Emerging Role of IL-4–Induced Gene 1 as a Prognostic Biomarker Affecting the Local T-Cell Response in Human Cutaneous Melanoma

Jan Philipp Ramspott[1,2,3,13], Fériel Bekkat[1,2,3], Lloyd Bod[1,2,3], Maryline Favier[1,2,3], Benoit Terris[4], Anne Salomon[5,6], Lounes Djerroudi[5], Kurt S. Zaenker[7], Yolande Richard[1,2,3], Valérie Molinier-Frenkel[8,9,10], Flavia Castellano[8,9,11], Marie-Françoise Avril[1,2,3,12] and Armelle Prévost-Blondel[1,2,3]

Several studies have emphasized the importance of immune composition of the melanoma microenvironment for clinical outcome. The contribution of IL4I1, a phenylalanine oxidase with immunoregulatory functions, has not been yet explored. Here we studied a primary cutaneous melanoma series from stage I–III patients to investigate the association between in situ IL4I1 expression and clinical parameters or tumor-infiltrating T-cell subsets. IL4I1 was detected in 87% of tumors and was mainly expressed by tumor-associated macrophages and very rare FoxP3$^+$ regulatory T cells. The proportion of IL4I1$^+$ cells was higher in patients with an ulcerated melanoma or with a positive sentinel lymph node and tended to correlate with a rapid relapse and shorter overall survival. This proportion also correlated positively with the presence of regulatory T cells and negatively with the presence of cytotoxic CD8$^+$ T cells. The location of IL4I1$^+$ cells may also be relevant to predict prognosis, because their presence near tumor cells was associated with sentinel lymph node invasion and higher melanoma stage. Collectively, our data show that IL4I1$^+$ cells shape the T-cell compartment and are associated with a higher risk of poor outcome in melanoma, supporting a key role for IL4I1 in immune evasion.

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INTRODUCTION

Cutaneous melanoma accounts for 15–25 cases per 100,000 people each year in Western countries. Approximately 20% of melanoma patients die of their disease, despite the early detection and prevention efforts made in recent years (Schadendorf and Hauschild, 2014). The American Joint Committee on Cancer for melanoma staging and classification includes criteria such as primary tumor thickness, ulceration, mitotic rate, status of the sentinel lymph nodes (sLNs) (either metastatic or not), and the presence of distant metastases to classify patients for assessment of prognosis and treatment (Amin et al., 2017). This staging system has been developed over time, and several biological parameters, including mutations of oncogenes, epigenetic alterations, and immunologic markers, have been proposed as prognostic tools; however, none are routinely used to evaluate survival, and they poorly predict patient outcomes (Weiss et al., 2015). New staging markers are still needed.

A recent meta-analysis highlighted that the profile of tumor-infiltrating leukocytes and immune-related genes can be important predictors of clinical outcome in cancer (Gentles et al., 2015). In primary melanoma, the density of tumor-infiltrating CD8$^+$ T and B lymphocytes may be an immunological marker of good prognosis, whereas infiltrates of macrophages and regulatory CD4$^+$ T cells (Tregs) have been associated with a worse prognosis. However, the prognostic value of these populations is still debated because of conflicting results (Barnes and Amir, 2017; Ladanyi, 2015). Immunosuppressive enzymes produced by tumor or immune cells of the tumor microenvironment participate in tumor escape and may thus help predict survival (Grohmann and Bronte, 2010; Molinier-Frenkel and Castellano, 2017). These enzymes modify the nutrient

[1]*INSERM, U1016, Institut Cochin, Paris, France;* [2]*CNRS, UMR8104, Paris, France;* [3]*Université Paris Descartes, Sorbonne Paris Cité, Paris, France;* [4]*AP-HP, Service de Pathologie, Hôpital Cochin, Paris, France;* [5]*Institut Curie, Department of Pathology, Paris, France;* [6]*Paris-Sciences-Lettres, INSERM, U934, Paris, France;* [7]*Institute of Immunology & Experimental Oncology, Center for Biomedical Education and Research, Witten/Herdecke University, Witten, Germany;* [8]*INSERM, U955, Equipe 09, Créteil, France;* [9]*Université Paris Est, Faculté de Médecine, Créteil, France;* [10]*AP-HP, Hôpital H. Mondor–A. Chenevier, Service d'Immunologie Biologique, Créteil, France;* [11]*AP-HP, Hôpital H. Mondor–A. Chenevier, Plateforme de Ressources Biologiques, Créteil, France; and* [12]*AP-HP, Service de Dermatologie, Hôpital Cochin, Paris, France*

[13]*Current address: Institute of Immunology & Experimental Oncology, Center for Biomedical Education and Research, Witten/Herdecke University, Witten, Germany.*

Correspondence: Armelle Prévost-Blondel, Institut Cochin, département Infection, Immunité, Inflammation, 27 rue du Faubourg Saint Jacques, 75014 Paris, France. E-mail: armelle.blondel@inserm.fr

Abbreviations: GrB, granzyme B; mAb, monoclonal antibody; OS, overall survival; sLN, sentinel lymph node; TAM, tumor-associated macrophage; Treg, regulatory T cell

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composition of the extracellular medium by catabolizing essential or semi-essential amino acids and producing toxic catabolites that block T-cell proliferation or induce their apoptosis. Among the most cited enzymes of this family, inducible nitric oxide synthase (Ekmekcioglu et al., 2000, 2006) and indoleamine 2,3-dioxygenase (Chevolet et al., 2014; de Lecea et al., 2017; Rubel et al., 2018), which catabolize arginine and tryptophan, respectively, have a negative effect on overall survival (OS) in patients with cutaneous primary melanoma and lymph node metastases. IL4I1 is a secreted L-amino acid oxidase that mainly catabolizes L-phenylalanine to produce hydrogen peroxide, ammonia, and phenylpyruvate. In the past 10 years, IL4I1 has been shown to be expressed by tumor-associated macrophages (TAMs) of numerous cancer types and to regulate T-cell properties both in vitro and in vivo (Aubatin et al., 2017; Boulland et al., 2007; Cousin et al., 2015; Lasoudris et al., 2011; Santarlasci et al., 2012).

Our aim was to investigate the pattern of IL4I1 expression in situ and its prognostic value in primary cutaneous melanoma from patients with stage I–III disease. Furthermore, we analyzed the impact of IL4I1$^+$ cells on the composition of tumor-infiltrating T-cell subsets.

RESULTS

Accumulation of IL4I1 in the primary tumor of patients with metastatic cutaneous melanoma

Primary cutaneous melanoma biopsy samples obtained from 39 melanoma patients were analyzed. Table 1 presents a detailed listing of the patients and their clinical characteristics. The median age was 58.4 years, and 41% were female. Among the patients, 82.1% underwent the sLN procedure. Stage 0–III primary melanomas were classified as superficial spreading melanoma, nodular melanoma, or acral lentiginous melanoma. Patients were followed for 7.3 ± 2.5 years. During the follow-up period, 38.5% (n = 15) of patients relapsed and 30.8% (n = 12) died, 8 (20.5%) of melanoma.

We first studied the pattern of IL4I1 expression in the primary tumor tissue from all melanoma patients using our rabbit monoclonal antibody (mAb). IL4I1 staining was detectable as intracytoplasmic granules in nontumor cells (Figure 1), as we observed with a commercial polyclonal antibody (Carbonnelle-Puscian et al., 2009). No IL4I1$^+$ cells were detected in only five patients (Figure 1a). We observed different patterns of IL4I1$^+$ cell localization: within the stromal area with no contact with tumor cells (referred hereafter as *peritumoral*) (Figure 1b) or as isolated stromal cells in close contact with HMB45$^+$ melanoma cells (referred hereafter as *intratumoral,* Figure 1c and d). In total, 46% of the biopsy samples displayed intratumoral IL4I1$^+$ cells, and 41% peritumoral IL4I1$^+$ cells (Figure 1e). Because IL4I1 is not expressed by melanoma cells (Carbonnelle-Puscian et al., 2009), we quantified the proportion of the respective areas of tumor and stroma within each sample, as shown in Supplementary Figure S1 online. Stromal area, negative for HMB45, represented 0%–84.6% of the surface of the biopsy samples, regardless of melanoma histological types (see Supplementary Figure S1e). The density of IL4I1$^+$ cells was heterogeneous, with a median of 65.7 cells/mm^2 of stroma (Figure 1f). The percentages of IL4I1$^+$ cells among stromal cells varied from 0%–38.5%, with a median of 2.2% IL4I1$^+$ cells (Figure 1g).

The presence of IL4I1 cells is associated with a poor prognosis in cutaneous melanoma

We further investigated the prognostic value of IL4I1 in melanoma by analyzing the relationship between IL4I1$^+$ cell infiltration and standard prognostic/clinical variables. We first focused on the proportions of IL4I1$^+$ cells among stromal cells in the 39 patients. There was no statistical difference associated with histological type, Breslow tumor thickness, sex, or age (Figure 2a and b and data not shown). In contrast, we noted a greater infiltration of IL4I1$^+$ cells in patients with an ulcerated melanoma ($P = 0.0240$) (Figure 2c). IL4I1$^+$ cells infiltrated more frequently Clark V than Clark I/II melanoma (Figure 2d). Patients with positive sLNs exhibited statistically more tumor-infiltrating IL4I1$^+$ cells with a mean of 11.6% ± 2.9% vs 4.6% ± 1.3% for patients with negative sLNs (Figure 2e) ($P = 0.0441$). The proportion of IL4I1$^+$ cells was more significant in most of the primary tumors from stage IIIB–D melanoma patients compared with those from patients with lower-stage tumors (Figure 2f). It also tended to be higher in patients who relapsed from the disease within 2 years after melanoma diagnosis compared with patients who did not relapse (Figure 2g) ($P = 0.0682$). Six of eight patients (75%) who died of melanoma during follow-up displayed more tumor-infiltrating IL4I1$^+$ cells at diagnosis compared with patients who remained alive (mean = 14.7 ± 3.5 vs. 7.3 ± 2.0) (Figure 2h). The OS curves consistently suggest that patients with a level over the median of IL4I1$^+$ cell infiltration tended to have worse survival (Figure 2i) ($P = 0.1218$).

We next analyzed whether the location of IL4I1$^+$ cells within the tissue had prognostic value and thus censored patients with no IL4I1$^+$ cell infiltrate. Similar proportions of patients with either peritumoral or intratumoral IL4I1$^+$ cells were observed, regardless of their melanoma histological type or the presence of an ulceration (Figure 3a and c). The analysis showed no statistical difference in the location of IL4I1 staining in the primary melanoma, regardless of their thickness or Clark levels (Figure 3b and d). Nevertheless, we detected peritumoral IL4I1$^+$ cells only in patients with a Breslow thickness less than 1mm (n = 4) or those with a Clark level II melanoma (n = 3). Furthermore, IL4I1$^+$ cells were directly localized in the tumor area for 10 of 12 (83%) patients with positive sLNs and only 7 of 20 (35%) patients with negative sLNs (Figure 3e) ($P = 0.0080$). We observed only peritumoral IL4I1$^+$ cells in the four primary tumors derived from stage IA melanoma patients, whereas most of the IL4I1$^+$ cells were near tumor cells in primary tumors derived from the 10 stage IIIB–D melanoma patients studied (Figure 3f) ($P = 0.0027$). Concurrently, intratumoral IL4I1$^+$ cells could be detected in five tumors from the seven patients (71.4%) who quickly relapsed (<2 years after diagnosis), whereas they were found in only 8 of 19 tumors (42.1%) from patients who did not relapse (Figure 3g). Consistently, 85.7% of patients who died of melanoma during follow-up exhibited intratumoral IL4I1$^+$ cells compared with such a location in only 54.5% patients who remained alive (Figure 3h). Intratumoral IL4I1 expression also tended to be associated with shorter OS (Figure 3i) ($P = 0.0877$). Collectively, our

Table 1. Clinical Characteristics of the Patients at Baseline

Characteristics	n	%	Median
Sex			
Male	23	59.0	
Female	16	41.0	
Age, years			
Median (range)			58.4 (30.2–91)
<50	11	28.2	
>50 to <65	12	30.8	
≥65	16	41.0	
Disease stage			
0	1	2.6	
IA	4	10.3	
IB	6	15.4	
IIA	7	17.9	
IIB	4	10.3	
IIC	3	7.7	
IIIA	4	10.3	
IIIB	6	15.4	
IIIC	3	7.7	
IIID	1	2.6	
Histological type			
ALM	7	17.9	
SSM	18	46.2	
NM	12	30.8	
Spitzoid[1]	1	2.6	
Dubreuilh[2]	1	2.6	
Ulceration			
Yes	13	33.3	
No	26	66.7	
Breslow thickness, mm			
Median (range)			1.88 (0–20)
<1.00	5	12.8	
1.01–2.00	18	46.2	
2.01–4.00	7	17.9	
>4.00	9	23.1	
Clark levels[3]			
I	1	2.6	
II	3	7.7	
III	7	17.9	
IV	18	46.2	
V	9	23.1	
Lymph node status			
sLN negative clinically (sLN procedure not applicable[4])	5	12.8	
sLN negative	18	46.2	
sLN positive	14	35.9	
Unknown (Breslow >1 mm, but sLN procedure not performed)	2	5.1	
Relapse			
No	24	61.5	
Yes	15	38.5	
Time to relapse, years			
>2	7	46.7	
<2	8	53.3	
Median (range), months from diagnosis date			23.2 (3.4–63.0)
Death			
No	27	69.2	
Death of melanoma	8	20.5	
Death of other cause	3	7.7	
Unknown (melanoma and concomitant cancer)	1	2.6	
Breslow thickness regarding the sLN status, mean (range), mm			
In sLN negative	18		3.5 (1.1–10.0)
In sLN positive	14		3.7 (1.1–20.0)

Abbreviations: ALM, acral lentiginous melanoma; NM, nodular melanoma; sLN, sentinel lymph node; SSM, superficial spreading melanoma.
[1]Considered as NM.
[2]Considered as ALM in Figures 2 and 3.
[3]One patient was censured because of missing data.
[4]Breslow < 1 mm.

data support a relationship between the percentage of IL4I1$^+$ cells and/or their location near tumor cells and the tumor aggressiveness.

IL4I1 is expressed by CD14$^+$ macrophages and Tregs

Next, we focused on characterizing IL4I1-expressing cells in melanoma, based on what have been described in TAM and in vitro Tregs—two crucial contributors to tumor escape. We selected eight tumors that exhibited a significant proportion of CD14$^+$ TAM and stained them for IL4I1 (Figure 4a). After quantification of the full biopsy samples (see Supplementary Figure S2e, left panel online), we found approximately 4% IL4I1$^+$ cells (see Supplementary Figure S2e, right panel), in line with our previous data (Carbonnelle-Puscian et al., 2009). In contrast, very few CD11c$^+$ dendritic cells were also IL4I1$^+$ (data not shown).

It was recently found that IL4I1 mRNA induced Tregs in vitro (Scarlata et al., 2015). Here, we assessed whether melanoma-infiltrating Tregs express IL4I1. By immunofluorescence, we detected up to ten Tregs positive for IL4I1 in three out of nine biopsy samples tested (Figure 4b). Therefore, IL4I1 expression is not restricted to TAMs but can be also detected in rare Tregs.

IL4I1 expression is related to a tumor microenvironment enriched in Tregs and poor in granzyme B-positive CD8$^+$ T cells in primary melanoma

A meta-analysis reported a significant correlation between high FoxP3$^+$ Treg infiltration and shorter OS in melanoma (Shang et al., 2015). We recently showed that IL4I1 potentiates the in vitro differentiation of human naïve CD4$^+$ T cells into Tregs (Cousin et al., 2015). We thus hypothesized that IL4I1 contributes to Treg accumulation in the tumor microenvironment. Biopsies poor in IL4I1$^+$ cells had few Tregs (FoxP3$^+$ cells), whereas those rich in IL4I1$^+$ cells exhibited massive Treg infiltration (Figure 5a). Accordingly, the quantification highlighted a direct correlation between the proportion of IL4I1$^+$ and FoxP3$^+$ cells in the 39 patients' biopsy

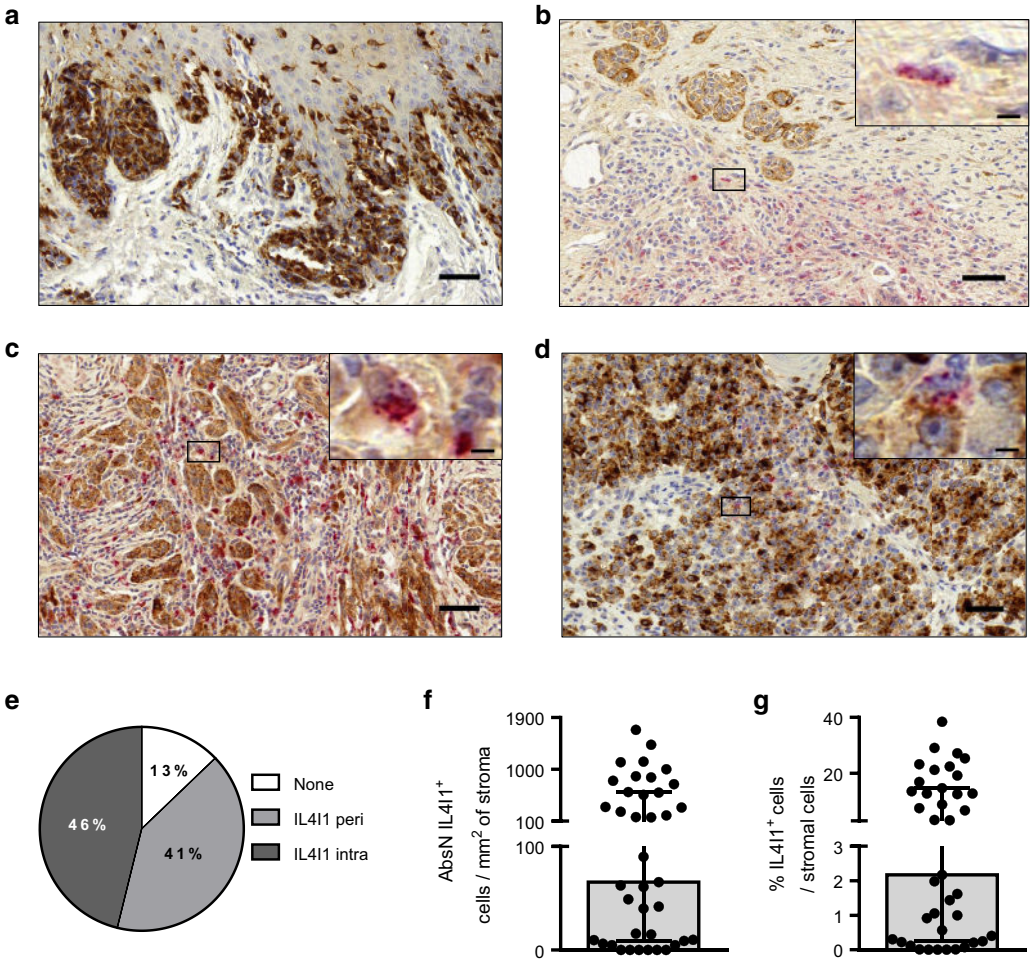

Figure 1. Localization and quantification of IL4I1$^+$ cells in primary cutaneous melanoma. Thirty-nine primary melanoma biopsy samples were stained with anti-IL4I1 (red) and anti-gp100 (brown) monoclonal antibodies. Representative immunohistochemistry according to the presence and location of IL4I1$^+$ cells: (**a**) no IL4I1 infiltrate, ALM; (**b**) IL4I1$^+$ cells localized mainly in the stromal area (peritumoral), SSM; (**c**) IL4I1$^+$ cells adjacent to tumor cells (intratumoral), NM; and (**d**) IL4I1$^+$ cells adjacent to tumor cells (intratumoral), SSM. Inset in **b**–**d**: IL4I1 granules in detail. (**a**–**d**) Scale bars = 50 μm, inset scale bars = 5 μm. (**e**) Proportion of patients according to location of IL4I1 staining. (**f**) Absolute number of IL4I1$^+$ cells/mm^2 of stromal tissue and (**g**) proportion of IL4I1$^+$ cells among stromal cells. (**f, g**) Data are shown as the median with the interquartile range. AbsN, absolute number; intra, intratumoral; NM, nodular melanoma; peri, peritumoral; SSM, superficial spreading melanoma.

samples (Spearman r = 0.7492) (Figure 5b). The co-localization of FoxP3 and IL4I1 signals illustrated by visible immunohistochemistry staining in Figure 5a (lower panel) further supports a direct role of IL4I1 in Treg recruitment in situ.

We previously showed, in mouse models of melanoma, that IL4I1 inhibits proliferation and anti-tumor function of CD8$^+$ T cells (Bod et al., 2017; Lasoudris et al., 2011). In humans, cutaneous melanomas highly infiltrated by IL4I1$^+$ cells exhibit a low density of CD8$^+$ T cells (Bod et al., 2017), suggesting that IL4I1 reduces anti-tumor CD8$^+$ T-cell activity. This complicated the analysis of the impact of IL4I1 in CD8$^+$ T-cell function. To circumvent this difficulty, we selected nine patients exhibiting both significant IL4I1 and CD8 signals on visible immunohistochemistry analysis. Then we used granzyme B (GrB) expression as a readout for CD8$^+$ T-cell cytotoxicity and compared the proportion of GrB$^+$ CD8$^+$ T cells by immunofluorescence staining in areas either poor or rich in IL4I1 (Figure 5c). As expected, we did not detect any CD8$^+$ T cells in the IL4I1-enriched area from two patients. After quantification (see Supplementary Figure S2f, left panel), we found that cytotoxic CD8$^+$ T cells were significantly less frequent in IL4I1-enriched areas (n = 14, 7 patients, 1–3 areas/patient) compared with IL4I1-poor areas (n = 15, 9 patients, 1–3 areas/patient) (P = 0.0016) (Figure 5c and d). We observed this difference for six of the seven patients for whom both rich and poor areas can be compared (see Supplementary Figure S2f, right panel).

DISCUSSION

Here, we focused our investigation on the putative role of IL4I1 expression in primary melanoma, in the search for a new predictive marker for patient outcome. Our findings, obtained retrospectively on 39 patients, show that patients either with ulcerated, Clark V level, or stage IIIB–D (the worst in our series) melanoma or with a positive sLN (i.e., advanced melanoma) displayed the highest proportion of tumor-infiltrating IL4I1$^+$ cells. Those who died of melanoma exhibited twice as many IL4I1$^+$ cells than those who were still alive at the end of follow-up. Statistical analysis did not

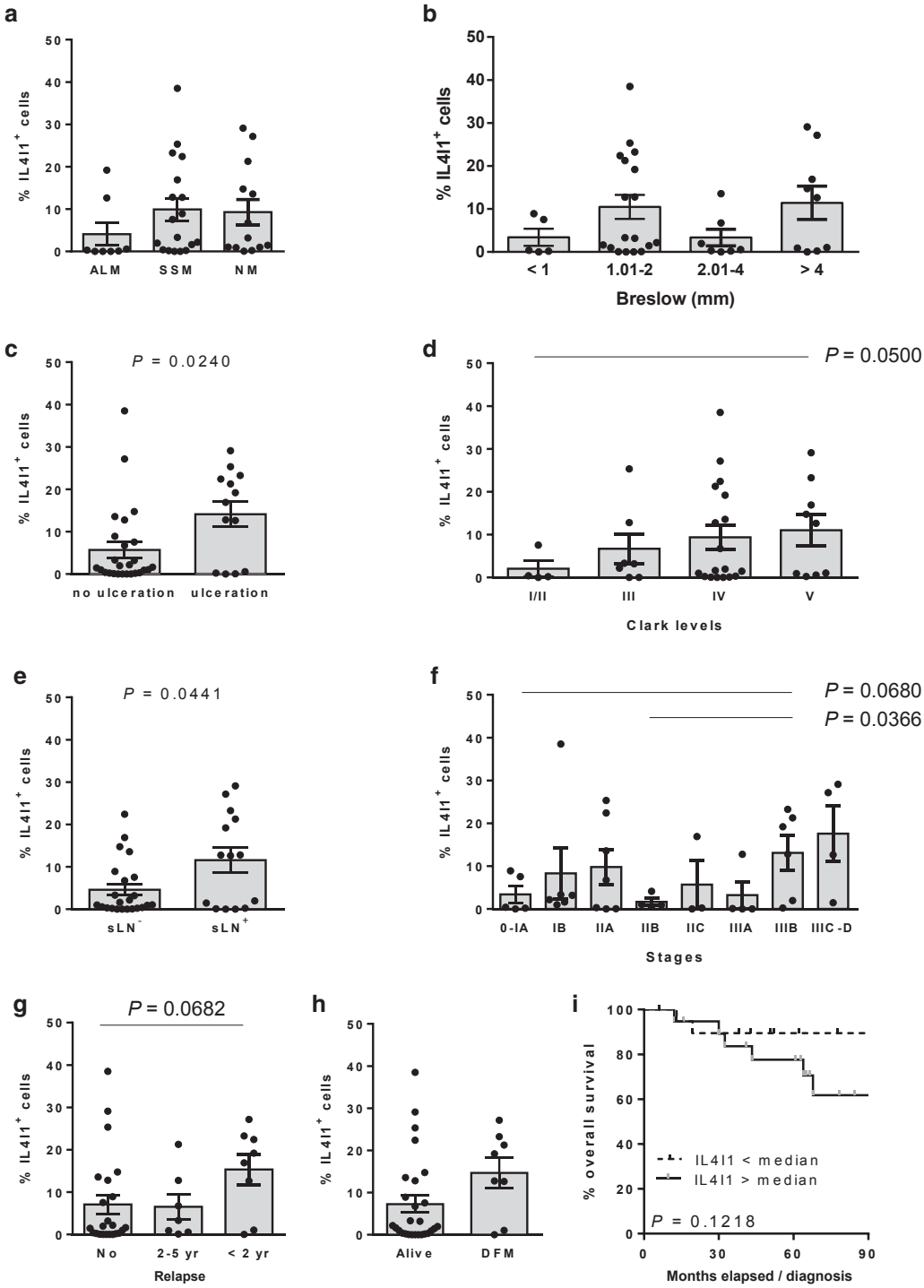

Figure 2. Correlation between clinical data and proportion of IL4I1$^+$ cells. Proportion of stromal IL4I1$^+$ cells (n = 39) according to (**a**) histological type, (**b**) Breslow thickness, (**c**) ulceration, (**d**) Clark levels, (**e**) sLN status, (**f**) stage, (**g**) time to relapse, (**h**) death from melanoma, and (**i**) overall survival. Patients (**e**) with unknown sLN status or (**h, i**) who died of causes unrelated to melanoma are censored. Data in **a**–**h** are shown as the mean ± standard error of the mean. *P*-values were calculated by (**c, d, e, f, h**) unpaired *t* test, or (**i**) log-rank statistics. DFM, death from melanoma; sLN, sentinel lymph node; yr, year.

show a significant relationship with Breslow thickness, which is the main pathological prognostic factor. This may be related to the fact that the mean thickness was similar in patients with positive and negative sLNs (Table 1). In addition, patients with sLN$^+$ and stage IIIB–D melanoma had more IL4I1$^+$ cells near tumor cells than those with negative sLNs and stage I–IIIA primary tumors. Altogether, our data suggest that increased proportions of IL4I1$^+$ cells and their intratumoral location are associated with a poorer prognosis within primary melanoma.

Previous studies have pointed to the dual role of IL4I1 in human cancer. Indeed, high IL4I1 transcript levels were associated with a poorer prognosis in breast cancer (Finak et al., 2008), particularly in its most aggressive form (triple

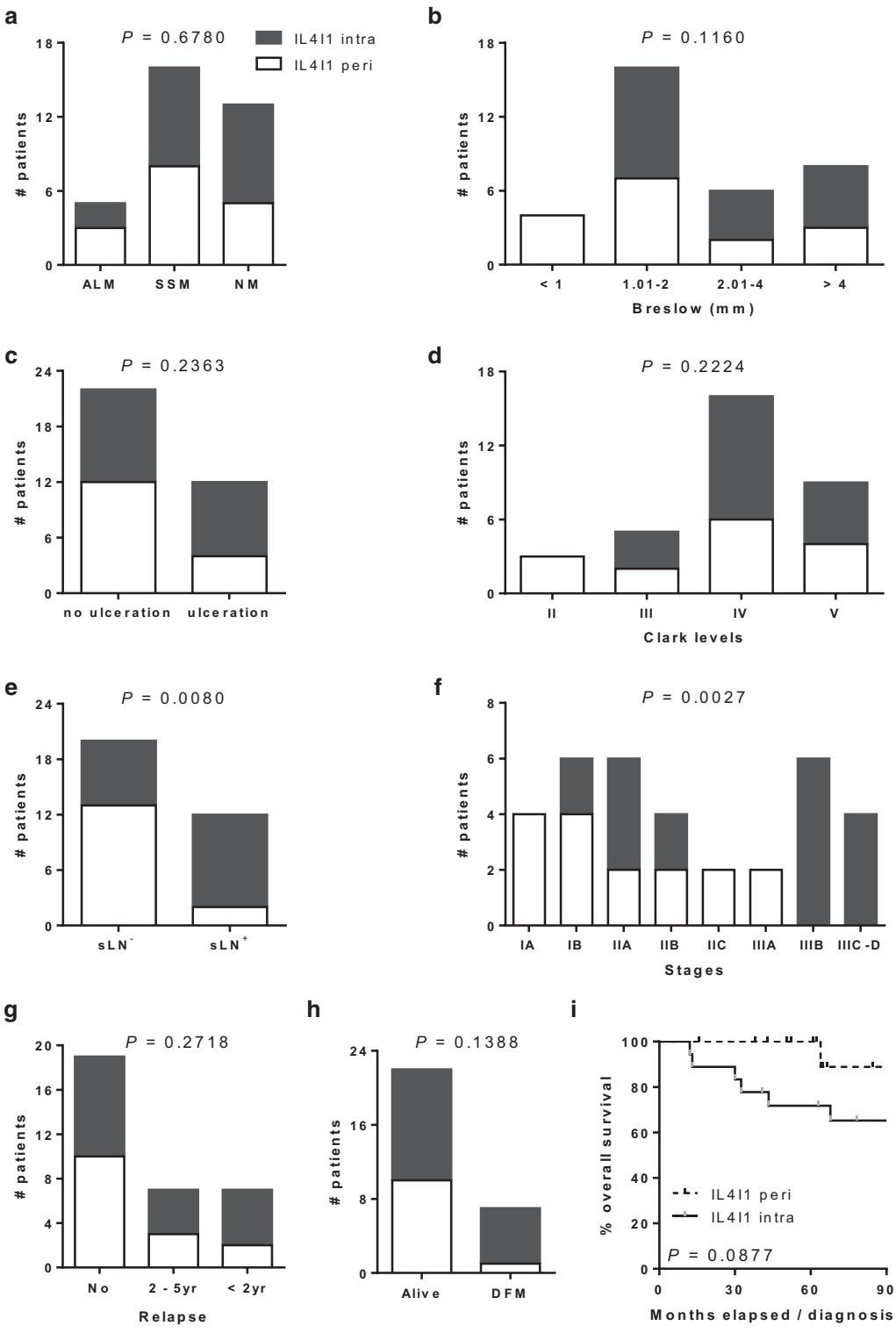

Figure 3. Intratumoral IL4I1$^+$ cell localization correlates with a poor outcome. Proportion of patients (n = 34) with either peritumoral or intratumoral localization of IL4I1$^+$ cells according to (**a**) histological type, (**b**) Breslow index, **(c)** ulceration, **(d)** Clark levels, (**e**) sLN status, (**f**) stages, (**g**) time to relapse, (**h**) DFM and (**i**) overall survival. Patients (**e**) with unknown sLN status or (**h, i**) who died from causes unrelated to melanoma are censored. *P*-values were calculated by (**a, c, e, h**) chi-square test, (**b, d, f, g**) chi-square test for trend, or (**i**) log-rank statistics. DFM, death from melanoma; intra, intratumoral; peri, peritumoral.

negative) (Komatsu et al., 2013). We showed that high IL4I1 levels were associated with a better outcome in follicular B-cell lymphoma (Carbonnelle-Puscian et al., 2009; Copie-Bergman et al., 2003). This protective effect is likely due to IL4I1 expression by tumor B cells, the neoplastic counterpart of normal germinal center B cells also overexpressing IL4I1 messengers (Caron et al., 2009). This result is consistent with our recent data showing that IL4I1 finely tunes B-cell physiology (Bod et al., 2018). In contrast, IL4I1 may essentially exert indirect inhibitory functions on the anti-tumor T-cell response in carcinoma, where it is among others expressed by TAM, as confirmed here in melanoma.

Expression of other amino acid-catabolizing enzymes, such as indoleamine 2,3-dioxygenase and inducible nitric oxide synthase, has been reported in primary cutaneous melanoma. Indoleamine 2,3-dioxygenase positivity in

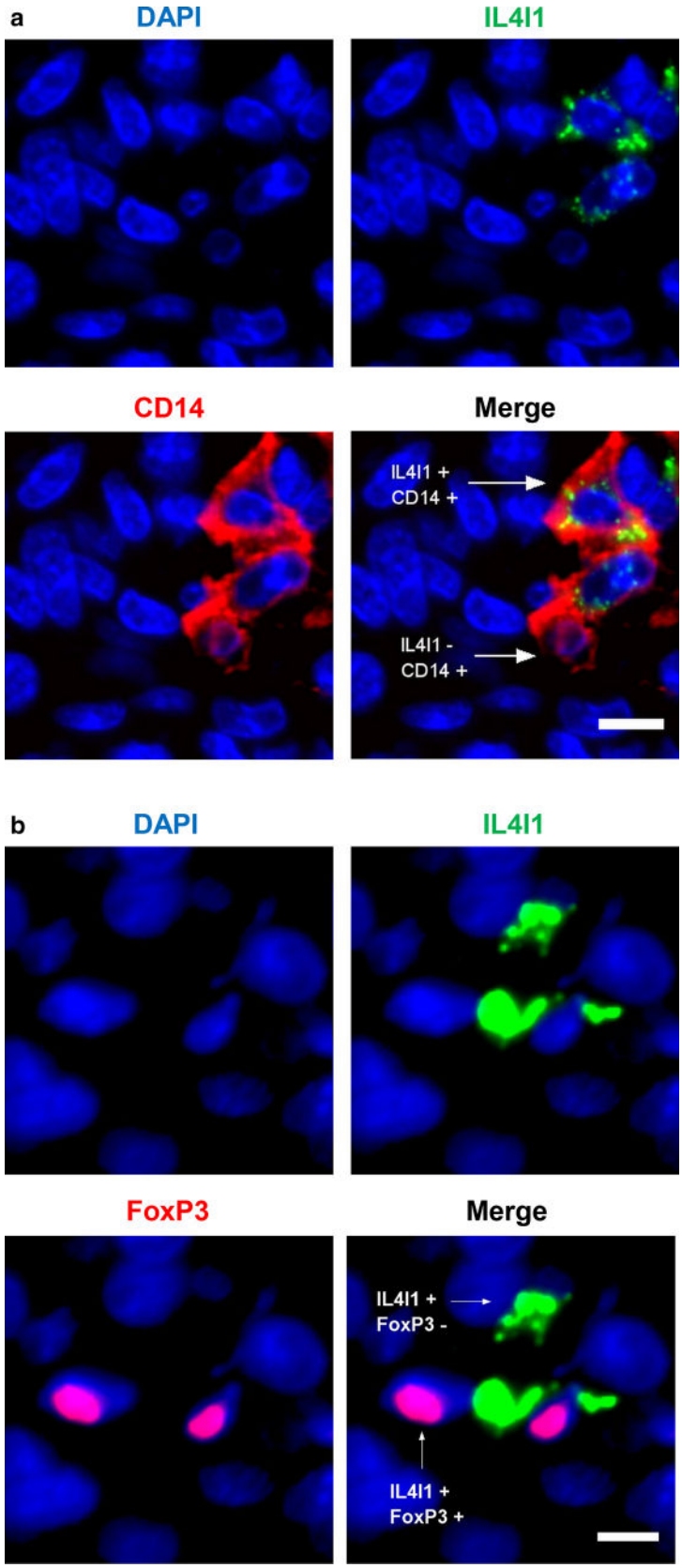

Figure 4. CD14$^+$ macrophages and Tregs express IL4I1. (**a**) Representative staining with anti-IL4I1 (green) and anti-CD14 (red) monoclonal antibodies. (**b**) Representative staining with anti-IL4I1 (green) and anti-FoxP3 (red) monoclonal antibodies. The white arrows show three IL4I1$^+$ cells, (**a**) positive for CD14 and (**b**) either positive or negative for FoxP3. Scale bars = 5 μm. Treg, regulatory T cell.

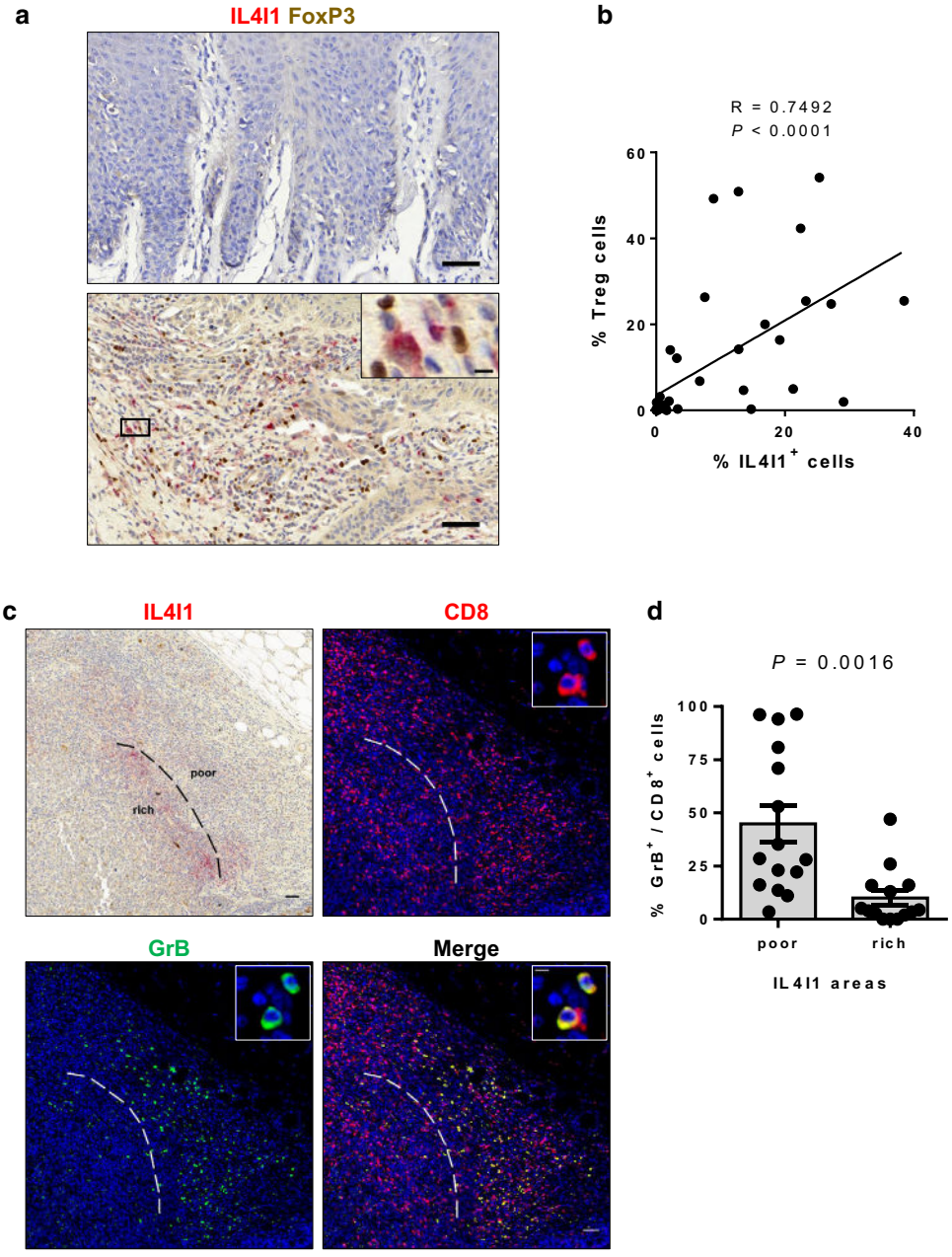

Figure 5. IL4I1 expression is related to a tumor microenvironment enriched in Tregs and poor in cytotoxic CD8$^+$ T cells. (**a**) Representative immunohistochemistry using anti-IL4I1 (red) and anti-FoxP3 (brown) monoclonal antibodies on melanoma biopsy samples poor (upper) and rich (lower) in IL4I1. (**b**) Correlation of FoxP3$^+$ T-cell and IL4I1$^+$ cell proportions among the stromal area. Each dot represents one patient's quantification (Spearman correlation). (**c**) Immunohistochemistry showed zones rich and poor in IL4I1 delimited by a line. Immunofluorescence was performed using anti-CD8 and anti-GrB monoclonal antibodies. (**a, c**) Scale bars = 50 μm, inset scale bars = 5 μm. (**d**) Quantification of CD8$^+$ T cells positive for GrB in IL4I1 poor (n = 15) and rich (n = 14) zones. Data are shown as mean ± standard error of the mean. *P*-value was calculated using unpaired *t* test. GrB, granzyme B; Treg, regulatory T cell.

peritumoral endothelium is associated with a negative prognosis (Chevolet et al., 2014). The high expression of inducible nitric oxide synthase in invasive primary melanoma is a strong predictor of disease-specific survival and OS for melanoma patients (Ekmekcioglu et al., 2000, 2006). Our findings strongly suggest that IL4I1 expression in primary cutaneous melanoma is associated with poor prognosis and could constitute a new (independent) prognostic factor. Data from a longer follow-up period and/or a larger series of patients would be helpful to strengthen this result.

Furthermore, our findings provide insights into the impact of IL4I1 on shaping two T-cell subsets (cytotoxic CD8$^+$ T cells and Tregs) in primary melanoma. Infiltration of melanomas by activated GrB$^+$ CD8$^+$ T cells has been associated with a positive prognosis (van Houdt et al., 2008). IL4I1-enriched zones in our biopsy samples were mostly infiltrated by GrB$^-$CD8$^+$ T cells. We have recently shown a low CD8$^+$ T-cell infiltrate in IL4I1-rich primary melanomas (Bod et al., 2017). These observations suggest that IL4I1 can affect both the proliferation and function of this subset. This is supported by several of our previous results: (i) IL4I1 inhibits in vitro human T-cell proliferation by down-regulating TCR signaling (Aubatin et al., 2017; Boulland et al., 2007), (ii) the in vivo escape of B16 melanoma overexpressing IL4I1 is accompanied by reduced expansion of cytotoxic anti-tumor CD8$^+$ T cells (Lasoudris et al., 2011), and (iii) genetic inactivation of IL4I1 in the mouse Ret model of spontaneous melanoma leads to an increased infiltration of cytotoxic CD8$^+$ T cells in primary tumors (Bod et al., 2017).

Despite conflicting results, a high density of tumor-infiltrating FoxP3$^+$ Tregs has generally been associated with shorter disease-free survival and OS (Gerber et al., 2014;

Knol et al., 2011; Miracco et al., 2007; Shang et al., 2015). Here, we show a correlation between the proportion of Tregs and IL4I1-expressing cells in primary melanoma. In addition, we detected rare IL4I1$^+$ Tregs, consistent with the recent identification of an ex vivo, naturally induced IL4I1$^+$ Treg subset (Scarlata et al., 2015). Our data support the contribution of IL4I1 to Treg accumulation within the tumor microenvironment. Thus, the intratumoral enrichment of IL4I1 may negatively affect the outcome of melanoma patients by affecting both tumor-infiltrating Tregs and CD8$^+$ T cells.

The cellular and molecular mechanisms responsible for IL4I1 expression in the tumor microenvironment remain unexplored. Consistent with previous studies showing that various inflammatory signals induce IL4I1 expression in macrophages/dendritic cells (Marquet et al., 2010) and Tregs (Scarlata et al., 2015), cancer-associated inflammation might contribute to IL4I1 expression. Similarly to what happens for indoleamine 2,3-dioxygenase and PD-L1, chronic activation of a T helper type 1 response in the tumor microenvironment may lead to IL4I1 expression by TAMs. Nevertheless, the characterization of the mechanisms (i.e., cell-cell contact, inflammatory cytokines) inducing IL4I1 in the melanoma microenvironment requires further investigations.

Although based on 39 patients, this study suggests that IL4I1 modifies the composition and antitumor properties of the immune infiltrate of melanoma, thus facilitating tumor escape from the immune response. Investigation of IL4I1 expression in primary melanoma at diagnosis may represent a new tool for predicting patient prognosis and should be addressed in further studies. The future development of adjuvant therapies targeting IL4I1 may also be considered.

MATERIALS AND METHODS

Patient and biopsy sample characteristics

Primary cutaneous tumors were retrospectively obtained from 39 melanoma patients recruited between 2006 and 2012 at the Cochin Hospital (Paris, France). Patients with stage I–III disease were included. Biopsy samples from melanoma exereses were routinely fixed and embedded in paraffin (n = 34) or frozen (n = 5). The study (Protocol IMMUMELA) was approved by the Ethics Committee Ile de France Comité de Protection des Personnes (CPP) Ile de France III (Am5937-3-2834) (EUDRACT 2010-A00838-31). The Declaration of Helsinki protocols were followed. All included patients or their families gave written informed consent. The clinical characteristics of the patients are detailed in Table 1.

Histological staining and microscopy

Sections of melanoma biopsy samples (4 μm thick) were mounted on slides (Superfrost plus, Microm Microtech, Brignais, France). Slides were stained with the following antibodies: rabbit anti-human IL4I1 (clone 43.7, developed by FC and VMF), anti-gp100 (clone HMB45; Dako, Carpinteria, CA), anti-FoxP3 (clone 259D; BioLegend, San Diego, CA), anti-CD14 (clone BS9; Nordic BioSite, Stockholm, Sweden), anti-CD8α (clone 4B11; Novocastra, Newcastle upon Tyne, UK), and anti-GrB (clone GrB-7, Dako).

Briefly, after deparaffinization, heat-induced epitope retrieval in a solution of high pH, blocking of endogenous peroxidase activity, and immunostaining were performed by a fully automated Leica (Hesse, Germany) BondIII stainer, according to the manufacturer's recommendations. Double staining (IL4I1/gp100, IL4I1/FoxP3) was shown by poly-horseradish peroxidase (brown) and poly-alkaline phosphatase (red) (chromoplex Leica).

We observed no staining of parental HEK 293 cells with the mAb specific for IL4I1, whereas strong granular staining was detected in these cells transfected with the human *IL4I1* gene (see Supplementary Figure S3a and b online). IL4I1 was shown with high sensitivity in a human reactive lymph node where tingible body macrophages of germinal centers were labeled (see Supplementary Figure S3c). We next verified the sensitivity and specificity of clone 43.7 using normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Dallas, TX) as control on primary melanoma biopsy samples, as illustrated in Supplementary Figure S3d and e.

For immunofluorescence staining, slides were incubated with primary antibodies for 1 hour at room temperature. Double staining (IL4I1/CD14, IL4I1/FoxP3, CD8/GrB) was shown using appropriate secondary antibodies conjugated to a fluorochrome (goat anti-rabbit AF488 and goat anti-mouse AF647 [Jackson ImmunoResearch, West Grove, PA] and goat anti-mouse AF546 (Thermo Fisher Scientific, Waltham, MA) for 30 minutes at room temperature. Slides were counterstained with DAPI (Thermo Fisher Scientific).

Image acquisition was performed on a PerkinElmer (Waltham, MA) Multilabel Lamina Slide Scanner, either in the fluorescence (magnification, ×40) or brightfield (magnification, ×20) scan mode.

Immunohistochemical analysis

Visible HMB45 signal was semi-automatically counted in the full biopsy samples using the Trainable Tissue Segmentation algorithm (tissue segmentation map) of inForm software (PerkinElmer) to score the density of HMB45$^-$ stromal area and of HMB45$^+$ tumoral area, as illustrated in Supplementary Figure S1a–d.

Next, visible IL4I1, FoxP3, and nuclear signals were semi-automatically counted in the full biopsy samples using the Counting Objects algorithm (cell segmentation map) of inForm without knowledge of the clinical data. The density of IL4I1$^+$ cells corresponds to their absolute cell number per mm^2 of stromal tissue. The proportion of Tregs and IL4I1$^+$ cells corresponds to the ratio between the absolute number of cells of interest and absolute number of stromal cells (nuclei count) × 100.

IL4I1$^+$ cells were located either within intratumoral (mainly in close contact with tumor cells) or peritumoral (mainly in the tumor stroma) areas. Fluorescent CD14 and IL4I1 signals were semi-automatically counted in the full biopsy samples of eight patients to score the proportion of double-positive cells using the Counting Objects algorithm of inForm, as shown in Supplementary Figure S2. Biopsy samples from nine patients were selected for the presence of IL4I1$^+$ and CD8$^+$ cells, and fluorescent CD8, GrB, and nuclear signals were counted in two to six fields per section using the approach mentioned. The proportion of GrB$^+$CD8$^+$ T cells was quantified with respect to the density of IL4I1$^+$ cells.

Statistics

Analyses were performed using Prism 6 software (GraphPad, La Jolla, CA). Associations between clinical data and IL4I1 categories (location or percentage in stroma) were analyzed by the chi-square test or the chi-square test for trend and unpaired *t* test. Patients for whom the sLN procedure was not applicable (Breslow thickness < 1 mm) were considered to be sLN negative (n = 5) (Table 1), whereas patients with a Breslow thickness greater than 1 mm, but unknown sLN status (n = 2), were censured in Figure 2e and 3e. The time to first relapse was defined from the time of diagnosis to that of the first

detected metastasis. Only deaths due to melanoma were considered. Deaths from other or unknown causes were treated as censored events at the date of death for the log-rank analysis. The correlation of CD8, GrB, and IL4I1 infiltrates was analyzed using unpaired *t* tests. The correlation between the proportion of IL4I1 and Tregs was analyzed using the nonparametric Spearman test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.06.178.

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