**Collapse of the Plasmacytoid Dendritic Cell Compartment in Advanced Cutaneous Melanomas by Components of the Tumor Cell Secretome**

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**Abstract**

Melanoma is an immunogenic neoplasm infiltrated by T cells, although these adaptive T cells usually fail to eradicate the tumor. Plasmacytoid dendritic cells (PDCs) are potent regulators of the adaptive immune response and can eliminate melanoma cells via TLR-mediated effector functions. The PDC compartment is maintained by progressively restricted bone marrow progenitors. Terminally differentiated PDCs exit the bone marrow into the circulation, then home to lymph nodes and inflamed peripheral tissues. Infiltration by PDCs is documented in various cancers. However, their role within the melanoma immune contexture is not completely known. We found that in locoregional primary cutaneous melanoma (PCM), PDC infiltration was heterogeneous, occurred early, and was recurrently localized at the invasive margin, the site where PDCs interact with CD8⁺ T cells. A reduced PDC density was coupled with an increased Breslow thickness and somatic mutations at the NRAS p.Q61 codon. Compared with what was seen in PCM, high numbers of PDCs were found in regional lymph nodes, as also identified by in silico analysis. In contrast, in metastatic melanoma patients, PDCs were mostly absent in the tumor tissues and were significantly reduced in the circulation, particularly in the advanced M1c group. Exposure of circulating PDCs to melanoma cell supernatant (SN-mel) depleted of extracellular vesicles resulted in significant PDC death. SN-mel exposure also resulted in a defect of PDC differentiation from CD34⁺ progenitors. These findings indicate that soluble components released by melanoma cells support the collapse of the PDC compartment, with clinical implications for refining TLR agonist-based trials.

**Introduction**

Localized primary cutaneous melanoma (PCM) can be successfully cured by surgical excision. However, a large fraction of the patients with locally advanced PCM progress to metastatic melanoma (MM). Advances in the knowledge of the genomic landscape and immune microenvironment of melanomas have led to the introduction of effective target therapies (BRAF and MEK inhibitors for BRAF-mutated MM) and immunotherapies (anti–CTLA-4 and anti–PD-1) in the clinical treatment of MM (1). These approaches have affected the prognosis of metastatic patients, with an improvement in the clinical responses and survival.

Melanoma is an immunogenic tumor, and its immune-mediated spontaneous regression has been documented. A large fraction of tumors are hypermutated and infiltrated by immune cells, particularly proliferating T cells (2). These T cells can mount an antigen-specific response (3), but during progression, they become ineffective due to a local immunosuppressive milieu (4). Immune-escape mechanisms in melanoma include activation of the PD-1/PD-L1 pathway, which can be bypassed by checkpoint inhibitors such as nivolumab and pembrolizumab (5). Target therapy with BRAF inhibitors (BRAFi) can also enhance melanoma immunogenicity. Accordingly, the clinical response to BRAFi is partially mediated by the immune system, and posttreatment biopsies contain an increased frequency of tumor-infiltrating lymphocytes including CD8⁺ T cells (6).

All these findings highlight the relevance of the spontaneous and drug-induced immune contexture in the various clinical settings of melanoma. Immune-mediated cancer cell
elimination requires the combined activation of adaptive and innate immunity. Among innate immune cells, plasmacytoid dendritic cells (PDCs) might exert an important role in shaping the antitumor immune responses (7). Activated PDCs may exert potent antitumor activity through TLR7/9-dependent cytokine production, such as IFNα, and activation of effector functions, including granzyne B production (8) and TRAIL (9). PDC recruitment has been reported in several types of cancers, including melanoma (10), head and neck cancer (11), ovarian carcinoma (12), and breast cancer (13), and high PDC density has been associated with disease progression (14). Studies have identified PDCs in small PCM cohorts (10, 15). The analysis of circulating PDCs in early-stage PCM revealed that their frequencies and pattern of chemokine receptors are comparable to healthy donors (16), whereas data from patients with systemic disease are limited and showed a decreased PDC frequency (17).

In this study, we sought to understand the dynamics of PDCs during melanoma progression from early skin-limited PCM to advanced metastatic disease. By combining digital microscopy with flow cytometry, we provide evidence that the PDC compartment collapsed during melanoma progression. We next addressed the mechanism and found that exposure of PDCs to the melanoma secretome affected the viability of terminally differentiated PDCs. In the same experimental conditions, we uncovered that PDC generation from CD34+ precursors was also impaired. These findings indicated that soluble factors released by melanoma cells could significantly impair the PDC compartment. Because the residual PDC component in advanced melanomas has likely adopted an immune-suppressive program (14, 15, 18), approaches to enhance PDC output and activation should be considered to potentiate spontaneous and drug-mediated immune responses.

Materials and Methods

Patient cohorts

For this study, three distinct cohorts (CH) of melanoma patients were used, as illustrated in Supplementary Fig. S1. Patients with histologically confirmed and genotyped (for BRAF and NRAS) metastatic melanoma were included in the study. Exclusion criteria included immune-deficiency (steroid administration), bone marrow transplant, and known history of HIV and hepatitis B/C infection. Specifically, CH1 included 101 stage ≤3 [American Joint Commission on Cancer (AJCC)] melanoma patients, CH2 included 60 metastatic stage 4 (AJCC) melanoma patients, and CH3 included 29 stage 4 (AJCC) melanoma patients.

Human tissue samples

Tissues were represented by a cohort of 101 PCMs (obtained from CH1) of superficial spreading type 60 benign nevi (NV), 60 MM (obtained from CH2), and 33 sentinel lymph nodes (SLN, obtained from CH1; Supplementary Fig. S1). PCMs with Breslow thickness index ≥2 were considered thick. Clinical and pathologic features of the PCM cases are reported in Supplementary Table S1. The local ethics committee provided formal approval to this project (WV-Immunocancer 2014 to WV, institutional review board code NP006).

IHC

The set of primary antibodies used for this study is listed in Supplementary Table S2. The identification of PDCs was performed with the CD303/BDCA-2 antibody (Supplementary Fig. S2). The primary immune reaction was assessed using the MACH 4 TM Universal AP Polymer Kit (Biocare Medical; cat. no. M41536 1) followed by Fast Red (Dako REAL Detection System, Alkaline Phosphatase/RED Rabbit/mouse, Dako; cat. no. K500511-2) as chromogen or the Novolink Polymer Detection System (Leica Biosystem; cat. no. RE7280-CE) followed by DAB (Dako; cat. no. K346811-2) as chromogen, according to the manufacturer’s protocol.

Digital microscopy and image analysis

Image analysis was performed by using a digital microscopy approach. The absolute cell count of CD303+ PDCs and CD45R0+ leukocytes (LK) was quantified using a custom-programmed script in Cognition Network Language based on the Definiens Cognition Network Technology platform (Definiens AG). A manual region of interest selection and classification tool was used to identify the regions of interest. Melan-A-stained sections were used in all PCM cases as reference for the identification of the tumor area. The automated scoring was performed by using Definiens Tissue Studio 2.0 software (Definiens AG). The quantitative scoring algorithm was customized using commercially available templates and was created to match the requirements of the staining parameter in the skin. Cell parameters inputed for cell identification included nuclear curvature threshold, intensity thresholds, size, roundness, compactness, cell size, nuclear size, cell radius, and roundness. Modified settings were optimized for each marker in our image sets (Supplementary Figs. S3–S5).

Cell-to-cell interactions between PDCs and T cells were measured by applying a custom algorithm with the analysis software ImageJ 1.47 version (https://image.nih.gov) on sections double stained for CD303 (DAB chromogen) combined with CD3 or CD8 (Ferangi Blue chromogen, cat. no. FB813 H). Nuclei were counterstained with colloidal iron (Bio-Optica; cat. no. 04-180809). Images were acquired with an Olympus DP70 camera mounted on an Olympus Bx60 microscope using Cell software (Version F, Matrix Optics). Captured images were analyzed with ImageJ software. The algorithm was custom created and validated on 100 processed images. Briefly, the algorithm starts from a color deconvolution process. The plugin takes an RGB image and returns three 8-bit images [magenta filter (MF) = for nuclei; blue filter (BLF) = CD3 and CD8; brown filter (BRF) = CD303]. BLF and BRF images were processed to a marker area detection while the MF image was analyzed to identify nuclear structures. Subsequently, a processed MF image was merged with BLF or BRF to define the cell structure as an object. During this step, the cellular density was defined for each population. Finally, the three images were merged and cellular contacts detected (Supplementary Fig S6). The algorithm outputs included the number of positive cells for each marker, the extent of tissue area, and the number of contacts between two populations.

Human subjects and blood specimen processing

Whole blood (10 mL) was collected from 29 MM patients and 25 age-matched healthy donors (HD). Blood was drawn directly into S-Monovette 2.7 mL K3E tubes containing EDTA.
(1.6 mg/ml; Sarstedt; cat. no. 05.1167.001) and gently rocked at room temperature until processing within 1 hour from the collection. MM patients were classified according to the "Final Version of the 2009 AJCC Melanoma Staging and Classification" criteria. Briefly, patients with metastases to distant skin or subcutaneous sites or distant lymph nodes were categorized into group M1a, whereas patients with metastases to the lungs were categorized into group M1b. Finally, patients with metastases to any other visceral sites were categorized into M1c. Two or more elevated lactate dehydrogenase (LDH) levels drawn more than 24 hours apart upgraded a patient to group M1c, regardless of the site of metastases (19, 20). Clinical and pathologic features of the MM patients are reported in Supplementary Table S3.

Generation of melanoma-conditioned medium

All cell lines (obtained from 1993 to 1997) were kindly provided by Michele Maio’s laboratory (University Hospital of Siena) and shown to be Mycoplasma free by routine testing using the Universal Mycoplasma detection kit (ATCC; cat. no 30-1012K). MCLs were validated by IHC using a panel of melanoma-specific markers and molecularly tested for a panel of melanoma genes (Supplementary Fig. S7 and Supplementary Table S4). The cell lines were thawed and underwent at least three passages in culture before their use.

For the generation of melanoma-conditioned medium (melanoma supernatant, SN-mel), MCLs (Mel 146, Mel 252, Mel 327, Mel 336, and Mel 346) were seeded in RPMI 1640 medium supplemented with 10% FBS and human IL3 (20 ng/mL; Miltenyi Biotec; cat. no. 130-097-415). Blood PDCs (5 × 10^5 cells/mL) were cultured in RPMI 1640 with 10% FBS and 1% penicillin, LEV, and SEV pellets were resuspended in MilliQ water and diluted 1:100 with sterile MilliQ water. Five microliters was spotted onto freshly cleaved mica sheets (grade V-1, thickness 0.15 mm, size 15 × 15 mm; Nanoandmore GmbH; cat. no. 50-S-15-15-1). Samples were air dried and imaged in tapping mode with a NanoeAFM (Nanosurf AG) equipped with Multi75AI-G (Budget Sensors; Supplementary Fig. S8; ref. 21).

Purification and culture of peripheral blood PDCs

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats by Ficoll gradient. Peripheral blood PDCs were magnetically sorted with the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec; cat. no. 130-097-415). Blood PDCs (5 × 10^5 cells/mL) were cultured in RPMI 1640 with 10% FBS and human IL3 (20 ng/mL; Miltenyi Biotec; cat. no. 130-098-448). AP, LEV, and SEV pellets were resuspended in 80 µL 75 µL, and 70 µL of sterile distilled water, respectively, and were diluted at ratios of 1:20 and 1:100 in RPMI 1640 medium with 10% FBS and human IL3 (20 ng/mL) to PDC conditioning. PDCs (1 × 10^5) were cocultured for 24 hours with Mel 252 and Mel 327 at different PDC/melanoma cells ratios (1:2 and 2:1, respectively) in RPMI 1640 medium supplemented with 10% FBS and human IL3 (20 ng/mL). Cells were then harvested and surface-labeled with anti–BDCA-2 and anti–CD123 (Supplementary Table S2) and assayed for cell viability using LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (Thermo Fisher Scientific; cat. no. L34973).

RNA extraction

Total RNA was extracted from 5 × 10^5 melanoma cells from all cell lines, unstimulated and stimulated, with recombinant human IFNα (100 U/mL; cat. no. 300-02-AA), recombinant human IFNγ (100 U/mL; cat. no. 300-02), and a cocktail of recombinant human TNFα (20 ng/mL; cat. no. 300-01A), recombinant human IL6 (20 ng/mL; cat. no. 200-06), and recombinant human IL1β (20 ng/mL; cat. no. 200-01B; all from PeproTech) for 12 hours using the miRNeasy Mini kit (Qiagen; cat. no. 217004). DNase digestion was performed using RNase-Free DNase Set (Qiagen; cat. no. 79254). Quantitation of the RNA samples was determined by absorbance at 260 nm, and the purity (1.9 < A260/A280 < 2.0) and concentration were confirmed using NanoDrop Lite Spectrophotometer (Thermo Fisher).

Reverse transcription and real-time quantitative PCR

Expression analysis of 11 chemokines (CXCL9, CXCL10, CXCL11, CXCL12, CCL3, CCL4, CCL5, CCL8, CCL20, CCL19, and CCL21) and chemerin (RARRES2) coding genes was performed by real-time (RT) PCR technology using the custom RT2 PCR Array (Qiagen; product no. 330171) in combination with RT2 SYBR GREEN ROX qPCR Mastermix (Qiagen; cat. no. 330500) following the manufacturer’s instructions. Total RNA was treated with genomic DNA elimination mix and CDNA was synthesized from 1 µg total RNA using RT2 First Strand Kit (Qiagen; cat. no. 330404). The results were analyzed using StepOne v2.3 software (Applied Biosystems).
Purification and differentiation of human peripheral CD34+ hematopoietic progenitor cells (HPCs)

HPCs were differentiated following published protocols (22). Briefly, aliquots of 2 mL of mobilized CD34+ cells from HDS were obtained from the Laboratory for Stem Cells Manipulation and Cryopreservation, ASST Spedali Civili. Mononuclear cells were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, cat. no. GE17-1440-02) at 25°C, 1410 rpm. CD34+ cells were purified using the CD34 Microbead Kit Ultralpume human (cat. no. 130-100-453) and LS MACS magnetic columns (cat. no. 130-042-401; Miltenyi Biotec). For validation purposes, cells were stained with a panel of antibodies before the differentiation procedure (Supplementary Table S2). Cytokines used for differentiation culture include FLT3L (Miltenyi Biotec; cat. no. 130-096-479) at 100 ng/mL, SCF (R&D Systems; cat. no. 255SC050/CF) at 20 ng/mL, and GM-CSF (Miltenyi Biotec; cat. no. 130-093-865) at 20 ng/mL. Cells were cultured for 1 week before use (described in the following sections).

Stromal cell culture conditions

M55 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and prepared following previously published protocols (22). M55 stromal cells were maintained in complete alpha MEM medium supplemented with 10% heat-inactivated FBS, and cells were passaged at 90% confluency. Twenty-four hours prior to coculture with CD34+ HPCs (1 × 10^6 cells), M55 were incubated with mitomycin C (10 μg/mL; Sigma-Aldrich; cat. no. M4287-2MG) for 3 hours at 37°C and washed with PBS. Cells were subsequently plated at 2.5 × 10^5 cells per 100 μL onto a 96-well plate in complete alpha MEM medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin.

Flow-cytometric analysis

For whole blood staining, 200 μL of whole blood was incubated with a panel of fluorochrome-conjugated antibodies (Supplementary Table S2) for 15 minutes in the dark at 4°C. Red blood cells were lysed, and leukocytes were fixed by adding FACS Lysing Solution (BD Bioscience; cat. no. 349202) following the manufacturer’s instructions. A minimum of 2 × 10^5 PBMCs was acquired according to the forward light scatter versus side light scatter profile. Gating strategies are reported in Supplementary Figs. S9 and S10.

For the analysis of DC differentiation from CD34+ HPCs, GMDPs (granulocyte, monocyte, and dendritic cell progenitors), MDPs (monocyte-dendritic progenitors), and CDPs (common dendritic cell progenitors) were gated within the Lin−/CD34+ cell fraction, whereas pre-cDCs (precursor of conventional dendritic cell progenitors) were gated within the Lin−/CD34+ cell populations, based on previously published protocols (22). Samples were read on MACS Quant Cytometry (Miltenyi Biotec) or on FACSCanto II system (Becton Dickinson). Cells were subsequently stained for 8 minutes with 5 μM SYTOX Blue and washed with PBS. Cells were subsequently fixed by adding FACS fixation buffer (BD Bioscience; cat. no. 550914). Stimulation with staurosporine (1 μM; Alexis Biochemicals; cat. no. ALX-380-014-M005) for 6 hours was used as a positive control (Supplementary Fig. S11).

To test the proliferation index of HPCs after differentiation, cells were stained for 8 minutes with 5 μM of carboxy-fluorescein-diacetate-succinimidyl-ester (CFSE; Molecular Probes, Invitrogen; cat. no. C1165) in PBS at room temperature and then washed with PBS and plated onto 96-well plate before culture with differentiation conditions.

DNA extraction and integrity

For human tissues, formalin-fixed paraffin-embedded blocks were reviewed for quality control and tumor cell content. Sufficient material (at least 6 slides of 6 μm) and neoplastic cellularity (at least 70%) were considered as enrollment parameters. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen; cat. no. 56404). For cell lines, genomic DNA was extracted using QIAamp DNA mini kit (Qiagen; cat. no. 51304). Quality of extracted DNA was considered suitable with a NanoDrop 260/230 ratio over 0.5.

Next-generation sequencing (NGS) and mass spectrometry analysis

For NGS analysis, a multigene custom panel (that included the following genes: ARID2, BRAF, CDK4, EPHB6, GRIN2A, KIT, KRAS, MAP2K1, MET, NF1, NRAS, PIK3CA, PREX2, PTEN, TP53, and RAC1) was developed using AmpliSeq designer software v2.1. For library preparation, 20 ng of DNA was used for each multiplex PCR amplification. Emulsion PCR, to construct the libraries of clonal sequences, was performed with the Ion OneTouch OT2 System (Thermo Fisher). Sequencing was run on the Ion Personal Genome Machine (Thermo Fisher) loaded with Ion 318 Chip v2. Data analysis was done using the Torrent Suite Software v5.0 (Thermo Fisher). Filtered variants were annotated using a custom pipeline based on vcf2p (https://github.com/ekg/vcf2p). SnpSift, the Variant Effect Predictor software, and NCBI RefSeq database. For mass spectrometry analysis, the Sequenom MassARRAY system (Sequenom) was used in conjunction with the MelaCARTA panel. Mutant and wild-type alleles were identified using the Sequenom MassARRAY Analyzer 4 platform.

The Cancer Genome Atlas (TCGA) data

Data were downloaded from TCGA harmonized data repository using R/Biocductor package TCGAdata (version 2.8.2) accessing “TCGA-SKCM” project and selecting both primary and metastatic cutaneous melanomas. Gene-expression data were downloaded as raw counts. Comparison of gene expression between conditions was performed using Linear Modeling read counts transformed to log-CPM values. Observation-level weights were computed to account for mean–variance count relations using the voom function in the limma package (24). Gene expression was computed as RPKM and transformed on log scale. The global-test method was used to evaluate differences in expression profiles of gene signatures between the different groups. Clinical and subtype data were retrieved from curated sources (23). Three groups were identified based on tumor site and
included distant metastasis (n = 87), primary tumor (n = 43), and regional lymph nodes (n = 159). Only samples with confirmed sites of origin were considered. The effect of each gene on prognosis was evaluated using univariate Cox proportional hazard (Cox PH) models with gene expression treated as a continuous predictor. To account for potential nonlinear effect on hazard ratios, we tested for nonlinearity using restricted cubic splines with 3 knots. Because no gene showed a significant nonlinear trend, we modeled a simple linear effect. Multiple testing correction was performed using FDR adjustment.

Statistical analysis
In all analyses, all output variables were modeled on the log2 scale. Due to the substantial proportion of zero values (12%), analysis involving PDCs was performed using a censored regression model. Leukocyte density was, on the other hand, modeled using standard OLS. The relation between PDC density and mean numbers of PDCs and leukocytes were significantly correlated with the occurrence of PCM regression (Fig. 2E and F) and a greater percentage of cells involved in the interactions (Fig. 2G–J) between PDCs and T cells (Supplementary Fig. S6) in the invasive margin compared with the tumor center. These data suggest a role for PDCs in modulating T cells, including regulatory T cells, through cell contact at the invasive margin of PCM. However, by triple staining, only a small fraction of CD8+ T cells coexpressed FOXP3 (1.72% on total CD8+ T cells), showing limited interaction with PDCs (Supplementary Fig. S12).

Heterogeneity of PDC infiltration in PCM molecular subgroups
The molecular profile of cancer cells can define immunogenicity, and its therapeutic modulation can modify that immunogenicity (27) and the immune microenvironment (28). Blockade of the BRAF cascade in BRAFV600-mutated melanomas affects the immunogenicity of melanoma cells (6). We analyzed the genomic landscape of a set of 50 PCM including PDChigh and PDClow (Supplementary Table S6). A panel of melanoma genes (29) was tested by combining NGS with mass spectrometry analysis. The PDChigh/PCMc group was significantly enriched in PREX2 mutations (Fig. 3A; Supplementary Table S6). Samples from these patients contained a higher mean PDC content compared with the wild-type counterpart. On the contrary, mutations in NRAS p.Q61 were significantly enriched in PDClow/PCMc samples and showed a significantly lower PDC density, as was also evident for mutations in PTEN and NFI1 (Fig. 3A; Supplementary Table S6). No differences were observed for other genes.

TCGA described a four-group genomic classification of melanomas with clinical relevance (23). In our cohort, we identified the four groups: the BRAF+, NRAS+, NFI1+, and the triple-negative groups. Patients in the NRAS+ subgroup were older, as expected (Fig. 3B, Supplementary Table S6), and showed a significant decrease in PDC density, particularly when compared with the BRAF+ subgroup (Fig. 3C). Next, we analyzed CD8+ T-cell content. The density of CD8+ T cells correlated with the density of CD303+ PDC, and PDClow/PCMc contained a significantly lower density of CD8+ T cells compared with PDChigh/PCMc samples (unpaired t test; CI PDChigh = 151.7–1372; CI PDClow = 169.8–370.2; P = 0.0099; Fig. 3D). No differences were observed in CD8+ T-cell infiltration for all genes tested (Fig. 3E) and in TCGA subgroups (Fig. 3F). Altogether, these data indicate that the genomic landscape of melanomas could alter the PDC and CD8+ T-cell contexture.

Collapse of PDC infiltration in distant melanoma metastasis
Transformed melanocytes exit the primary tumor site and reach the draining SLN. We analyzed PDC in SLNs from a cohort of 33 melanoma patients (stage ≤III AICC; Supplementary Table S7). Ten SLN cases were positive for micrometastasis (N1a, AICC), and six SLN cases for macrometastasis (N1b, AICC). The remaining 17 SLN cases were negative for metastasis, confirmed by subserial sectioning and staining for S100
Figure 1.
Distribution of PDCs and leukocytes in PCM and NV. A–L, Sections from FFPE human PCM (n = 101) and NV (n = 60) cases and stained with the indicated markers. Sections were counterstained with hematoxylin. Magnification, 200×. A–H, Representative cases of PCM with (A–D) high and (E–H) low CD303+ PDCs and relative CD45+ leukocyte cells. I–L, Representative cases of NV. M–O, Scatter plot graphs showing the (M) density of CD303+ PDCs, (N) CD45+ leukocytes, and (O) PDC/leukocyte ratio within PCMs and NVs. P–R, The distribution of CD303+ PDCs, CD45+ leukocytes, and PDC/leukocyte fraction in PCM with different Breslow thickness. Mean and SEM are shown. The statistical significance was calculated by a Student t test.

\[ P < 0.05; \quad **, P < 0.01; \quad ***, P < 0.001. \]
Figure 2.

PDC localization and interaction with T cells in PCM. **A–D**, Graphs representing the distribution of CD303⁺ PDCs, CD3⁺ and CD8⁺ T cells at the invasive margin (IM) and tumor center (CT) in PCM (n = 10). **E–F**, Scatter plots illustrating the distribution of cell contacts between CD303⁺ PDCs and CD3⁺ or CD8⁺ T cells at the IM and CT. **G–H**, The percentage of CD3⁺ or CD8⁺ T-cell contacts with CD303⁺ PDCs calculated on total of CD3⁺ or CD8⁺ T cells present at the IM or CT. **I–J**, The percentage of CD303⁺ PDCs interacting with CD3⁺ or CD8⁺ T cells on total CD303⁺ PDCs detected in IM or CT. Means with SEM are plotted. The statistical significance was calculated by a Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
protein or MART-1. On the basis of double-staining for CD303 coupled with CD31 and Heca-452, we identified PDCs in the nodal parenchyma and were found around and within the high endothelial venules (HEVs), but not in lymphatic sinuses (Fig. 4A–D). PDC density was significantly higher in SLNs compared with PCMs (Fig. 4E). However, we found no difference between SLN with or without metastasis, suggesting that the homing of PDCs to lymph nodes and their survival was preserved, at least in stage I–III AJCC, regardless of the nodal status (Fig. 4F). The analysis of each patient also demonstrated

Figure 3.
CD303⁺ PDCs and CD8⁺ T-cell immune contexture in molecular subgroups of PCM. CD303⁺ PDC and CD8⁺ T-cell density in PCM (n = 50) reported based on the genomic profile of a panel of melanoma genes and identified in the four groups: the BRAF⁺ (n = 27), NRAS⁺ (n = 13), NF1⁺ (n = 2), and the triple-negative group (n = 11). A, Frequency of distribution of CD303⁺ PDC density in mutated (mut) and wild-type (wt) PCM. B–C, The frequency of distribution of (B) age and (C) CD303⁺ PDCs within the TCGA molecular subgroups of PCM. D, The frequency of distribution of CD8⁺ T cells in PDChigh and PDClow PCM. E, The frequency of distribution of CD8⁺ density in mutated (mut) and wild-type (wt) PCM. F, The frequency distribution of CD8⁺ T cells within the TCGA molecular subgroups of PCM. Mean with SEM or SD are plotted. The statistical significance was calculated by a Student t test and Student t test with Welch correction. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
that the content variation of PDCs in PCMs was not dependent on the PDC density found in SLNs (linear regression analysis; Pearson $r$ 95% CI = 0.13 to 0.69; $R^2 = 0.2049$; $P = 0.0082$; Fig. 4G).

Metastatic disease (MM) represents a challenge that could be treated with targeted and immunotherapy approaches. We extended our analysis to MM (stage IV, AJCC) including cutaneous (MCMC) and noncutaneous body sites (MCMNC; Fig. 4H–V; Supplementary Table S8). Both PDC density (Fig. 4T) and leukocyte density (Fig. 4U) were significantly lower in MM compared with PCM for both MCMC and MCMNC sites. Linear regression analysis demonstrated lack of a correlation between PDCs and leukocyte density in MM (Fig. 4V), indicating that the PDC reduction was selective. These data...
indicated a collapse of the PDC compartment in MM tissues, likely resulting from a defect of PDC differentiation, survival, or recruitment to the metastatic sites.

Chemokine production by melanoma cells and tissues

The tissue recruitment of PDCs is mediated by various chemoattractants. We analyzed the mRNA expression profile of a panel of chemokine coding genes and the gene encoding the PDC chemoattractant RARRES2 (alias chemerin) in a set of melanoma cell lines (Fig. 5A and B). Except for CCL20, all cell lines expressed low amounts of chemokines and RARRES2 transcripts under resting conditions. However, when exposed to a cocktail of proinflammatory stimuli (including TNFα, IL6, and IL1β), the cell lines showed significantly increased expression of CCL3, CCL4, CCL5, and CCL20 (P < 0.001). CCL5 (P < 0.001) and CCL8 expression (P < 0.05) was induced by both IFNα and IFNγ, whereas CCL4 was only enhanced by IFNγ (P < 0.05). CXCL9, CXCL10, and CXCL11 were significantly upregulated by IFNα and

Figure 5.
Chemokine expression profile in melanoma cell lines and PDC chemokine receptor expression in HDs and MM patients. A and B, Chemokine and chemerin (RARRES2) gene expression was measured by real-time RT-PCR in five cell lines unstimulated and stimulated with proinflammatory cytokines, IFNx (100 U/mL) or IFNy (100 U/mL) for 12 hours (n = 3). A, The mRNA relative expression (2−ΔΔCt) in unstimulated cell lines. The means of technical replicates (n = 3) for each cell line are shown. B, Heat map of the log2 fold increase (red color) or fold decrease (green color) of the gene expression in cell lines under stimuli, normalized to unstimulated control. The means are shown. C and D, Chemokine receptor expression on PDCs identified by flow cytometry on peripheral whole blood samples. C, Percentages and (D) MFI of chemokine receptor-positive PDCs from HDs (n = 25) and MM (n = 29). Mean and SEM are shown. The statistical significance was calculated by a Student t test. *, P < 0.05.
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cohort of chemotherapy-naïve patients and obtained from cryopreserved cells. Using

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patients. These findings indicated that interferons and proinflammatory stimuli can modulate the immune microenvironment of PCM and MM, including PDCs.

We expanded this analysis on human melanoma samples by using an in silico approach. Chemokine production was tested on a TCGA data set (n = 331; ref. 23) containing the transcriptome of PCMs, metastatic regional lymph nodes, and distant metastasis. To this end, we generated signature A, composed by genes coding for PDC-attracting chemokines (Supplementary Table S9). As a surrogate marker of the PDC content, we also generated signature B, which included seven PDC transcripts (Supplementary Table S9). Components of signatures A and B were significantly more expressed in regional lymph nodes (Supplementary Figs. S13 and S14; Supplementary Table S9). However, the two signatures in primary tumors were unable to predict AJCC stage and patient survival. In contrast, components of signature A expressed in regional lymph nodes predicted good prognosis (Supplementary Fig. S14 and Supplementary Table S9).

Retained expression of chemokine receptors in PDCs from MM patients

A reduced frequency of PDCs in MM might also depend on their defective migratory properties. Fully differentiated circulating PDCs expressed several chemokine receptors. However, only CXCR3 and CXCR4 are functional (30, 31). Upon activation, PDCs also upregulate functional CCR7 (30, 32). CCR6 is also expressed more on PDCs from PCM patients and is likely involved in their migration in response to CCL20 produced in the melanoma microenvironment (16). Therefore, we examined expression of CCR6, CXCR3, CCR7, CXCR4, and CCR5 in circulating PDCs from whole blood of chemotherapy-naïve MM patients (stage IV, AJCC) and HDs. We found most of the chemokine receptor repertoire was heterogeneous, with no significant differences between MM patients and HDs, in terms of percentage of positive cells and median fluorescence intensities, except for CXCR3 and CXCR7, which were decreased in patients (% of CXCR3+: PDC: HD vs. MM; mean 97.65 vs. 91.04, P value = 0.0166; MFI of CXCR7+: PDC: HD vs. MM, mean 1,862 vs. 711.8, P value = 0.0378; Fig. 5C and D; Supplementary Fig. S15; Supplementary Tables S10 and S11). CCR6 expression remained low and unchanged in our cohort of MM patients. These findings suggest that MM patients exhibited a chemokine receptor repertoire comparable with HDs.

Decreased PDC frequency in the peripheral blood of MM patients

Previous studies in PCM report that the frequency of circulating PDCs and myeloid (M)DCs decreases significantly in stage IV (16, 17). However, the data are limited to a small cohort of patients and obtained from cryopreserved cells. Using flow cytometry on fresh whole blood, we tested the PDC frequency as well as the myeloid DC (MDC) frequency, in a cohort of chemotherapy-naïve MM patients (Supplementary Fig. S9). Data were consistent with a significant reduction of PDCs (mean ± SD%: 0.25 ± 0.16 vs. 0.45 ± 0.19; n = 29 vs. n = 27, respectively, P value = 0.006) and MDC frequency (mean ± SD%: 0.35 ± 0.19 vs. 0.53 ± 0.21; n = 27 vs. n = 15, respectively, P value = 0.03) in MM patients compared with HDs (Fig. 6A–F). No differences were observed based on the molecular profile of the tumors (Fig. 6G and H). In MM, the tumor burden predicts patient survival (33). The site of metastasis and LDH concentration characterize the M1a, M1b, and M1c categories, with the latter category showing an increased LDH and the worst prognosis (19). We found that M1c patients showed a significant decrease in circulating PDC frequency (mean ± SD%: 0.18 ± 0.15 vs. 0.33 ± 0.14; n = 13 vs. n = 14, respectively, P value = 0.003) and MDC (mean ± SD%: 0.23 ± 0.09 vs. 0.47 ± 0.18; n = 13 vs. n = 14, respectively, P value = 0.02) compared with M1a + b patients (Fig. 6I and J). This finding was confirmed by absolute cell counts (Supplementary Table S12). Altogether, these data suggest that the circulating pool of PDCs and MDCs is significantly reduced in advanced melanoma, particularly in the more systemic M1c group.

Supernatants from melanoma cells impair viability of differentiated PDCs

The significant reduction of circulating and tissue PDCs in MM patients could be explained by an unfavorable interaction of terminally differentiated PDCs with melanoma cells or their secreted products. In PCM, the melanoma cell/PDC ratio was variable (ranging from 3.42 to 267.44) and was high in thick PCM (P < 0.01; Supplementary Fig. S5). We, thus, exposed PDCs from HDs to melanoma cell supernatant (SN-mel) from five cell lines. The percentage of dead cells was higher after 24 hours of SN-mel conditioning compared with the control, although significant only in Mel 327 cells (Fig. 7A). We found a significant increase in early apoptotic cells in four cell lines (Fig. 7B), and the fraction of late apoptotic/necrotic cells was unchanged (Fig. 7C). Data on PDC apoptosis were also validated by caspase-3 staining via flow cytometry and on tissue sections by IHC (Supplementary Fig. S11). Overall, these data suggest that components of the melanoma supernatant affect PDC viability and activate the apoptotic pathway.

SN-mel impair PDC differentiation from CD34+ HPCs

We hypothesized that the frequent collapse of the circulating PDC compartment in advanced MM stems from the effects (local and systemic) of the melanoma secretome on differentiation of PDCs from CD34+ HPCs. We, thus, exposed peripheral blood-mobilized CD34+ HPCs from HDs to SN-mel during in vitro differentiation. Significantly reduced cell numbers in 4 SN-mels tested was seen (Fig. 7D; Supplementary Fig. S16). Viability and proliferation rate were also evaluated. Early apoptotic cells were increased in 3 SN-mels (Fig. 7E), as was the fraction of late apoptotic/necrotic cells (Fig. 7F). We also detected a significant decrease in the proliferation of HPCs after SN-mel conditioning (Fig. 7G–H). Phenotypic analysis (22) indicated that exposure to SN-mel induced significant changes in precursor frequencies (Fig. 7I–L), including a significant decrease of GMDP (Fig. 7I), CDP (Fig. 7K), and pre-cDC fractions (Fig. 7L). These data demonstrated that the melanoma supernatants impair the differentiation and replication potential of bone marrow PDC progenitors.
Figure 6. Frequency of PDC and MDC subsets in MM patients. Flow cytometry analysis was performed on peripheral whole blood from HDs (n = 25) and MM patients (PT; n = 27). A–D, Representative plots of PDC and MDC subsets obtained from (A–B) HDs and (C–D) PT. E–F, The frequency of (E) PDCs and (F) MDCs in PT and HDs. G–J, Subgroup analysis of the PT cohort illustrating the frequency of distribution of PDCs and MDCs based on the (G–H) molecular subtype and (I–J) M1a–c categories. Two technical replicates for each sample were made. Mean percentages and SEM are shown. The statistical significance was calculated by a Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 7.
SN-mel affects the PDC viability and differentiation from CD34⁺ HPCs. A–C, Purified PDCs (5 × 10⁵ cells/mL) were conditioned with SN-mel, and cell viability was analyzed with Annexin V/SYTOX AADvanced (Ann V/SYTOX AAD) flow cytometry after 24 hours. Scatter plots illustrate (A) the percentage of death (Ann V⁺); B, early apoptotic (Ann V⁺/SYTOX AAD⁺); or (C) late apoptotic/necrotic (Ann V⁺/SYTOX AAD⁺) PDCs cultured in RPMI 1640 medium or SN-mel. Bars represent the mean of biological replicates (n = 3–7). D–L, Purified CD34⁺ HPCs from HDs were conditioned with SN-mel during in vitro differentiation. D–H, The total number of cells, (E) the fraction of early apoptosis, or (F) late apoptosis/necrosis, as well as the (G) proliferation and (H) division index of cells obtained after in vitro differentiation and SN-mel conditioning of CD34⁺ HPCs. Bars represent the mean of technical replicates (n = 3). I–L, The frequency of early precursors of DC lineage after 7 days differentiation protocol in control alpha MEM medium or SN-mel. Bars represent mean and SD of technical replicates (n = 5). M–R, PDCs purified from buffy coat of HDs were cultured for 24 hours in control RPMI 1640 medium or SN-mel from Mel 146 or the corresponding SN1, SN2, and SN3 fractions (M–O) and with different dilution of the AP, LEV, and SEV (P–R). Cell viability was analyzed with Annexin V/SYTOX AADvanced staining and flow cytometry quantification. Column bar graphs illustrate (M) the percentage of PDC death, (N) early apoptosis, and (O) late apoptosis/necrosis, as previously indicated. Bars represent the mean and SD (n = 6). Scatter plots illustrate (P) the percentage of PDC death, (Q) early apoptosis, and (R) late apoptosis/necrosis, as previously indicated. Bars represent the mean (n = 3). The statistical significance was calculated by a paired Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Soluble components of the melanoma secretome affect the viability of differentiated PDCs

Tumor cells, including melanoma cells, produce and release factors that suppress the function of immune cells or induce their apoptosis (34). Molecular components of tumor-derived EVs can suppress antitumor immune responses (35). Tumor supernatants are composed of secreted molecules and EVs that can be isolated by centrifugation. EVs include apoptotic bodies, large EVs, and small EVs. Using centrifugation, we serially depleted EVs from the SN-mel 146, which induced PDC death (Fig. 7A–C). PDC death, especially early apoptosis, was significant after 24 hours of exposure to all EV-depleted SN-mel fractions (Fig. 7M–O), suggesting a role played by soluble factors. To confirm our hypothesis, we exposed PDCs to different dilutions of different EVs derived by SN-mel 146. EVs alone did not affect PDC viability (Fig. 7P–R). Validation of the EV components was obtained by AFM (Supplementary Fig. S8). Together, these data support the hypothesis that soluble components of the melanoma secretome affect the viability of differentiated PDCs.

Discussion

The PDC compartment is maintained by progressively restricted bone marrow progenitors that require Flt-3 ligand (36) for their terminal differentiation (37). Differentiated PDCs exit the bone marrow, circulate, and home to lymph nodes and inflamed peripheral tissues in various diseases (38). Here, we report the analysis of the PDC compartment in the tissue of PCM and MM and in the blood of MM patients. First, we found that the full transformation of melanocytes was coupled with the recruitment of PDCs, whereas benign common-type nevi containing senescent melanocytes were mostly devoid of PDCs. Second, progression to metastatic disease was associated with a collapse of the PDC compartment. These two main findings are consistent with other studies (14, 39, 40). Differences in terms of the clinical significance likely depend on the tissues analyzed (primary vs. metastatic), on the analysis method (digital microscopy vs. flow cytometry), as well as on the PDC markers (CD303 vs. CD123) used. These variables should be considered for further prospective studies.

PDCs were recruited in the early stages of PCM, particularly at the invasive margin. CD8+ T-cell density was positively correlated with PDC density, and cell-to-cell interaction between these two cell types was increased at the invasive margin, occurring less frequently in the tumor center. However, PDC density was low in the tumor center, suggesting an unfavorable environment. The tumor center of PCM is characterized by a decreased availability of nutrients and oxygen deprivation due to reduced vascularization (41, 42). We found that the PDC density was significantly decreased in thick melanomas, characterized by an expanded tumor center due to a consistent vertical growth phase. Melanoma cells in the vertical growth phase increase their mitotic activity and nutrient requirements, outcompeting other cells of the microenvironment, including PDCs. The inherent molecular profile of cancer cells can modify cancer cell immunogenicity (27) and the surrounding immune contexture (28, 43). We found a significant reduction in PDC density in NRAS p.Q61 mutated PCM that might depend on the limited ability of melanoma cells to chemotact PDCs. We found that melanoma cells were proficient in chemokine production only under proinflammatory stimuli and in response to interferons. However, we could not detect significant differences between NRAS- and BRAF-mutated cell lines. These findings suggest that PDCs can be modulated by interactions between melanoma cells and the existing microenvironment, likely resulting in differences in the local availability of proinflammatory signals. Alternatively, biological properties of NRAS p.Q61, rather than chemokine production, may also alter the contexture (23).

The density of PDCs correlated with CD8+ T cells at the invasive margin, suggesting shared mechanisms of recruitment. The invasive margin is a relevant compartment in melanomas. Melanomas responding to PD-L1 blockade are enriched in proliferating T cells at the invasive margin (26). PDCs can induce antigen-specific CD8+ T cells (44) and shape the antitumor immune response by producing type 1 interferons (45). PDC activation could amplify the CD8+ T-cell responses and could be combined with other approaches such as PD-1/PD-L1 blockade (46) to improve clinical benefit. Human PDCs can also generate regulatory T cells (47). However, our data suggest that this is not the case in the PCM environment, where CD8+FOXP3+ cells were mostly absent.

The PDC compartment during melanoma dissemination is poorly investigated. We found that in N3 stage 3 PCM, PDCs were maintained in a high density in SLNs. Circulating PDCs efficiently homed to SLNs via HEVs, whereas PCM-infiltrating PDCs represented a nonmigratory compartment, being absent from the lymphatic vessels of the involved skin and the draining lymph nodes. Our in silico approach strengthened the notion that the PDC pool of regional lymph nodes is fueled by nodal chemokines (signature A), and their high density predicted good prognosis. On the contrary, we observed a decrease in PDC infiltration in distant metastasis to different body sites, including the skin. PDC of chemotherapy-naïve MM patients exhibited a chemokine receptor repertoire comparable to that of HDs. These findings rule out tissue migration defects of PDCs in advanced melanomas. However, our analysis performed in a cohort of MM revealed a significant reduction of circulating PDC and MDC frequencies, particularly in M1c patients characterized by visceral metastasis or high LDH. We found that the collapse of the circulating PDC compartment was likely due to an effect on the survival of differentiated PDCs or their bone marrow precursors.

One of the main findings of this study was that the exposure of PDCs to SN-mel induced PDC apoptosis, even after depletion of the EV compartment, indicating that soluble components released by melanoma cells affected PDC survival, consistent with other DCs (48). Gangliosides from human melanoma impair DC differentiation and promote apoptosis of monocye-derived DCs (34). Alternatively, nutrient deprivation or metabolic abnormalities could impair PDC survival. Immunometabolism is crucial for human PDCs to execute innate immune functions (49). However, inhibition of glycolysis does not interfere with viability of human PDCs (50). Our results demonstrated that MM patients with elevated serum LDH had a reduction of circulating PDCs and MDCs. LDH plays a main role in the glycolytic pathway, converting pyruvate to lactate, with subsequent acidosis. Lactate and acidosis leads to a local suppressive effect on various immune cells, including macrophages (51), MDSCs (52), and DCs (53). This metabolic switch and the corresponding acidosis might interfere with the PDC compartment by reducing bone marrow output and survival. In

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melanoma patients, high serum LDH predicts poor survival (54) and poor clinical response to anti-PD-1 treatment (55), suggesting LDH affects cancer cell elimination by adaptive immune cells (56).

In conclusion, this study demonstrated that the PDC compartment fluctuates with melanoma progression. Malignant transformation of normal melanocytes was associated with local infiltration of terminally differentiated PDCs. However, during the local progression of PCM, the PDC compartment collapses, particularly in NRAS-mutated PCM. Progression to metastatic disease, especially in the M1c stage, reduced the circulating and tissue pool of PDCs. At this time point, the effect observed in vitro on PDC differentiation from CD34+ HPCs was likely the main mechanism sustaining the PDC collapse. These findings identify a restricted clinical window of intervention, in which PDC-dependent immune responses could be potentiated. PDC activation by TLR agonists may have clinical benefit in cancer immunity. Preclinical findings have proposed TLR9 activation of PDCs by intratumoral injection of CpG-A oligonucleotides (46, 57, 58). Numerous studies have proposed TLR9 activation of PDCs by intratumoral injection of CpG-A oligonucleotides (46, 57, 58). Numerous studies have proposed TLR9 activation of PDCs by intratumoral injection of CpG-A oligonucleotides (46, 57, 58).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Correction: Collapse of the Plasmacytoid Dendritic Cell Compartment in Advanced Cutaneous Melanomas by Components of the Tumor Cell Secretome

In the original version of this article (1), the axes labels were unintentionally inverted for CD123 and BDCA2 in Fig. 6A. The error has been corrected in the latest online HTML and PDF versions of the article. The authors regret this error.

Reference

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