Plasmacytoid Dendritic Cells Support Melanoma Progression by Promoting Th2 and Regulatory Immunity through OX40L and ICOSL

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Abstract

Even though melanoma is considered to be one of the most immunogenic solid tumors, handling its development remains a challenge. The basis for such escape from antitumor immune control has not yet been documented. Plasmacytoid dendritic cells (pDC) are emerging as crucial but still enigmatic cells in cancer. In melanoma, the function of tumor-infiltrating pDCs remains poorly explored. We investigated the pathophysiologic role of pDCs in melanoma, both ex vivo from a large cohort of melanoma patients and in vivo in melanoma-bearing humanized mice. pDCs were found in high proportions in cutaneous melanoma and tumor-draining lymph nodes, yet associated with poor clinical outcome. We showed that pDCs migrating to the tumor microenvironment displayed particular features, subsequently promoting proinflammatory Th2 and regulatory immune profiles through OX40L and ICOSL expression. Elevated frequencies of interleukin (IL)-5-, IL-13- and IL-10–producing T cells in patients with melanoma correlated with high proportions of OX40L- and ICOSL-expressing pDCs. Strikingly TARC/CCL17, MDC/CCL22, and MMP-2 found in the melanoma microenvironment were associated with pDC accumulation, OX40L and ICOSL modulation, and/or early relapse. Thus, melanoma actively exploits pDC plasticity to promote its progression. By identifying novel insights into the mechanism of hijacking of immunity by melanoma, our study exposes potential for new therapeutic opportunities.

Introduction

Cancer cells deploy numerous strategies to escape from the host immune system taking advantage of its plasticity. In melanoma, despite a high immunogenicity, an adverse evolution is often observed due to establishment of an immunosuppressive microenvironment favoring tumor progression. The basis for such subversion of immunity in melanoma has not yet been documented. However, understanding the mechanisms underlying the lack of efficient antitumor immunity is crucial for the development of novel therapeutic approaches to prevent the negative outcome associated with metastatic disease.

Dendritic cells (DC) play an essential role in initiating and shaping antitumor immune responses (1–3). Among DC subsets, plasmacytoid dendritic cells (pDC) are key players in the regulation of immune responses (4, 5). Depending on their maturation state and on the microenvironment, pDCs can mediate immunity or tolerance. Indeed, pDCs are critical for the development of peripheral tolerance or induction of tolerance at mucosal sites (6). However, upon activation, pDCs have the ability to initiate protective immunity towards viruses through production of large amounts of type I IFN and proinflammatory cytokines. Such reactivity is based on the capacity of pDCs to sense viral nucleic acids through Toll-like receptors (TLR) 7 and 9 and trigger downstream signaling. Therefore pDCs display a high functional plasticity, being able to orient immunity towards multiple profiles depending on the surrounding signals (5, 7).

pDCs constitute an emerging intriguing immune cell in cancer. Indeed, pDCs play a pivotal role in antitumor immunity through their ability to process and cross-present tumor antigens to T cells (8), and subsequently induce adaptive immune responses (9). In the context of melanoma, pDCs have been shown to prime functional immune responses (9–11), display direct cytotoxic activity towards tumor cells through TRAIL expression (12) or lysozyme secretion (13), or once activated, potentially achieve melanoma tumor control through efficient priming of antitumor
responses (14–17). Infiltration of tumors by pDCs has been found in many types of cancers, yet associated with poor prognosis, especially in ovarian (18) and breast cancer (19). Functional alterations of pDCs have been identified (20–22) and related to the induction of immune tolerance leading to tumor progression (20, 23) and pDC-dependent angiogenesis induction (24). In melanoma, pDCs were found to be recruited to the tumor site and sentinel lymph nodes (9, 25–27). The infiltration of primary tumors by CD123+ cells has been linked with a poor prognosis (28). However, the mechanism by which pDCs promote the progression of melanoma tumors and the consequences of the specific features of pDCs on subsequent adaptive immunity remained unknown.

pDCs play a fundamental yet still enigmatic role in the control of tumor development. Their plasticity endorses them with a powerful ability to drive effective antitumor immunity but also with a potential to trigger tolerance and tumor progression. This challenging controversy prompted us to investigate the pathophysiologic role of pDCs in the context of melanoma. We delineated the role of pDCs both ex vivo from a large cohort of melanoma patients and in vivo in an innovative melanoma-bearing humanized mouse model (29). We showed that melanoma exploits pDCs for driving immune responses to its advantage. We identified for the first time a novel pDC-triggered pathway leading to tumor progression and early relapse in melanoma, which offers new opportunities to design effective antitumor therapies.

**Materials and Methods**

**Samples from melanoma patients and healthy donors**

Blood and tumor samples were obtained from 61 and 54 patients with melanoma respectively, stage I–IV. Clinical features are shown in Supplementary Tables S1 and S2. Blood samples were also obtained from 37 healthy donors. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque density-gradient centrifugation (Eurobio). Tumor samples were mechanically disintegrated and digested with 2 mg/mL collagenase-D (Roche) 20 U/mL DNase (Sigma). The resulting tumor-infiltrating cell suspensions were filtered and washed. Small whole tumor fragments (10 mm3) were incubated for 24 hours in complete RPMI-1640 10% FCS to generate tumor supernatants. These studies were carried out in accordance with European Union guidelines (86/609/CEE). French National Chart guidelines and protocols were approved by the Ethics Committee for Animal Experimentation of Grenoble.

**Humanized mice**

NOD-SCIDβ2m−/− immunodeficient mice (NOD.Cg-PrkdcScidβ2mTm1Wj/J) were purchased from Jackson ImmunoResearch Laboratories and bred at the Plateforme de Haute Technologie Animale (La Tronche, France). Humanized mice were constructed by intravenously xenotransplanting 1–2 × 106 human CD34+ HPCs into sublethally irradiated NOD-SCIDβ2m−/− mice (100–120 cGy). Four weeks later upon reconstitution of the human immune system, 10 × 106 human melanoma tumor cells were implanted subcutaneously into the flanks. Three days later, tumor, draining lymph nodes, control lymph nodes, spleen, and bone marrow were harvested. Organs were eventually digested with 2 mg/mL collagenase-D (Roche). Resultant cell suspensions were filtered and washed. Small whole tumor fragments (10 mm3) were also incubated for 24 hours in complete RPMI-1640 10% FCS to generate tumor supernatants. These studies were carried out in accordance with European Union guidelines (86/609/CEE). French National Chart guidelines and protocols were approved by the Ethics Committee for Animal Experimentation of Grenoble.

**Phenotypic analysis**

Cell suspensions were suspended in PBS 2% FCS and stained with anti-human antibodies (BD, Beckman). pDCs were identified as CD45+ HLA-DR+ BDCA2+ or Lin− HLA-DR− CD123+ and myeloid dendritic cells as Lin− HLA-DR− CD11c+ . The phenotype of pDCs was determined using anti-CD69, -OX40L, -GITRL, ICOSL, -PD-L1, -PD-L2, -CD40, -CD80, -CD86 antibodies and their isotype-matched controls. Suspensions were subjected to flow cytometry analysis using a FACSCalibur and CellQuest software (BD).

**Response to TLR-1 stimulation**

Cell suspensions were resuspended at 1 × 106/mL in complete RPMI-1640 10% FCS and cultured for 24 hours with 640U/HA/mL UV-formol–inactivated influenza virus strain A/H3N2/Wisconsin/67/05 (Sanofi-Pasteur) or CpG ODN-2336 (10 μg/mL, Coley Pharma). The activation phenotype of pDCs was analyzed using anti-CD40 and CD80, and -CD86 antibodies. Human soluble IFN-α, IP10, IL-6, and TNF-α production was measured in culture supernatants by a Cytometric Bead Array assay (CBA, BD). The proportion of pDCs was evaluated in the corresponding samples by flow cytometry using anti-HLA-DR and anti-BDCA2 labeling to calculate the relative cytokine production per pDC. The IFN-α production was also evaluated within pDCs after 6 hours of stimulation with TLR-1 in the presence of brefeldinA (1 μL/mL) for the last 3 hours followed by anti-HLA-DR and anti-BDCA2 surface labelling and intracellular IFN-α staining (BD).
Figure 1. pDCs are found in high proportions in cutaneous melanoma and DLN of tumor-bearing Hunice and melanoma patients and are associated with poor clinical outcome. A–D, cutaneous tumors, lymph node metastasis, and blood samples were obtained from melanoma patients or healthy donors (HD) and the proportion of pDCs was evaluated by flow cytometry. A, representative dotplots of HLA-DR⁺ BDCA2⁺ pDCs in each sample type (gated on CD45⁺ cells). B, percentages of pDCs found within CD45⁺ cells in cutaneous melanoma (n = 12), lymph node metastasis (n = 28), blood (n = 38) of patients and blood of healthy donors (n = 28). C, proportion of pDCs in blood of patients with melanoma corresponding to the stage of the disease at sampling time. D, comparative overall survival (OS) of patients with low (<0.4%) or high (>0.4%) tumor-infiltrating pDCs. Groups were separated using the median percentage of tumor-infiltrating pDCs: 0.4%. Comparison was done using log-rank test. E and F, humanized mice displaying a human immune system were transplanted with melanoma cells and analyzed 3 days later for the presence of pDCs at the tumor site, in DLN, control lymph nodes (CLN), spleen, and bone marrow (BM). E, representative dotplots of HLA-DR⁺ BDCA2⁺ pDCs in tumor and DLN (gated on CD45⁺ cells). F, percentages of pDCs found within CD45⁺ cells in tumor, DLN, CLN, spleen, and bone marrow (n = 46 Hunices). P values calculated using the Mann–Whitney test.
Figure 2. pDCs display a higher basal activation status at the tumor site compared with the DLN. The expression of CD62L (A and B) and costimulation molecules (C and D) was analyzed on pDCs found in cutaneous tumor, lymph node metastasis, and blood of patients with melanoma (A and D; n = 12, 28, and 38, respectively), blood of healthy donors (A, D; n = 20) as well as in the tumor, DLN, spleen, and bone marrow (BM) of melanoma-bearing Humice (B and C; n = 16). A and B, percentages of CD62L⁺ pDCs among pDCs in melanoma patients and healthy donors (A) and Humice (B). C, percentages of pDCs expressing CD40, CD80, and CD86 among pDCs in Humice. D, percentages of expression of CD40, CD80, and CD86 on pDCs in patients with melanoma compared with healthy donors. P values were calculated using the Mann–Whitney test.
Figure 3. pDCs from the melanoma environment responded to TLR-L stimulation. Tumor-infiltrating cells or PBMC from patients with melanoma or healthy donors (HD) were stimulated with TLR7-L (Flu virus) or TLR9-L (CpGA). Expression of costimulatory molecules and cytokine secretion was evaluated 24 hours later. A, percentages of expression (top) and mean fluorescence intensity (MFI; bottom) of CD40 and CD86 on pDCs from tumor (n = 13–15) or blood (n = 26) of patients with melanoma compared with healthy donors (HD; n = 19). B, IFN-α and IP10 were measured in the supernatants 24 hours later. Results were standardized by calculating the cytokine production per pDC in each sample (n = 33, 19, and 19, respectively). C, IFN-α intracellular labeling was performed within pDCs from melanoma patients or healthy donors (n = 25, 30, and 22, respectively). P values were calculated using the two-way RM ANOVA test (straight line) or the Mann–Whitney test (dotted lines). *, P < 0.05; **, P < 0.01; *** P < 0.001.
Figure 4. pDCs from the melanoma environment drive a Th2 proinflammatory and regulatory profile. A and B, cell suspensions (A, \(n = 15–21\)) or purified pDCs (B, \(n = 15–21\)) from tumor, DLN, spleen, and bone marrow (BM) of melanoma-bearing Humice were cocultured with allogeneic naïve CD4 T cells. The cytokine profile was evaluated 5 days later in the supernatants. C, pDCs purified from lymph node (LN) metastasis (\(n = 17\) tumor samples) and blood samples (\(n = 28\)) of patients with melanoma were cocultured with allogeneic naïve CD4 T cells. The cytokine profile was evaluated 5 days later in the supernatants. \(P\) values were calculated using the Mann–Whitney test.
A

LN metastasis patient

Blood patient

Blood HD

CD4

IL-5 1.41% 5.85%

IL-13 0.46% 0.09%

IL-10 0.02% 0.16%

CD8

IL-5 1.86% 11.7%

IL-13 2.07% 2.02%

IL-10 1.1% 0.68%

CD3

0.02%

IL-10

0.16%

IL-5

0.09%

IL-13

0.41%

CD8

0.17%

IL-10

0.15%

IL-5

0.46%

IL-13

1.29%

CD4

0.96%

IL-10

5.85%

B

CD4 T cells

% IL-5+ CD4 T cells

% IL-13+ CD4 T cells

% IL-10+ CD4 T cells

CD8 T cells

% IL-5+ CD8 T cells

% IL-13+ CD8 T cells

% IL-10+ CD8 T cells

LN metastasis patient

Blood patient

Blood HD

C

% FoxP3+ CD4+ CD8+ T cells

LN metastasis patient

Blood patient

Blood HD

(Legend for Fig. 5 on following page.)
Coculture of pDCs/naive CD4 T cells for determination of Th profile

pDCs were purified from patients’ or healthy donors’ cell suspensions using the EasySep Human pDC enrichment kit (StemCell; purity >92%) and from cell suspensions from humanized mice using the human BDC4+ selection kit (Miltenyi; purity 70–98%). Human naïve CD4 T cells were purified from cord blood using the Human Naïve Enrichment Kit (StemCell; purity 97–99.5%). pDCs and naïve CD4 T cells were cultured in a 1:10 ratio for 5 days. T cells (1 × 10^6 cells/mL) were restimulated for 16 hours with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (Sigma). In some experiments, anti-OX40L blocking antibodies, goat IgG control antibodies (10 μg/mL; R&D systems), anti-ICOSL blocking antibodies, or mouse IgG control antibodies (1 μg/mL; eBiosciences) were added during the pDC/T cocultures. Culture supernatants were collected at day 5 and after 16 hours of restimulation. IL-5, IL-13, TNF-α, IL-10, and IFN-γ cytokines were quantified by a Cytometric Bead Array assay (BD).

Intracellular cytokine staining of T cells

Cell suspensions (1 × 10^6/mL) were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin (Sigma) for 5.5 hours in the presence of brefeldinA (1 μM/L) for the last 3 hours. Cells were then surface labeled with anti-CD4, -CD8, and -CD3 antibodies, and intracellular cytokine staining was performed using anti-IL-5, IL-13, -IL-10 and IFN-γ antibodies (BD).

Quantification of chemokines/Th2-prone factors in tumor supernatants

CCL17/thymus and activation-regulated chemokine (TARC), CCL22/macrophage-derived chemokine (MDC), thymic stromal lymphopoietin (TSLP), and matrix metalloproteinase-2 (MMP-2) were quantified in tumor supernatants derived from patients with melanoma or melanoma-bearing humic by ELISA (R&D systems).

Modulation of pDCs by rhCCL17/TARC, rhCCL22/MDC, and rhMMP-2

pDCs were purified from blood of healthy donors and cultured in the presence of IL-3 (10 ng/mL) and increasing concentrations of rhTARC, rhMDC, or rhMMP-2. The level of expression of OX40L and ICOSL on pDCs was assessed 24 hours later using flow cytometry.

Statistical analysis

The statistical analyses were performed by Prism software using the Mann–Whitney test, the nonparametric U test, the Wilcoxon matched t test, the one-way ANOVA, the two-way RM ANOVA, the Spearman correlation, and the log-rank test.

Results

pDCs are found in high proportions in cutaneous tumors and tumor-draining lymph nodes and are associated with a poor prognosis

We first evaluated the proportion of pDCs in tumors and blood samples of patients with melanoma (Supplementary Tables S1 and S2) compared with healthy donors. We found that pDCs infiltrated melanoma tumor tissue, with a higher proportion of pDCs in cutaneous tumors compared with lymph node metastasis and blood (Fig. 1A and B). Moreover, when classified according to disease stage, patients at advanced stage III–IV melanoma displayed a lower frequency of circulating pDCs compared with patients at early stage I–II (Fig. 1C), suggesting a preferential recruitment of pDCs to the tumor site. Strikingly, we found that tumor-infiltrating pDCs were significantly associated with a poor clinical outcome, as the proportion of tumor-infiltrating pDCs was correlated with the Breslow level (Supplementary Fig. S1), and most importantly, a high proportion of pDCs was correlated with shorter survival (Fig. 1D). To better investigate the crucial role of pDCs in human melanoma, we developed a melanoma-bearing humanized mouse model (29) based on immunodeficient mice reconstituted with a human immune system following HCD34+ HPC transplantation, and subsequently engrafted with human melanoma (Supplementary Fig. S2). In this model, human pDCs were able to rapidly and massively migrate to human melanoma tumors and tumor-draining lymph nodes (DLN; Fig. 1E and F). In contrast, myeloid dendritic cells (mDC) represented a minor population among infiltrating cells (Supplementary Fig. S3A and S3B). Together, these data illuminated a preferential attraction of pDCs by melanoma and a relationship between the presence of pDCs at the tumor site and poor clinical outcome.

pDCs from the draining lymph nodes display a poor basal activation status compared with the cutaneous tumor site but exhibit an unaltered functionality with respect to TLR triggering

To understand how tumor-infiltrating pDCs could lead to early relapse, we examined the migratory profile and basal activation status of melanoma-infiltrating pDCs. Interestingly, we observed in patients with melanoma a higher proportion of CD662L-expressing pDCs in blood compared with sites of tumor metastasis or with circulating pDCs of healthy donors (Fig. 2A), suggesting the mobilizing potential of circulating pDCs in patients. In melanoma-bearing humice, allowing a more precise
Figure 6. OX40L and ICOSL expression by pDCs is respectively responsible for the Th2-biased and regulatory-prone immunity in melanoma. A, percentages of expression of OX40L, ICOSL, GITRL, PDL1, and PDL2 on pDCs from tumor, DLN, spleen, and bone marrow (BM) of melanoma-bearing Humice (n = 9–17 Humice). B, percentages of expression of OX40L and ICOSL on pDCs from cutaneous tumors and lymph node (LN) metastasis or blood of melanoma patients compared with pDCs from healthy donors (HD; n = 22–33 TIL, 27–49 PBMC patients, 17–20 PBMC healthy donors).
analysis of the tumor site and its associated DLN, we found a higher proportion of CD62L-expressing pDCs at the tumor site compared with the DLN and spleen (Fig. 2B), suggesting a mobilization of pDCs to the tumor site followed by their migration to the DLN. Moreover, a higher proportion of pDCs expressing CD40, CD80, and CD86 was found at the tumor site compared with DLN and spleen in melanoma-bearing Humice (Fig. 2C) and in cutaneous tumors compared with sites of lymph node metastasis in patients with melanoma (Fig. 2D). Together, these results clearly indicate that pDCs display a poor activation status when reaching DLN compared with the tumor site.

We further investigated whether melanoma could affect the ability of pDCs to respond to TLR-L stimulation, potent activators revealing pDCs’ fitness. pDCs from tumors and blood of patients with melanoma upregulated the costimulatory molecules CD40 and CD86 in response to TLR7-L and TLR9-L stimulation in a similar manner to samples from healthy donors (Fig. 3A). pDCs from the tumor site appeared slightly impaired in their capacity to secrete IFN-α and IP10 in response to TLR9-L stimulation compared with pDCs from patients’ blood, but their response to TLR7-L was not affected (Fig. 3B). In addition, when we performed intracellular staining of IFN-α within pDCs, the frequency of cytokine-producing pDCs at the tumor site was higher than that found in the blood of patients or of healthy donors (Fig. 3C).

Therefore, whatever their activation status, pDCs from the melanoma microenvironment remain fully functional with regard to TLR triggering.

pDCs from the melanoma environment drive a Th2 proinflammatory and regulatory profile

Next, we determined how such tumor-induced features of pDCs affected subsequent aspects of adaptive immunity. In melanoma-bearing Humice, we observed a dramatic production of IL-5, IL-13, and TNF-α in cocultures of naïve CD4 T cells with DLN suspensions (Fig. 4A). We confirmed that such a profile was directly driven by pDCs as this response was observed using purified pDCs (Fig. 4B). Notably, in patients with melanoma, only pDCs isolated from tumor metastasis caused naïve CD4 T cells to secrete IL-5, IL-13, TNF-α, and IL-10 (Fig. 4C). Furthermore, such an immune profile could be seen directly in vivo as we found higher frequencies of IL-10- and IL-13-producing CD4 and CD8 T cells in tumor metastasis and blood of melanoma patients compared with that of healthy donors, and a higher frequency of IL-5–producing CD8 T cells in blood of patients with melanoma compared with that of healthy donors (Fig. 5A and 5B). In addition, we observed a high frequency of FoxP3+CD25+ regulatory CD4 T cells (Treg) in tumor samples and an increased circulating Treg frequency in patients with melanoma compared with healthy donors (Fig. 5C).

Together, these results strongly suggest that melanoma instructs pDCs to trigger proinflammatory and regulatory immune profiles.

OX40L and ICOSL expression by pDCs is responsible for the Th2- and regulatory-prone immunity, respectively, in melanoma

Next, we investigated the mechanism by which tumor-polarized pDCs induce such an immune profile. In melanoma-bearing Humice, by analyzing a panel of molecules known to modulate immunity towards Th2 or regulatory profiles, we observed significantly higher proportions of pDCs expressing OX40L and/or ICOSL at the tumor site and DLN compared with pDCs from spleen or bone marrow (Fig. 6A). In contrast, no differential expression of GITRL, PD-L1, and PD-L2 by pDCs was observed. Notably, by assessing the relevance of these observations in patients with melanoma, we found elevated proportions of OX40L-expressing pDCs in tumor metastasis and blood of patients with melanoma compared with healthy donors, and higher proportions of ICOSL-expressing pDCs in tumor metastasis than in the blood of either melanoma patients or healthy donors (Fig. 6B). Remarkably, the proportions of OX40L- and ICOSL-expressing pDCs correlated with the frequencies of IL-13–producing CD4 and CD8 T cells (Fig. 6C) and IL-10–producing CD4 T cells (Fig. 6D), respectively. To directly investigate the role of OX40L and ICOSL in the pDC-induced polarization of immune responses, we assessed the ability of tumor-derived pDCs to drive naïve T cells towards this profile in the presence of OX40L- or ICOSL-blocking antibodies. Strikingly, the blocking of OX40L during pDC/T cocultures abrogated IL-5, IL-13, and TNF-α production, whereas the blocking of ICOSL specifically inhibited IL-10 secretion (Fig. 6E). Furthermore, patients with advanced melanoma showed higher levels of OX40L+ pDCs and Th2 T cells in circulation compared with patients at an early stage of the disease (Supplementary Fig. S4). Thus, tumor-modulated pDCs seem to drive immunity preferentially towards Th2 and regulatory profiles through OX40L and ICOSL expression.

TARC/CCL17, MDC/CCL22, and MMP-2 factors found in the melanoma microenvironment are associated with pDC accumulation, OX40L and ICOSL upregulation, and clinical outcome

To get further insights into the mechanism of pDC modulation by melanoma tumors, we examined the presence of Th2- and regulatory-modulating factors in the tumor microenvironment. We looked for the presence of TARC, MDC, TSLP, and MMP-2 in tumor supernatants, as these factors are associated with Th2 immunity (30, 31) and/or attraction of Th2 (32) and Treg cells (33). In both melanoma-bearing Humice and patients with melanoma, we found the presence...
Figure 7. MDC/CCL22 and MMP-2 factors are found in the melanoma microenvironment and correlate with pDC accumulation and early relapse. A and B, the factors TARC, MDC, TSLP, and MMP-2 were quantified in tumor supernatants collected from melanoma-bearing Humice (A, n = 49) and melanoma patients (B, n = 20). C, correlation between the frequency of tumor-infiltrating pDCs and the level of MDC (left) or MMP-2 (right) found within the same patient’s tumors (n = 15). D, correlation between the PFS and the level of MDC found in patients’ tumors (n = 10).
of TARC, MDC, and MMP-2 at the tumor site (Fig. 7A and B). Importantly, the levels of MDC and MMP-2 correlated with the proportions of pDCs found in the corresponding samples (Fig. 7C). Furthermore, MDC, MMP-2, and TARC favored the upregulation of OX40L and ICOSL respectively on pDCs (Supplementary Fig. S5). Strikingly, we found that MDC was inversely associated with the length of progression-free survival (PFS; Fig. 7D), as a high level of MDC at the tumor site correlated with early relapse. Together, our data strongly suggest that TARC, MDC, and MMP-2 promote pDC accumulation and modulation at the tumor site, subsequently triggering immunity preferentially towards Th2 and regulatory profiles and ultimately leading to tumor progression and early relapse.

Discussion

pDCs are emerging as crucial but still enigmatic cells in melanoma. Under specific conditions, pDCs are able to induce potent tumor-specific responses, but the tumor microenvironment may compromise such potential and promote tumor progression. By combining the in vivo analysis of melanoma-bearing humanized mice and the ex vivo study of a large cohort of patients with melanoma, we identified the mechanism of immunity hijacking by melanoma, bridging the observations of the presence of tumor-infiltrating pDCs and of Th2 and regulatory immune profiles.

We showed in both melanoma-bearing Humice and patients with melanoma that pDCs rapidly and massively accumulated at the tumor site and in DLN, confirming earlier studies performed by immunohistochemistry analysis on tissue sections (9, 25, 26). The combined analysis of pDC frequency and migratory profile suggests a depletion of pDCs from blood and an accumulation at the cutaneous tumor site and DLN during the course of the disease. This is in line with the increased pDC frequency observed in the invaded sentinel lymph node in patients with melanoma (27) and the attraction of circulating pDCs to the tumor site through the CCR6/CCL20 pathway (25). Moreover, infiltrating pDCs appeared to be important for melanoma development because extensive pDC infiltration was associated with poor clinical outcome. A negative impact of pDCs on patient outcomes has also been demonstrated in breast and ovarian cancers (19, 21). In melanoma, our observations are in accordance with a recent study describing a relation between pDC infiltration (defined as CD123+ cells) on primary melanoma sections and early relapse (28), yet without elucidating the cellular and molecular networks involved.

Strikingly, we demonstrated that pDCs at the tumor site triggered IL-5–/IL-13–secreting CD4 and CD8 Th2 cells and IL-10–secreting regulatory T cells through OX40L and ICOSL expression, respectively. Remarkably, a proinflammatory Th2 microenvironment has been identified by gene expression profile in the sentinel lymph node (34) of patients with metastatic melanoma and further supported by a systemic Th2-driven inflammation characterized by high plasma concentrations of IL-4, IL-5, and IL-13 cytokines (35). Such an immune profile has been described in breast cancer but was found to be driven by myeloid dendritic cells (29, 36). Th2 cytokines may lead to melanoma tumor progression in several ways. Indeed, IL-13 has been involved in the suppression of immune surveillance and inhibition of cytotoxic T-cell functions (37). Moreover, IL-13 may also directly favor melanoma growth through signalling via IL-13Rα as melanoma tumor cells express IL-13Rα (38). A strong association between pSTAT3 expression by melanoma cells and infiltration by pDCs has been described (28), suggesting a helping role of pDCs in tumor progression through the triggering of Th2 responses. IL-10+ regulatory T cells may lead to immune suppression through the suppression of Th1 and cytotoxic T cells (39). Infiltrating ICOS+ regulatory T cells have been identified in melanoma (40), and these cells display potent immunosuppressive functions in the context of cancer (33, 41). The local interaction of ICOS+ regulatory cells with ICOSL+ pDCs may lead to their expansion within the tumor microenvironment.

We found TARC/CCL17 and MDC/CCL22 in the melanoma microenvironment, chemokines that are known to be involved in the recruitment of Th2 (31, 32, 42) and Treg to the tumor site (33, 43). Furthermore, high quantities of the proteolytic enzyme MMP-2 are also found within the melanoma microenvironment. Dysregulation of antitumor immunity toward an inflammatory Th2 profile could be attributed to MMP-2 through myeloid dendritic cells conditioning to express OX40L (30, 44). We also demonstrated the ability of TARC, MDC, and MMP-2 to upregulate the expression of OX40L and ICOSL on pDCs. The level of MDC/CCL22 at the tumor site is inversely correlated with PFS, reinforcing its role in the tumor microenvironment. In line with these data, the level of MDC/CCL22 reflects the disease activity in patients with atopic dermatitis (45), an inflammatory skin disease characterized by high serum levels of Th2-type cytokines. Therefore, melanoma developed strategies to simultaneously polarize pDCs to elicit Th2 and immunosuppressive pathways, and to promote the recruitment of pDC-primed T cells that in turn will facilitate tumor progression.

Even if pDCs displayed particular features within the melanoma microenvironment evidenced by a marked defect in their activation level in lymph node in contrast with the tumor site, we showed no impairment in their capacity to upregulate coactivation molecules or to produce cytokines in response to TLR7- and TLR9-L stimulation. The intact ability of melanoma-infiltrating pDCs to respond to TLR L stimulation is consistent with the potent antitumor responses following imiquimod (TLR7-L) therapy in patients with melanoma (46–48) or after CpG (TLR9-L; refs. 14, 15) treatment, both of which involved the recruitment and activation of pDCs (16, 17, 46–48). Despite the role of pDCs on tumor progression under steady state conditions, remodeling of pDCs using TLR agonists represents a promising means to achieve melanoma tumor control by reversing the functional hijack of pDCs.

On the basis of our previous work (25) and work from others (30), the present study leads to a better understanding of the hijacking of immunity by melanoma. pDCs expressing CCR6 are recruited to melanoma tumor through CCL20.
pDCs are then induced to express OX40L and ICOSL that, under MMP-2 conditioning, could drive IL-5- and IL-13-producing Th2 CD4 and CD8 T cells and IL-10–producing regulatory T cells that might migrate to the tumor site through TARC/CCL17 and MDC/CCL22. This sequence leads to tumor progression and early relapse. Melanoma actively circumvents the immune system to facilitate its progression. By delineating the mechanisms underlying melanoma-driven plasticity of pDC in the melanoma microenvironment concomitant with their unaltered ability to respond to TLR-mediated signals, we have identified novel therapeutic options for patients with metastatic melanoma.

Disclosure of Potential Conflicts of Interest
The authors disclose no conflicts of interest.

Authors’ Contributions
Conception and design: C. Aspord, M.-T. Leccia
Development of methodology: C. Aspord
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Aspord, J. Charles, J. Plumas

References
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Plasmacytoid Dendritic Cells Support Melanoma Progression by Promoting Th2 and Regulatory Immunity through OX40L and ICOSL

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