IDO expression (IDO+++ and with no IDO expression (IDO-) in peritumoral APCs showed a fast decline during the first 2 years of follow up. The difference between the Kaplan-Meier curve for patients with IDO+++ APCs compared with all other groups was statistically highly significant ($P = 0.0095$). In contrast, there was no significant difference in PFS for patients with IDO- APCs compared with all other groups ($P = 0.3078$). Overall, the between-group differences in PFS related to the level of IDO expression in peritumoral APCs was highly significant ($P = 0.02$).

The level of IDO expression in primary melanoma cells shows a strong trend toward correlation with OS

Next, we evaluated the correlation of OS with IDO expression levels in primary melanoma and peritumoral APCs (Figures 6a and b). The point of median OS was not reached in any subgroup. Probability for OS decreased fastest during the first 5 years in patients with IDO+++ melanoma cells (Figure 6a). Statistical analysis just failed to show significance of a between-group difference for OS regarding IDO expression in melanoma cells ($P = 0.08$). Accordingly, Cox regression analyses did not show independent correlation of IDO expression in melanoma cells with OS, although a strong trend was observed ($P = 0.08$).

OS in patients with respect to varying IDO expression in APCs did not differ much within the first 5 years of follow-up but was lowest in the IDO+++ APC group after 6 years (80%) (Figure 6b). There was no significant difference between the varying degrees of IDO expression in APCs on OS ($P = 0.33$).
DISCUSSION
In this study, we show a significant and positive correlation of the level of IDO expression in primary cutaneous melanoma cells with Breslow tumor thickness. In addition, the level of melanoma cell IDO expression was strongly correlated to well-known prognostic histopathologic parameters such as ulceration, mitotic rate, lymphangioinvasion, microsatellitosis, and the presence of TILs (Rose, 2017). Furthermore, IDO in melanoma cells carried a significant prognostic association with PFS, which was independent of Breslow thickness and tumor stage. Thus, our data further substantiate the literature showing the important immunoregulatory role of IDO in melanoma.

Earlier observations showed that IDO expression in the sentinel node and in peritumoral endothelium of melanoma patients is associated with a lower PFS (Speeckaert et al., 2012; Chevolet et al., 2014). In different cancer entities, IDO expression within tumor cells has been increasingly...
identified to be associated with poor prognosis (Jia et al., 2015; Kim et al., 2016; Zhang et al., 2017). Brody et al. (2009) showed strong melanoma cell IDO expression in metastatic lymph nodes and its association with poor survival (Brody et al., 2009).

The presence of TILs in primary melanoma remains controversial regarding its prognostic value (Weiss et al., 2016). The composition of the TIL infiltrate, either immunosupportive or immunosuppressive, may be one determinant of the prognosis of primary melanoma. In our study, the level of melanoma IDO expression, but not APC IDO expression, positively correlated with the gradation of TILs. APC IDO expression (and melanoma IDO expression), however, did correlate with the peritumoral infiltrate, which we determined as being located around the tumor.

These results may have two implications. First, the quality of TILs, either immunosupportive or immunosuppressive, might be reflected by the expression of IDO in melanoma cells. In other words, the level of IDO expression in cancer cells could be an important factor to predict immunosuppressive TILs and indicate that loss of immune control has been initiated (Inozume et al., 2016).

Figure 4. CD68⁺ macrophages and CD11c⁺ DCs express IDO in the peritumoral inflammatory infiltrate of primary cutaneous melanomas.

Double immunofluorescence of primary melanoma for IDO with (a) CD11c and (b) CD68. Representative photographs of the single stainings are shown, as is the overlay of IDO (green) with individual markers (red). Double-positive cells are shown in yellow. Scale bar = 50 μm. DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase.

Figure 5. The level of IDO expression in melanoma cells and APCs correlates with PFS. All 99 patients are shown. Of these, 26 patients showed progression during follow-up. (a) Patients with IDO+++ melanoma cells (20%) showed fast and early progression. Patients with progression and IDO++ (19%), IDO+ (23%), and IDO- (27%) melanoma cells had slower tumor progression. \( P = 0.02 \). (b) Disease in patients with IDO+++ APCs (34%) progressed significantly faster compared with all other groups. \( P = 0.0095 \). The between-group difference in PFS related to the intensity of APC IDO expression was significant. \( P = 0.02 \). APC, antigen-presenting cell; IDO, indoleamine 2,3-dioxygenase; PFS, progression-free survival.
IDO in Melanoma Correlates to Breslow Thickness and Is Prognostic for Progression-Free Survival

Second, IDO in cancer might have two components: IDO expression in cancer cells and IDO expression in peritumoral inflammation. Induction of IDO in melanoma cells is probably through direct effects of IFN-γ from adjacent TILs (Brandacher et al., 2006). IDO expression in APCs, however, might be induced by factors such as neoantigen presentation, tumor cell death, or IL-6/TNF-α from bystander macrophages in the peritumoral infiltrate (Grohmann et al., 2002; Huang et al., 2013; Von Bubnoff et al., 2011a). IDO induction of peritumoral APCs would therefore describe the recognition of the tumor by the immune system rather than directly influence tumor growth. This is supported by the fact that we did not detect a correlation of Breslow tumor thickness with the level of APC IDO expression in our study.

In our patient population, we mainly detected IDO in peritumoral myeloid CD11c⁺ DCs and CD68⁺ macrophages by double immunofluorescence. A very strong IDO expression of APCs was linked to a significantly shorter PFS compared with all other IDO-expressing APC groups. One interpretation is that IDO⁺ APCs may account for local and systemic immunosuppression in specific situations (Johnson and Munn, 2012; Lemos et al., 2016; von Bubnoff et al., 2011b). Increased serum IDO activity and increased numbers of circulating IDO⁺ monocytes may be a prerequisite for tumor development. In melanoma phase III studies, no effect was seen as monotherapy using an IDO inhibitor such as epacadostat or indoximod (Zakharia et al., 2017). However, when an IDO-inhibitor was combined with anti-PD1 antibodies, response rates were significantly increased to 52–59% compared with anti-PD1 therapy only (30–40% response rates) (Indoximod combo triggers responses in melanoma, 2017). It will be very interesting to correlate IDO expression in melanoma cells and APCs with therapy response in these groups.

Our study implicates that investigations targeting the expression of IDO in melanoma may be of pivotal future interest. The use of IDO inhibitors may be of particular use in combination with immune-stimulatory protocols such as PD-1 antibodies to subvert melanoma-associated antigen tolerance and to sustain T-cell effector function for the eradication of melanoma cells.

MATERIALS AND METHODS

Patients
We analyzed samples from 99 patients with primary cutaneous melanomas with tumor thickness of less than 0.75 mm (n = 32, group 1, low-risk group), tumor thickness of 0.75–1 mm (n = 31, group 2, intermediate-risk group), and tumor thickness of 4 mm or
greater (n = 36, group 3, high-risk group) collected at the Department of Dermatology—Medical Center University of Freiburg from 1998 through 2013. These groups were used to investigate correlation of IDO expression with tumor thickness. Written consent was obtained from all patients at the time of tissue rejection in accordance with the study protocol approved by the local ethics committee of the University Medical Center Freiburg. All patients came to the clinic for surgical treatment of primary melanoma. Clinical data (sex, age) and follow-up information in 2016 were obtained from medical follow-up care records. The study was performed according to local ethical guidelines and approved by the local regulatory committee.

Histology and immunohistochemistry
Serial sections were prepared from formalin-fixed, paraffin-embedded skin biopsy samples. Standard hematoxylin and eosin staining and periodic acid Schiff reactions were performed for diagnostic purposes. Tumor thickness, ulceration, mitotic rate, lymphangioinvasion, regression, and microsatellite were determined by a dermatopathologist (FM) (Jia et al., 2015; Kim et al., 2016). Anti-IDO monoclonal antibody (mAb) (clone 10.1, dilution 1:200; Millipore, Billerica, MA) labeling was performed on paraffin-embedded tissue sections (1 μm) after heat pretreatment for 10 minutes in Target Retrieval Solution, pH 9 (DAKO, Hamburg, Germany). Appropriate isotype-matched controls were included. Additional antibodies for immunohistochemistry included anti-CD11c mAb (5D11, dilution 1:100; Novocastra, Newcastle, UK), anti-S100 antibody (Z0311, dilution 1:1,000; DAKO), anti-CD68 mAb (PGM1, dilution 1:50; DAKO), anti-CD1a mAb (O10, dilution 1:10; Zytomed Systems, Berlin, Germany), and anti-CD3 mAb (F7.2.38, dilution 1:50; DAKO). Visualization was performed using the REAL detection system (4th edition, DAKO) with fast red as the chromogen. Photographs of stainings (microscope: Eclipse 80i, Nikon, Düsseldorf, Germany) were visualized with the program AxioVision (Zeiss, Feldbach, Switzerland).

Immunofluorescence staining
Immunofluorescence double staining was carried out using formalin-fixed, paraffin-embedded sections after heat antigen retrieval following standard protocols. The following primary antibodies were used: anti-ID0 polyclonal antibody (AHB33, dilution 1:100; Serotec, Düsseldorf, Germany), anti-CD11c mAb (5D11, dilution 1:50; Novocastra), and anti-CD68 mAb (PGM1, dilution 1:50; Novocastra). As secondary antibody we used a FITC-conjugated donkey-anti-sheep-IgG (dilution 1:100; Serotec, Düsseldorf, Germany) to detect the IDO antibody. To detect the CD11c and CD68 antibodies we used Alexa Fluor 568 (dilution 1:500; Invitrogen, Waltham, MA). Controls included staining with isotype-matched antibodies. Analysis was performed using a microscope (Eclipse 80i; Nikon, Düsseldorf, Germany) interfaced with a 12-V, 100-W halogen lamp (Osmar, München, Germany). Photographs (camera JVC KY-F75U; JVC Professional Products, Wayne, NJ) were visualized with the program AxioVision (Zeiss).

Scoring of IDO+ melanoma and APCS
IDO+ melanoma cells or IDO+ APCS were scored as IDO negative (IDO−), IDO+ (<25%, mild), IDO++ (25–50%, strong), or IDO+++ (>50%, very strong) if these percentages of melanoma cells or APCS in 10 representative high-power fields (magnification ×200) were IDO+.

Scoring of the density of the peritumoral inflammatory infiltrate
Cells of 10 representative high-power fields (magnification ×200) around the melanoma (vertical and horizontal growth phase) were calculated. The density of the peritumoral inflammatory infiltrate was scored as follows: none, if less than 25% of all cells were inflammatory cells in all high-power fields; mild, if 25–50% of all cells were inflammatory cells; moderate, if 50–75% of all cells were inflammatory cells; or intense, if nearly all (>75%) cells were of inflammatory origin and only a few stromal cells were seen. All samples were evaluated independently by two investigators (DvB and FM).

Grading of TILs
The Clark method for grading TILs was used (Fukuno et al., 2015). TILs were graded as brisk when present throughout the vertical growth phase or infiltrating the entire base thereof, nonbrisk if present in one or more foci of the vertical growth phase, or absent if lymphocytes had no contact with the vertical growth phase but were present in perivascular or fibrotic areas.

Statistical methods
Statistical analyses were performed using Microsoft Excel 2010 (Redmond, CA) and IBM SPSS Statistics Version 24 (IBM, Chicago, IL). To compare clinical and histopathological parameters between groups (Table 1), the analysis of variance test was used. The correlations of IDO to Breslow thickness, histopathologic parameters, and the density of the peritumoral inflammatory infiltrate (Table 2) were performed with the linear-by-linear trend test and analysis of variance test. Follow-up time was defined from the date of primary excision to the date of last follow-up or death (OS). Survival analyses included only deaths caused by melanoma. Multivariate Cox regression analyses with IDO expression and tumor thickness as covariates was performed to show significance of IDO on PFS. Kaplan-Meier analyses were performed to estimate OS and PFS. Probabilities of PFS and OS are shown as contingent upon IDO expression in melanoma cells and APCS in the peritumoral inflammatory infiltrate. The log rank test was applied to determine significance between the varying IDO-expressing survival curves of patients (between-group difference) or to differ between two selected survival groups. A P-value less than 0.05 was considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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