Identification and Functional Analysis of Tumor-Infiltrating Plasmacytoid Dendritic Cells in Head and Neck Cancer

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ABSTRACT

The antitumor activity of IFN-α is well established. However, the role of the plasmacytoid dendritic cell (PDC), the major producer of IFN-α upon viral infection, in tumor biology is unknown. We sought to study the presence and function of PDC in a human solid tumor. Here, we demonstrate that PDCs infiltrate tumor tissue of patients with head and neck squamous cell carcinoma (HNSCC). Functional activity of PDC was examined by using CpG motif containing oligonucleotides, a defined microbial stimulus for PDCs (recognized via toll-like receptor 9). We found that HNSCC diminished the ability of PDC to produce IFN-α in response to CpG motif containing oligonucleotide. Tumor-induced down-regulation of toll-like receptor 9 was identified as one mechanism likely contributing to impaired PDC function within the tumor environment. In tumor-draining lymph nodes, suppression of CpG-induced IFN-α production was less pronounced than in single-cell suspensions of primary tumor tissue. In these lymph nodes, CpG-induced IFN-α production was associated with increased levels of interferon-induced protein 10 and IFN-γ and activation of CD4 and CD8 T cells. These results show for the first time the presence of PDCs in human solid tumor tissue and that tumors suppress the capacity of PDCs to produce IFN-α. PDCs, which in the absence of appropriate stimulation are reported to promote regulatory CD8 T cells, may contribute to an impaired T-cell-mediated immune response in HNSCC.

INTRODUCTION

HNSCC is the sixth most frequent tumor in the world. Standard treatment of HNSCC stage III and IV combines surgery and subsequent locoregional radiotherapy and chemotherapy. The 5-year survival in patients with HNSCC stage III and IV could not be improved over the past 30 years and remains low at 15–30%, highlighting the urgent need for an improved therapy (1–3). Although HNSCC is infiltrated by different immune cells, patients with HNSCC show a profound impairment of cellular immune responses (4–7). There are a number of indications from the literature that head and neck cancer is sensitive to immunotherapy (4–14).

IFN-α is one of the most potent antitumor cytokines that is routinely used for the treatment of several malignant diseases, including hairy cell leukemia, cutaneous T-cell leukemia, chronic myeloid leukemia, non-Hodgkin’s lymphoma, adjuvant therapy of malignant melanoma, and AIDS-related Kaposi’s sarcoma (15). The major cellular source of IFN-α is the so-called “natural type I IFN,” a cell type that has been described for many years as a rare CD4+/MHC II+ population (1:1000 within PBMCs) capable of synthesizing extremely high amounts of type I IFN upon viral infection (16–18). The IFN is identical with one of the two DC subsets present in human peripheral blood, which is now called the PDC (also termed DC2; Refs. 19–21). It was not until recently that the murine counterpart of the human PDC has been identified (22–24). In humans, PDCs have been found in peripheral blood (19, 21), in tonsils (25), in cerebrospinal fluid (26) and under pathological conditions such as systemic lupus erythematosus (27), malignant ascites of patients with ovarian carcinoma (28), and in nasal mucosa of allergic subjects after topical allergen challenge (29).

There is evidence that in the absence of appropriate stimulation, PDCs promote the development of regulatory CD8 T cells (30). Besides viral infection, a well-defined microbial stimulus for PDC is the recognition of CpG motifs within microbial DNA. CpG motifs are unmethylated CG dinucleotides within certain sequence contexts. The vertebrate immune system is able to detect CpG motifs via TLR9 (31). Thus far, only two cell types in the human immune system have been identified that are capable to detect CpG motifs based on the expression of TLR9: B cells and PDCs (32–34). However, the presence of even small numbers of PDC potently modulates the activity of other immune cell subsets such as T cells, natural killer cells, and MDCs (35–37). On the basis of the ability to stimulate IFN-α production in PDC and to activate B cells, three distinct classes of CpG ODN have been identified: CpG-A (also termed D-type; high IFN-α production in PDCs but lack of B cell stimulation) CpG-B (also termed K-type; low at inducing IFN-α but strong B-cell stimulation), and CpG-C (high IFN-α production in PDCs and strong B cell stimulation; Refs. 38–40).

Antitumor activity of CpG ODN has been demonstrated in a number of different tumor models (41–44). We and others (45–47) recently demonstrated that the local injection of CpG ODN into the tumor area is superior to injection of CpG ODN into a site distant of the tumor. Head and neck cancer is one of the tumor entities that provides access for local therapeutic intervention. Repeated local injections of CpG ODN into the tumor area of patients with head and neck cancer may promote an antitumor response. To support the rationale for such an approach, we sought to study the presence of the primary target cell for CpG ODN, the PDCs, in tumor tissue of patients with head and neck cancer.

MATERIALS AND METHODS

Human Tissue Samples and Patient Characteristics. Tumor tissue specimens from the oral cavity, the pharynx, or the larynx of patients with head and neck cancer were obtained during standard surgical procedure. At the same time, tumor draining lymph nodes were obtained by neck dissection and examined by a pathologist. Specimens of nasopharyngeal tonsils were obtained...
from children undergoing surgery of nasopharyngeal hyperplasia, and healthy
nasopharyngeal mucosa was obtained from patients undergoing plastic surgery of
the nasal septum. Specimens of normal oropharyngeal mucosa and lymph nodes were
obtained from patients with sleep-related breathing disorders undergoing uvulo-
pharyngoplasty. Skin biopsies of normal looking human skin obtained from
surgical specimens served as a nonmucosa control. Tissue specimens were
transported in sterile saline and processed immediately after excision.

The characteristics of patients with head and neck cancer are provided in Table
1. The use of human tissues for research purposes was approved by the ethics
committee of the University of Munich.

**Preparation of Single-Cell Suspensions.** Tumor specimens with no adja-
cent normal tissue and lymph node tissue were washed several times and
carefully minced into small pieces in sterile serum free RPMI medium (RPMI
1640 supplemented with 100 units/ml penicillin, 1 mM glutamine, and 100
units/ml streptomycin). Tumor tissue was digested with collagenase type VIII
(1.5 mg/ml; Sigma) and DNase type I (1.0 µg/ml) for 120 min at 37°C with
gentle agitation. Tumor-draining lymph nodes and nasopharyngeal tonsillar
tissue were dissociated using a syringe plunger through a 100-µm nylon
 cell strainer. The resulting cell suspensions were washed in PBS, resuspended
in PBS containing 10% FCS, and filtered through a 40-µm nylon cell
strainer (Falcon; Becton Dickinson Labware) into cold RPMI medium con-
taining 10% FCS.

**Isolation of PDCs.** Human PBMCs were prepared from buffy coats pro-
vided by the blood bank of the University of Greifswald. Blood donors were
18–65-year-old healthy donors who were tested to be negative for HIV,
hepatitis B virus, and hepatitis C virus. Additional exclusion criteria were
manifest infections during the last 4 weeks: fever; symptomatic allergies;
abnormal blood cell counts; increased liver enzymes; or medication of any
kind, except vitamins and oral contraceptives. PBMCs were obtained from
buffy coats by Ficoll-Hypaque density gradient centrifugation (Bio-Rad)
as described previously (48). PDCs were isolated by magnetically activated
cell sorting using the BDCA-4 dendritic cell isolation kit from Miltenyi Biotec
(Bergisch-Gladbach, Germany). Briefly, PDCs were labeled with anti-
BDCA-4 antibody coupled to colloidal paramagnetic microbeads and passed
through a magnetic separation column twice (LS and RS column; Miltenyi
Biotec). The purity of isolated PDCs (lineage-negative, HLA II-positive,
and CD123-positive cells) was >90%. Viability was >95% as determined by
trypan blue exclusion. PDCs were cultured at a final concentration of 5 × 10³
cells/ml in 96-well plates.

**Cell Culture.** Single-cell suspensions from tumor and tumor-draining
lymph nodes were cultured in RPMI-medium (RPMI 1640 supplemented with
10% FCS, 100 units/ml penicillin, 1 mM glutamine, and 100 units/ml strepto-
mycin). All compounds were purchased from Miltenyi Biotec. Samples were
incubated at a final concentration of 1.5 mg/ml (38). Single-cell suspensions of tumor
and lymph nodes were incubated at a final concentration of 1.5 × 10⁶ cells/ml
in 48-well plates. The tumor cell lines FADU (American Type Culture Col-
lection) and PCI-1 (gift from Theresa Whiteside, Pittsburgh, PA) and the
fibroblast cell lines WI38 (American Type Culture Collection, normal human
lung fibroblast cell line) were cultured in supplemented RPMI 1640 at a final
concentration of 5 × 10⁵ cells/ml.

**Flow Cytometry.** Surface antigen staining was performed as described
previously (49). Cells were stained with FITC-, phycoerythrin-, PerCP-, or
adenomatous polyposis coli-conjugated antibodies by incubation on ice for 15
min followed by washing with PBS. Fluorescence-labeled monoclonal anti-
body against CD3, CD4, CD8, CD11c, CD14, CD16, CD19, CD20, CD40,
CD56, CD69, CD80, CD86, CD123, and HLA-DR were purchased from
Becton Dickinson (Heidelberg, Germany). Anti-BDCA-2 and anti-BDCA-4 were
purchased from Miltenyi Biotec. Samples were analyzed on a FACS-
Calibur (Becton Dickinson) using TO-PRO-3 iodide (2 µm; Molecular Probes,
Leiden, the Netherlands) to exclude dead cells. Data acquisition and analysis
were performed using Cell Quest software.

**Immunohistochemistry.** For immunohistochemical staining, tissue speci-
mens were embedded in Tissue tek (Sakura Finetek, Torrance, CA), cryopre-
served in N₂, and stored at −20°C. Acetone-fixed cryosections (4 µm) were
incubated with the PDC-specific antibody BDCA-2 (50) for 1 h at room
temperature after blocking endogenous peroxidase activity with 0.3% H₂O₂.
The isotype antibody (mouse antihuman IgG; Dako A/S, Glostrup, Denmark)
was used as negative control. After incubation with biotinylated horse anti-
mouse IgG (1/300; Dako A/S) for 1 h at room temperature, sections were
rinsed and incubated with avidin-biotin-PO-complex (1/200; Sigma Chemi-
cals, Deisenhofen, Germany) for 30 min at room temperature. The chromogen,
0.01% 3-aminoo-9-ethyl-carbazol in 0.1 m sodium acetate buffer ([pH 5.5],
0.0015% H₂O₂, 6% DMSO) was used. PDCs were stained in red. Mayer’s
hemaluna was used to counterstain cellular elements (blue). Double immuno-
sta in ng was performed with mouse antihuman CD123 antibody (IgG2a κ;
PharMingen) using the avidin-biotin-PO method (red staining) as described
above and with mouse antihuman HLA-DR antibody (IgG1 κ; Dako) using
alkaline phosphatase-antialkaline phosphatase method (blue staining).
The antigen-antibody complex was visualized using secondary antibody (goat
antimouse IgG) followed by incubation with alkaline phosphatase-antialkaline
phosphatase complex (51) with commercial kits (Dako A/S). As chromogen,
we used fast blue (Sigma Chemicals, Munich, Germany).

**Detection of Cytokines.** The IFN-α module set, Bender MedSystems
detection range, 8–500 pg/ml), was used to detect IFN-α in cell culture
supernatants. This ELISA detects most of IFN-
and IFN-α F. IP-10 was measured by ELISA (R&D). IFN-γ and IL-10 were
were purchased from Miltenyi Biotec. Samples were analyzed on a FACS-
Calibur (Becton Dickinson) using TO-PRO-3 iodide (2 µm; Molecular Probes,
Leiden, the Netherlands) to exclude dead cells. Data acquisition and analysis
were performed using Cell Quest software.

**Real-Time Reverse Transcription-PCR.** Cells were lysed, and RNA was
extracted using the total RNA isolation kit (High Pure; RAS, Mannheim,

<table>
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<th>Patient</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>TNM</th>
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<th>PDC in %</th>
<th>MDC in %</th>
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</table>

*Age indicates the age of the patient in years at diagnosis; TNM, tumor node metastasis system of staging (Union International Contre Cancer); n.d., not determined.
An aliquot of 8.2 μl of RNA was reverse transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT) as primer (First Strand cDNA Synthesis kit; Roche, Mannheim, Germany). The obtained cDNA was diluted 1:25 with water, and 10 μl were used for amplification. Parameter specific primer sets optimized for the LightCycler (RAS) were developed by Search-LC GmbH (Heidelberg, Germany) and purchased from Search-LC. The PCR was performed with the LightCycler FastStart DNA Sybr Green kit (RAS) according to the protocol provided in the parameter specific kits and as described previously (35). The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions versus the PCR-cycle number (CP) at which the detected fluorescence intensity reaches a fixed value. Using >300 data points, the actual copy number was calculated as follows: \( X = e^{t - 0.6935 CP + 20.62} \). All used primer sets had an efficiency > 1.86. The data of two independent analyses for each sample and parameter were averaged. The copy number was normalized by the housekeeping gene cyclophilin B and is presented as number of transcripts per 10^3 copies of cyclophilin B.

Statistical Analysis. Data are expressed as means ± SE or as box diagrams. Statistical significance of differences was determined by the paired two-tailed Student’s t test (\( * \) indicates \( P < 0.05 \)) or as indicated. Statistical analyses were performed using StatView 4.51 software (Abacus Concepts, Inc., Calabasas, CA).

RESULTS

Identification of Tumor-Infiltrating PDCs in HNSCC. PDCs have been described in mucosa-associated lymphoid tissue (tonsils) and in experimentally induced allergic rhinitis (25, 29). In peripheral blood, PDCs can be identified by three-color staining and flow cytometry (lineage-negative, MHC II-positive, CD123-positive; Ref. 21). To establish a protocol that allows the detection of PDCs in primary tumor tissue, we tested whether this three-color-staining protocol can be used to detect PDCs in human mucosa-associated lymphoid tissue (tonsil). Single-cell suspensions of nasopharyngeal tonsils were prepared and stained with a mixture of lineage antibodies (CD3, CD14, CD16, CD19, CD20, and CD56), with MHC II and CD123. Dead cells were excluded from analysis by positive staining for To-Pro. This procedure revealed a distinct population of cells with the typical characteristics of PDCs (lineage-negative, MHC II-positive, CD123-positive, FSC, and SSC properties; Fig. 1A).

The antibody BDCA-2 has been described to specifically stain PDCs (50, 52). The use of BDCA-2 instead of CD123 in the three-color staining protocol revealed the same population of cells (frequency, FSC, and SSC properties), confirming the identity of PDCs (Fig. 1B). BDCA-4, another antibody that specifically stains PDCs in peripheral blood (52), was less specific for PDCs when used in lymphoid tissues (Fig. 1C: higher frequency of cells, different FSC and SSC properties as compared with Fig. 1B). MDCs were identified in single-cell suspensions of tonsils by three-color staining (Fig. 1D: lineage-negative, MHC II-positive, and CD11c-positive cells). MDCs presented a distinct population of cells with similarly low FSC and SSC characteristics as PDCs. On the basis of these results in tonsils, all subsequent analyses were performed with three-color stainings using lineage and MHC II in combination with CD123 (PDC) or CD11c (MDC).

To examine the presence of PDCs in HNSCC versus normal oropharyngeal mucosa, single cell suspensions of primary tumor specimens and of normal oropharyngeal mucosa were prepared and stained with the protocol as above. Flow cytometric analysis revealed the presence of both PDCs and MDCs in the tumor tissue (Fig. 2A). PDCs and MDCs showed the same FSC and SSC properties as observed in tonsils, confirming the identity of these cell subsets. In oropharyngeal mucosa, similar numbers of MDCs were found, but PDCs were hardly detectable (Fig. 2B).

The frequency of PDCs and MDCs was determined in tumor specimens obtained from 16 individual patients (Table 1). Although the mean frequency of PDCs and MDCs was similar (PDC 0.34%, Fig. 3, left panel; MDCs 0.40%, Fig. 3, right panel), we observed a considerable patient-to-patient variability of PDCs (range, 0.1–1.1% PDCs) and MDCs (range, 0.02–0.75%) numbers, and the frequencies of PDCs and MDCs were not linked to each other (e.g., patient no. 5: PDCs 1.1%, MDCs 0.3%; patient no. 14: PDCs 0.2%, MDCs 0.8%). Furthermore, no correlation was found between the tumor stage and the frequency of DC subsets (Table 1).

In tonsils, the frequency of PDCs was higher than the frequency of MDCs (PDCs: 0.8%; MDCs: 0.4%; \( n = 8; P = 0.009 \)), whereas equal numbers of PDCs and MDCs were found in tumor draining lymph nodes (Fig. 3, PDCs: range, 0.2–1.6%, mean, 0.4%; MDCs: range, 0.2–0.5%, mean, 0.4%; \( n = 4 \)). In normal oropharyngeal mucosa, PDCs were almost absent, whereas the number of MDCs was ~10-fold higher (Fig. 3, PDCs: range, 0.01–0.08%, mean, 0.03%; MDCs: range, 0.19–0.36%, mean, 0.27%; frequency of PDCs versus MDCs:...
ascites and in tumor tissue (28). We found that SDF-1 mRNA is expressed in primary HNSCC tumor tissue (332 ± 14 copies; n = 7) but at lower levels as compared with healthy mucosa (2087 ± 319 copies; n = 10). In the two tumor cell lines FADU and PCI-1 SDF-1 mRNA could not be detected.

**Localization of PDCs in HNSCC and Lymphoid Tissues.** The antibody BDCA-2 has been used to specifically stain PDCs in cryosections of nasopharyngeal tonsils (50). By using BDCA-2, we found that PDCs are located in the perifollicular T-cell areas near high endothelial venules (Fig. 4, A–B, PDCs indicated by arrows). This observation confirmed earlier results by Summers et al. (25) and by Olweus (53) who detected PDC by a combination of CD123 and CD45RA. Single staining with CD123 in our hands was not appropriate for the identification of PDCs in tonsils because besides PDCs, also high endothelial venules stained positive for CD123 (data not shown).

BDCA-2 staining of frozen sections of HNSSC tumor specimens revealed that PDCs are distributed throughout the solid tumor tissue (Fig. 4, C–D, PDCs indicated by arrows). Consistent with flow cytometric analysis, no PDCs were found in skin biopsies stained with BDCA-2 (data not shown).

**HNSCC Suppresses CpG-Induced IFN-α Production in PDC.** Because single-cell suspensions prepared from primary tumor specimens contained a comparable frequency of PDCs as peripheral blood, we hypothesized that exposure of this cell suspension to CpG ODN may result in IFN-α production by PDCs and in IFN-α-mediated indirect activation of other immune cell subsets (32). We used ODN 2216 (CpG-A type ODN) that is known to induce very high amounts of IFN-α in PDCs [10–30 ng/ml PDC-derived IFN-α within PBMCs containing 0.2–0.4% PDCs (38)]. To our surprise, IFN-α production could be detected only in three of seven single-cell preparations stimulated with ODN 2216 for 48 h (Table 2; Tu8, Tu12, and Tu14). Furthermore, the amount of IFN-α was very low (range, 16–61 pg/ml) as compared with the amount of IFN-α expected based on experiments with PBMCs (10–30 ng/ml) and given similar numbers of PDCs in both cell preparations. Nevertheless, in the three tumor preparations in which CpG-induced IFN-α was detectable (Table 2; Tu8, Tu12, and Tu14), increased levels of IP-10 were found as compared with the control without CpG (Table 2), whereas in the other tumor preparations, no major changes in IP-10 were observed (P = 0.066; Spearman rank correlation).

The weak IFN-α response of tumor-infiltrating PDCs suggested that HNSCC tumor cells or other cells present in tumor tissue actively suppress the ability of PDCs to produce IFN-α. To study this effect in more detail, PDCs or PBMCs were isolated from peripheral blood of healthy donors and were incubated in the presence of supernatants derived from cell cultures of single-cell suspensions of primary HNSCC tumors (Fig. 5A) and the two HNSCC cell lines FADU and PCI-1 (Fig. 5B). Indeed, the tumor-derived supernatants both of the primary tumor cells (Fig. 5A; n = 7; P = 0.0013) and of the tumor cell lines (Fig. 5B), but not the supernatant derived from normal human fibroblasts (Fig. 5C), inhibited IFN-α production of PDCs.

The function of PDCs is associated with their selective expression of TLRs that determine the sensitivity toward different microbial molecules (32). We hypothesized that the reduced ability of tumor-infiltrating PDCs to produce IFN-α in response to CpG ODN may be based on a tumor-induced down-regulation of TLR9 expression. PDCs were isolated from peripheral blood and incubated in the presence or absence of the supernatant of the HNSCC cell line PCI-1. The levels of TLR1 through TLR10 were determined by real-time PCR and given as number of transcripts per 10^3 copies of the housekeeping gene cyclophilin B. Consistent with our previous results (32), PDCs expressed high levels of TLR1, TLR7, and TLR9 mRNA and

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**Fig. 2. Identification of PDCs and MDCs in tumor tissue.** A single-cell suspension of freshly resected tumor of a patient with head and neck cancer (A) or of normal oropharyngeal mucosa (B) was prepared. PDCs were identified within To-Pro 3- and lineage-negative cells by positive staining for HLA-DR and CD123. MDCs were identified within To-Pro 3- and lineage-negative cells by positive staining for HLA-DR and CD11c. Numbers indicate the frequency of PDCs and MDCs (percentage) within all cells of the tumor (A) or the normal oropharyngeal mucosa (B) single-cell suspensions.

P = 0.0006; n = 6). Both PDCs and MDCs were low or absent in single-cell suspensions of human skin (Fig. 3).

It has been proposed that high expression of SDF-1 in ovarian carcinoma is responsible for the presence of PDCs in the malignant
low levels of TLR6 and TLR10 mRNA, whereas the other TLRs were at the detection limit (TLR2 < 17, TLR3 < 1, TLR4 < 3, TLR5 < 4, TLR8 < 1 copies of mRNA/1000 copies housekeeping gene; Fig. 6). In the presence of the supernatant of the tumor cell line PCI-1, all of these TLRs showed decreased levels of mRNA expression (Fig. 6).

Therefore, decreased levels of TLR9 may contribute to the reduced ability of PDCs to produce IFN-α in response to CpG.

Another mechanism by which IFN-α production of PDCs may be suppressed within tumor environment could be the presence of IL-10 (54). By using real-time PCR we compared the IL-10 mRNA expres-
Single-cell suspensions from tumors of individual patients with head and neck cancer were incubated with or without CpG ODN 2216 (2 μg/ml). After 48 h, cytokines were measured in the supernatant. Bold indicates samples that produce IFN-α in response to CpG ODN.

Table 2 Cytokines in the supernatant of tumor single-cell suspensions

<table>
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<tr>
<th></th>
<th>IFN-α (pg/ml)</th>
<th>IP-10 (pg/ml)</th>
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<tr>
<td></td>
<td>Medium</td>
<td>CpG</td>
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<tr>
<td>Tu6</td>
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* Single-cell suspensions from tumors of individual patients with head and neck cancer were incubated with or without CpG ODN 2216 (2 μg/ml). After 48 h, cytokines were measured in the supernatant. Bold indicates samples that produce IFN-α in response to CpG ODN.

In Tumor-Draining Lymph Nodes, CpG ODN Induce Th1 Cytokines and Chemokines and Activate PDCs, MDCs, and T Cells.

It is assumed that CpG ODN injected locally into the area of the tumor will be drained via the lymphatics to the corresponding lymphatic tissues. Although the activity of CpG ODN on tumor-infiltrating PDCs was low, the activity of CpG ODN in tumor-draining lymph nodes might still be intact. Therefore, we examined the effects of CpG ODN on single-cell suspensions of lymph nodes resected from the draining area of the site of the primary HNSCC (n = 6 patients). Single-cell suspensions were incubated with or without CpG ODN 2216 (2 μg/ml) for 48 h, and cytokines were measured in the supernatant by ELISA. Considerable levels of IFN-α were measured in three of the six lymph node preparations examined (Table 3). A major difference in the cytokine production was observed between IFN-α-positive and IFN-α-negative lymph node preparations. The cell suspensions that produced IFN-α in response to CpG ODN also showed a marked increase in the Th1 chemokine IP-10 and the Th1 cytokine IFN-γ, whereas inhibitory cytokine IL-10 was only slightly increased. In contrast, CpG ODN did not affect the levels of IP-10, IFN-γ, and IL-10 in the three lymph node preparations that lacked up-regulation of IFN-α upon CpG ODN stimulation (IP-10: P = 0.072; IFN-γ: P = 0.071; Spearman rank correlation). On the other hand, the IFN-α-negative lymph node preparations demonstrated high baseline levels of IL-10 (Table 3).

CpG-induced IFN-α known to be exclusively produced by PDCs may indirectly activate MDCs. We observed a strong CpG-induced up-regulation of the costimulatory molecule CD86 on PDC within 2 days (Fig. 8, A and C, P = 0.049), MDCs showed a spontaneous activation within 2 days (Fig. 8, B and D, P = 0.033) with a trend toward an additional increase in the presence of CpG (Fig. 8, B and D, P = 0.109).

Increased IFN-γ production in the CpG-stimulated single-cell suspensions of tumor-draining lymph nodes (Table 3) suggested that CpG-induced activation of DCs might be associated with T-cell activation. We found that in lymph node preparations incubated with CpG ODN for 48 h, CD69 is up-regulated in both CD4 T cells (Fig. 9A) and CD8 T cells (Fig. 9B).

**DISCUSSION**

The antiviral cytokine IFN-α is used in the treatment of hematological malignancies and solid tumors such as melanoma (15, 55). The recent identification of the major cellular source of IFN-α, the IFN-α-producing cell (IPC; identical with PDCs) prompted us to examine the role of this DC subset in the immunobiology of cancer. In the present study, we found that PDCs infiltrate solid tumor tissue of patients with head and neck cancer. Tumor-infiltrating PDCs exhibited a reduced capacity to produce IFN-α upon stimulation with CpG ODN. The decreased sensitivity of PDCs toward CpG ODN was associated with a tumor-induced down-regulation of TLR expression, including TLR9 in PDCs. CpG-induced IFN-α production was higher in tumor-draining lymph nodes than in primary tumor tissue and was associated with increased IP-10 and IFN-γ production indicating a CpG-induced bias toward Th1.

In the literature, tumor-infiltrating DCs in head and neck cancer have been evaluated by immunohistochemistry in paraffin-embedded or frozen tumor tissue sections using CD1a (56) or S100 staining (57–59). In the present study, we prepared single-cell suspensions

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from primary tumor tissue and examined the frequency of MDCs and PDCs by a three-color staining protocol (lineage, MHC II, CD11c, or CD123) that is well established to identify these two DC subsets in peripheral blood (35). We found that tumor tissue of head and neck cancer is infiltrated with similar numbers of MDCs and PDCs. The frequency of MDCs and PDCs varied between individual patients, and no positive or negative correlation of MDC and PDC numbers with the tumor stage was observed. When comparing the frequencies of MDCs and PDCs in single-cell suspensions of different tissues (in tumor, healthy mucosa, and skin versus lymphoid tissue and blood; Fig. 3), one has to take into consideration that the frequencies of MDCs and PDCs refer to cell preparations of different cellular composition. Single-cell suspensions of tumor tissue, mucosa, and skin contain a large number of nonimmune cells such as tumor cells, fibroblasts, epithelial cells, keratinocytes, and only a minor fraction of immune cells, whereas most cells obtained from blood or lymphoid tissues represent immune cells.

Considering the essential role of DCs in the induction of immunity, their presence within malignant tissues is in marked contrast with the relative absence of effective antitumor responses in vivo. It has been reported that tumor-infiltrating DCs lack adequate antigen-presenting function and might rather be mediators of tumor-induced tolerance (60, 61). Furthermore, the proper function of DCs is also impaired outside the tumor in lymphatic tissue and in peripheral blood (62–64). This systemic effect of the tumor has been linked to tumor-derived vascular endothelial growth factor (65, 66) or IL-6 and macrophage colony-stimulating factor (67). Tumor-induced down-regulation of TLRs in PDCs has not been described. Down-regulation of TLRs as demonstrated in our study may represent a novel mechanism by which tumors negatively affect the proper function of DCs.

It has been proposed that ovarian carcinoma cells attract PDCs via the chemokine SDF-1 (28). Despite the higher numbers of PDCs infiltrating head and neck cancer tissue in our study, the expression of SDF-1 was low as compared with healthy mucosa, and no SDF-1 could be detected in two head and neck tumor cell lines. Therefore, SDF-1 may be involved but is unlikely to be the only chemokine responsible for the migration of PDCs into head and neck tumor tissue. Although there is evidence from the literature that SDF-1 might be the only chemokine triggering PDC migration as a single agent (68), recent results suggest that PDCs respond to other chemokines if they are present in combination with SDF-1 (69).

PDCs infiltrating the tumor tissue of head and neck cancer patients theoretically could be in favor of the tumor or in favor of the host. In this context, it is interesting to note that in the absence of appropriate microbial stimulation such as virus or CpG DNA, PDCs activated by T-cell-derived CD40L support the development of regulatory T cells (30) and a Th2 response (DC2) (70). Because active viral infection or microbial stimulation such as virus or CpG DNA usually are not present in the tumor environment, PDCs in tumor tissue are expected to support regulatory T cells and a Th2 rather than a Th1 response (28, 71). Thus, the tumor may take advantage of recruiting PDCs to prevent an effective antitumor response.

From the therapeutic perspective, appropriate activation of PDCs will be critical for an effective antitumor immunotherapy. Because of the limited TLR expression by PDCs, only the corresponding ligands will be useful. Small synthetic antiviral molecules such as imiquimod...
stimulate PDCs via TLR7 (72). The natural counterpart of these molecules has not been identified. CpG ODN in combination with CD40L drive PDCs to produce both of the two Th1 cytokines IFN-$\gamma$ and IL-12 (35). In fact, PDCs might be the only cell type that is capable to produce considerable amounts of IFN-$\gamma$ and IL-12 at the same time. CCR7 is up-regulated in PDCs in response to CpG ODN (35). CCR7 renders PDCs responsive to 6Ckine (S. Rothenfusser et al., unpublished results) addressing them to the T-cell areas of lymphatic tissues. The presence of PDCs in tumor tissue together with the selective recognition of CpG motifs by PDCs reveal CpG ODN as a potential therapeutic agent for immunotherapy of head and neck cancer.

There is recent information from tumor models that local administration of CpG ODN into the area of the tumor is much more effective than injection of CpG ODN at a distant site (45–47). The need to activate tumor-infiltrating PDCs in situ may provide an explanation for the superior activity of peritumoral CpG administration. Early attempts of immunotherapy of cancer unknowingly may have provided the appropriate stimulation (microbial DNA) for tumor-infiltrating PDCs by inducing a local bacterial infection (73) or by administering bacterial lysates (74) into the area of the tumor. Toxicity was limiting in these early historic attempts of tumor therapy. On the basis of the recent advances in the understanding of the immunological mechanisms, selective targeting of PDCs by synthetic CpG ODN may markedly improve the design of such immunotherapeutic regimens.

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PLASMACYTOID DENDRITIC CELLS IN HEAD AND NECK CANCER


Identification and Functional Analysis of Tumor-Infiltrating Plasmacytoid Dendritic Cells in Head and Neck Cancer

Evelyn Hartmann, Barbara Wollenberg, Simon Rothenfusser, et al.


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