

Identification and Functional Analysis of Tumor-Infiltrating Plasmacytoid Dendritic Cells in Head and Neck Cancer¹

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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

HNSCC³ 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 leu-

kemia, 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 IPC," 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 IPC 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 potentially 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

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³ The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; DC, dendritic cell; IPC, IFN-producing cell; PBMC, peripheral blood mononuclear cell; PDC, plasmacytoid dendritic cell; MDC, myeloid dendritic cell; TLR, toll-like receptor; CpG ODN, CpG motif containing oligonucleotide; IL, interleukin; FSC, forward scatter; IP-10, interferon-induced protein 10; SDF-1, stromal-derived factor.

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 were obtained from patients with sleep-related breathing disorders undergoing uvulopalatopharyngoplasty. 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 adjacent 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 μ g/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- μ m nylon cell strainer. The resulting cell suspensions were washed in PBS, resuspended in PBS containing trypsin/EDTA, and filtered through a 40- μ m nylon cell strainer (Falcon; Becton Dickinson Labware) into cold RPMI medium containing 10% FCS.

Isolation of PDCs. Human PBMCs were prepared from buffy coats provided 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 (Biochrom) 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, MHC 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 5×10^5 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 streptomycin). All compounds were purchased endotoxin tested. ODNs were provided by Coley Pharmaceutical Group (Wellesley, MA): ODN 2216 (38) (CpG-A): 5'-ggGGGACGATCGTCgggggG-3' (lowercase letters: phosphorothioate linkage; capital letters: phosphodiester linkage 3' of the base; bold: CpG-dinucleotides). No endotoxin could be detected in ODN preparations using the *Limulus* amebocyte lysate assay (LAL assay, BioWhittaker; lower detection limit 0.03 EU/ml). On the basis of previous studies, ODN 2216 was used at a final concentration of 3 μ g/ml (38). Single-cell suspensions of tumor and lymph nodes were incubated at a final concentration of 1.5×10^6 cells/ml

in 48-well plates. The tumor cell lines FADU (American Type Culture Collection) and PCI-1 (gift from Theresa Whiteside, Pittsburgh, PA) and the fibroblast cell line WI38 (American Type Culture Collection, normal human lung fibroblast cell line) were cultured in supplemented RPMI 1640 at a final concentration of 5×10^5 cell/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 antibody 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 nM; Molecular Probes, Leiden, the Netherlands) to exclude dead cells. Data acquisition and analysis were performed using Cell Quest software.

Immunohistochemistry. For immunohistochemical staining, tissue specimens were embedded in Tissue tek (Sakura Finetek, Torrance, CA), cryopreserved in N₂, and stored at -20°C. Acetone-fixed cryosections (4 μ m) 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 Chemicals, Deisenhofen, Germany) for 30 min at room temperature. The chromogen, 0.01% 3-amino-9-ethyl-carbazole in 0.1 M sodium acetate buffer [(pH 5.5), 0.0015% H₂O₂, 6% DMSO] was used. PDCs were stained in red. Mayer's hemalaun was used to counterstain cellular elements (blue). Double immunostaining 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- α isoforms but not IFN- α B and IFN- α F. IP-10 was measured by ELISA (R&D). IFN- γ and IL-10 were determined by using the Bio-Plex Protein Array (Bio-Rad) according to the instructions provided by the manufacturer. The Bio-Plex cytokine assay allows the multiplexed quantitative measurement of multiple cytokines in a single small volume of cell culture supernatant. The principle of this assay is similar to a capture sandwich immunoassay but using spectrally addressed polystyrene beads coated with the corresponding antibodies. The protein array was analyzed by a specialized microplate reader system (Bio-Plex Array Reader; Bio-Rad), and the data were calculated by the Bio-Plex Manager software.

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

Table 1 Clinical data and frequency of PDCs and MDCs in patients with head and neck cancer^a

Patient	Age (yr)	Gender	TNM	Stage	Primary site	PDC in %	MDC in %
1	60	F	T ₄ N _{2a} M ₀	IV	Hypopharynx	0.1	n.d.
2	60	M	T ₁ N ₁ M ₀ G3	III	Tongue	0.1	n.d.
3	50	M	T ₃ N ₃ M ₀ R2 G3	IV	Tongue	0.1	n.d.
4	58	M	T ₄ N ₁ M ₀ R0 G2	IV	Hypopharynx	0.3	n.d.
5	52	M	T ₄ N ₀ M ₀	IV	Larynx	1.1	0.3
6	51	M	T ₂ N ₁ M ₀ G3	III	Hypopharynx	0.2	0.5
7	67	M	T ₃ N ₀ M ₀ R0	III	Larynx	0.2	0.3
8	55	M	T ₂ N ₀ M ₀ R0 G2	II	Oropharynx	0.6	0.6
9	66	M	T ₄ N _{2b} M ₀ R1 G3	IV	Larynx	0.1	0.0
10	60	M	T ₃ N _{2b} M ₀	IV	Oropharynx	0.7	0.5
11	75	M	T ₂ N _{2c} M ₀	IV	Oropharynx	0.0	0.1
12	62	M	T ₄ N ₀ M ₀ R0 G3	IV	Larynx	0.3	0.5
13	61	M	T ₄ N ₃ M ₀ R0 G3	IV	Hypopharynx	0.6	0.6
14	63	M	T ₂ N ₁ M ₀ R0 G2	III	Larynx	0.2	0.8
15	53	M	T ₃ N _{2b} M ₀ R0 G3	IV	Hypopharynx	0.4	0.4
16	66	M	T ₂ N ₁ M ₀ G3	III	Hypopharynx	0.4	0.1

^a 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.

Germany). An aliquot of 8.2 μ l 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 μ l 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: $X = e^{(-0.6553 \cdot CP + 20.62)}$. 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 $\pm$ 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 than PDC. 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

Plasmacytoid dendritic cells in human tonsils

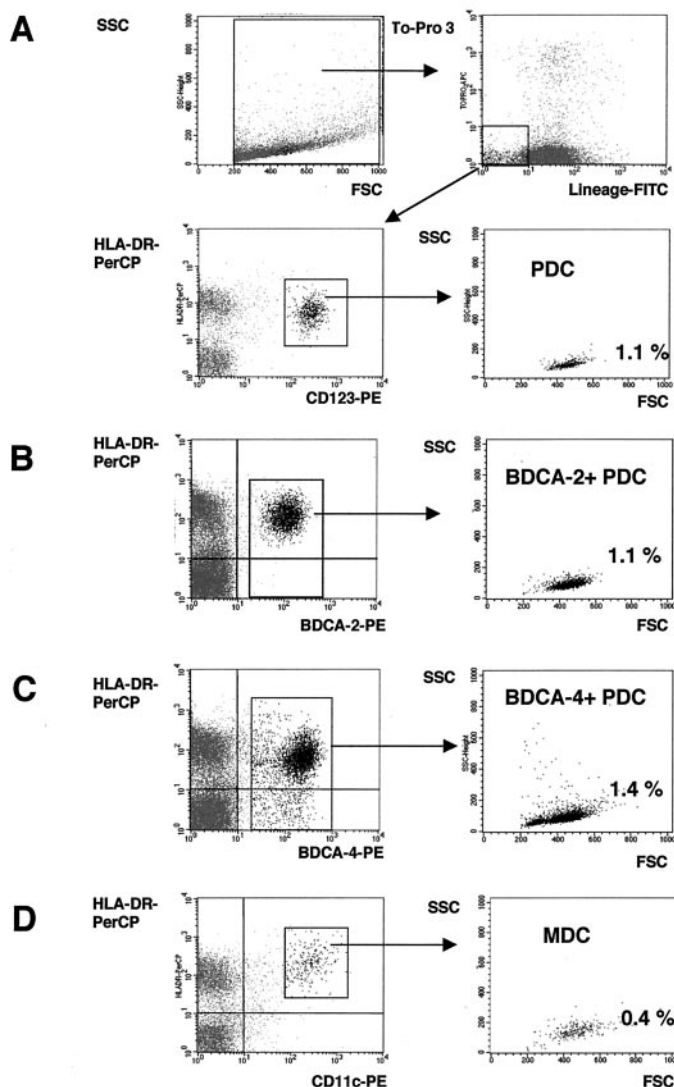
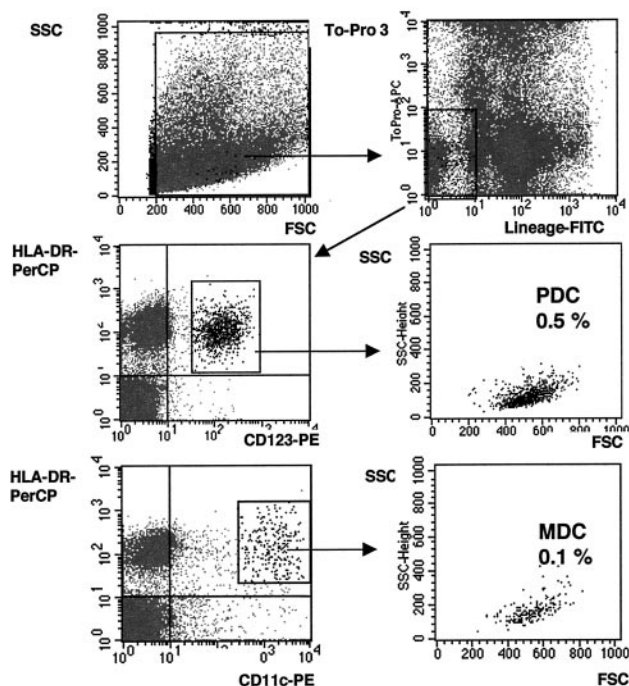


Fig. 1. Identification of PDCs and MDCs in human tonsils. A single-cell suspension from freshly resected human tonsils was prepared. PDCs were identified within lineage-negative and HLA-DR-positive cells by positive staining for CD123 (A), BDCA-2 (B), and BDCA-4 (C). MDCs were identified within lineage-negative (A) and HLA-DR-positive cells by positive staining for CD11c (D). Numbers indicate the frequency of PDCs and MDCs (percentage) within all cells of the single-cell suspension.

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:

A Tumor tissue



B Normal oropharyngeal mucosa

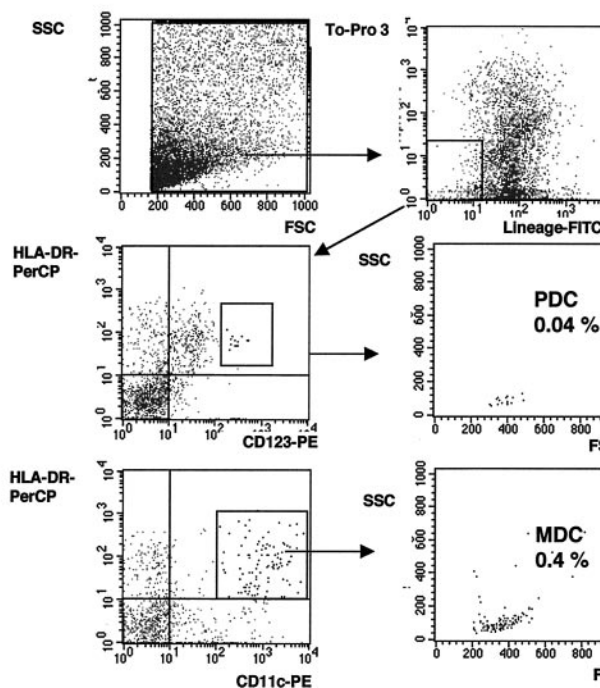


Fig. 2. Identification of PDCs and MDCs in tumor tissue. A single-cell suspension of freshly resected tumor of a patient with head and neck cancer (A) or of normal oropharyngeal mucosa (B) was prepared. PDCs were identified within To-Pro 3- and lineage-negative cells by positive staining for HLA-DR and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

ascites and in tumor tissue (28). We found that SDF-1 mRNA is expressed in primary HNSCC tumor tissue (332 ± 114 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 perfollicular 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).

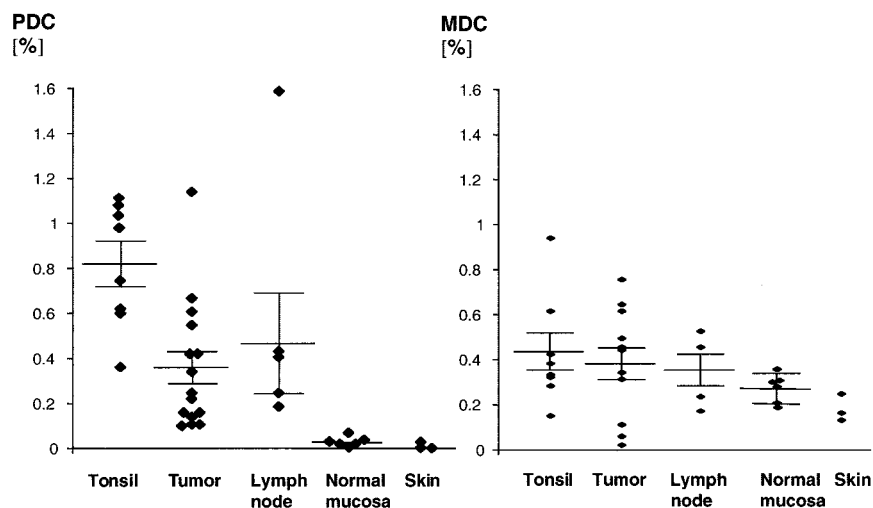
BDCA-2 staining of frozen sections of HNSCC tumor specimens revealed that PDCs are distributed throughout the solid tumor tissue (Fig. 4, C–D, PDCs indicated by arrows). Consistent with flow cytometric analysis, no PDCs were found in skin biopsies stained with BDCA-2 (data not shown).

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 tumor 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 house-keeping gene cyclophilin B. Consistent with our previous results (32), PDCs expressed high levels of TLR1, TLR7, and TLR9 mRNA and

Fig. 3. Frequency of PDCs and MDCs in different tissues. Single-cell suspensions of freshly resected human tonsils, tumors, and tumor-draining lymph nodes (LN; patients 5, 13, 14, and 16 in Table 1) from patients with head and neck cancer; of normal oropharyngeal mucosa; and of normal looking human skin were prepared. Within To-Pro 3- and lineage-negative cells, PDCs were identified by positive staining for HLA-DR and CD123 and MDCs by positive staining for HLA-DR and CD11c. The frequencies of PDCs (*left panel*) and MDCs (*right panel*) within all cells of the single-cell suspensions are indicated for tonsils ($n = 8$), tumor ($n = 16$), tumor-draining lymph nodes ($n = 5$), normal mucosa ($n = 6$), and normal skin ($n = 3$).



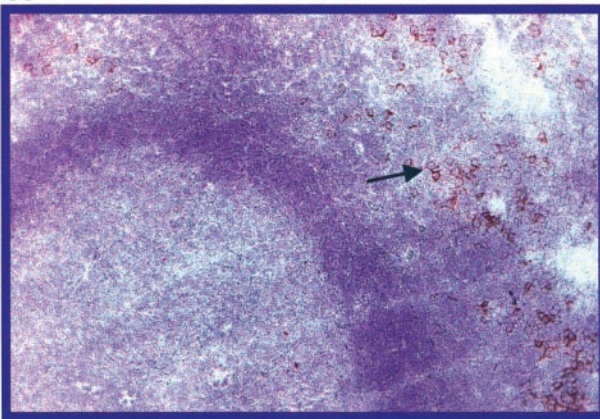
low levels of TLR6 and TLR10 mRNA, whereas the other TLRs were at the detection limit (TLR2 ≤ 17 , TLR3 ≤ 1 , TLR4 ≤ 3 , TLR5 ≤ 4 , TLR8 ≤ 1 copies of mRNA/1000 copies housekeeping gene; Fig. 6). In the presence of the supernatant of the tumor cell line PCI-1, all of these TLRs showed decreased levels 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.

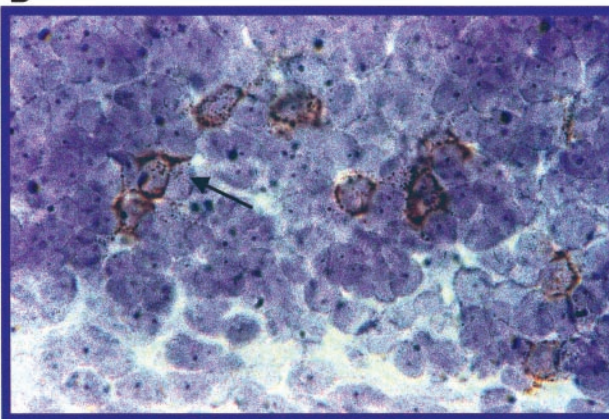
Another mechanism by which IFN- α production of PDCs may be suppressed within tumor environment could be the presence of IL-10 (54). By using real-time PCR we compared the IL-10 mRNA expres-

PDC in adenoid tissue

A

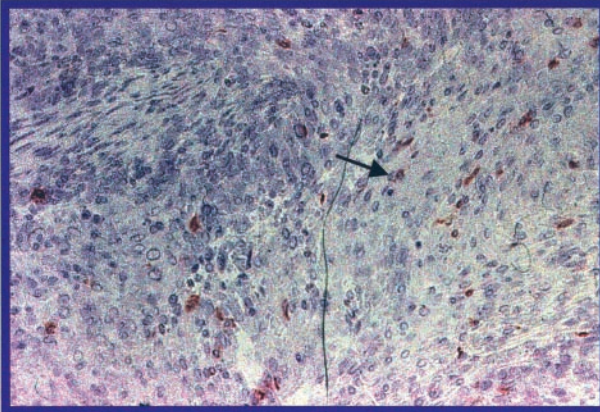


B



PDC in tumor

C



D

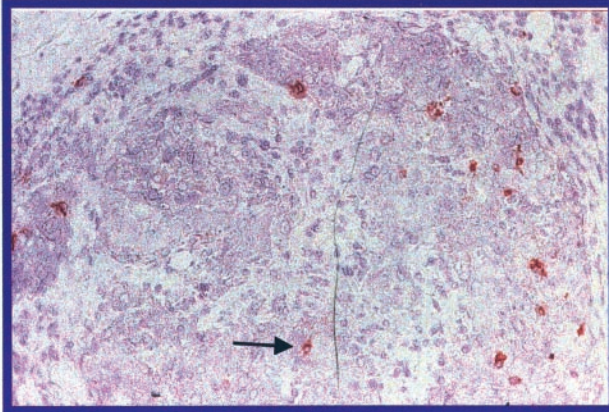


Fig. 4. Localization of PDC in lymphatic tissue and in tumor tissue. Sections of frozen tissue was stained with the PDC-specific antibody BDCA-2 (see "Materials and Methods"). In adenoids (A and B), PDCs are located around high endothelial venules in perifollicular T-cell areas (PDCs indicated by arrow). In tumor (C and D), PDCs can be identified throughout the solid tumor tissue (arrows). Two representative sections of tumor tissue are shown.

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

	IFN- α (pg/ml)		IP-10 (pg/ml)	
	Medium	CpG	Medium	CpG
Tu6	0	0	353	590
Tu8	0	32	0	144
Tu10	0	0	182	98
Tu12	0	61	313	2127
Tu14	0	16	156	465
Tu15	0	0	1624	842
Tu16	0	0	194	136

^a Single-cell suspensions from tumors of individual patients with head and neck cancer were incubated with or without CpG ODN 2216 (2 μ g/ml). After 48 h, cytokines were measured in the supernatant. Bold indicates samples that produce IFN- α in response to CpG ODN.

sion in primary tumor tissue and in two HNSCC cell lines to the IL-10 mRNA expression in nasopharyngeal mucosa of healthy individuals. We found that IL-10 was expressed in tumor tissue (Fig. 7C; $P = 0.012$), but it was weak in both tumor cell lines and in healthy mucosa.

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 μ g/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 the 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

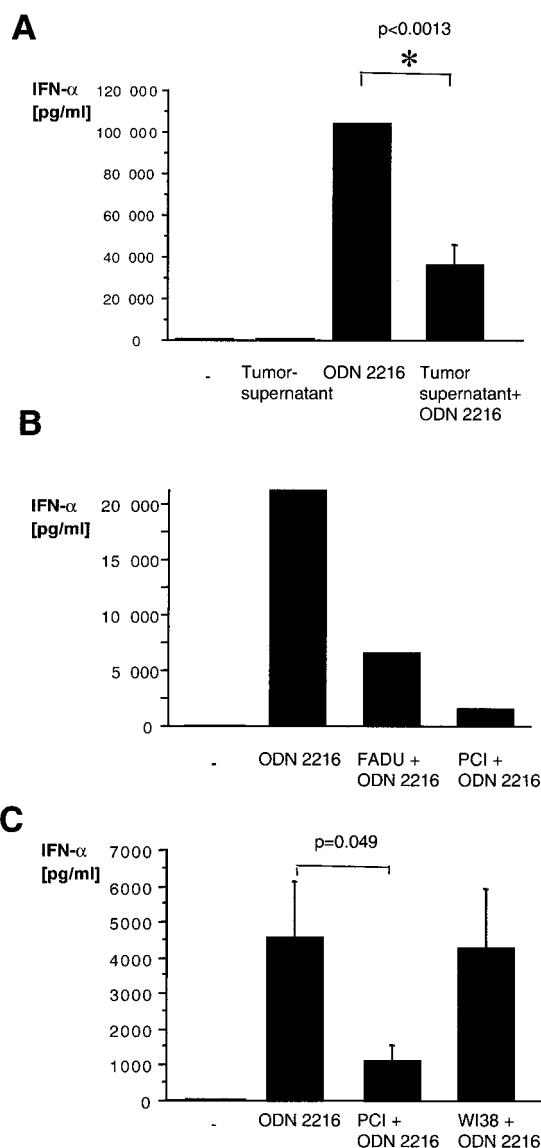
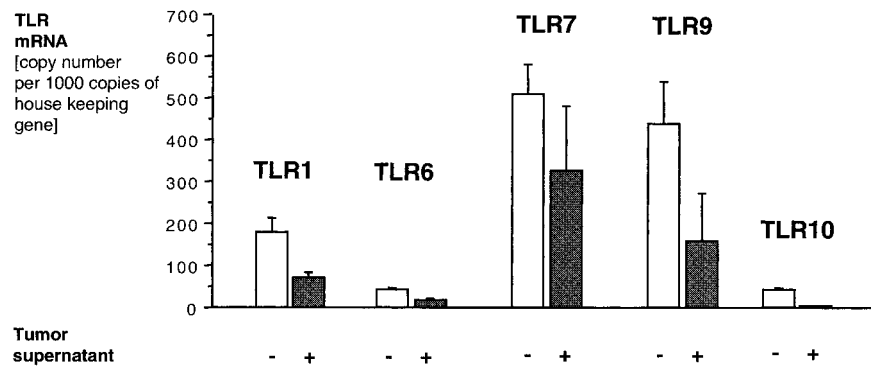


Fig. 5. Effect of tumor on CpG-stimulated IFN- α production in PDCs. Supernatants of single-cell suspensions from primary tumor tissue of six individual patients (Tu8, Tu10, Tu11, Tu12, Tu14, Tu15, and Tu16; Table 1) and from the two HNSCC tumor cell lines FADU and PCI-1 or the fibroblast cell line WI38 were collected after 48 h and frozen. PDCs (A and B) or PBMCs (C) isolated from a healthy donor were incubated in the presence or absence of ODN 2216 (6 μ g/ml) with or without supernatants. After 48 h, IFN- α production was measured by ELISA. A, PDCs were cultured with or without the supernatant derived from primary tumor tissue. B, PDCs were cultured with or without the supernatant of tumor cell lines (medium to supernatant: 1:1; FADU and PCI-1). C, the supernatant of the tumor cell line PCI is compared with the fibroblast cell line WI38 (normal fibroblasts as control).

Fig. 6. Tumor-derived factors down-regulate TLR expression in PDCs. PDCs isolated from a healthy donor were incubated in the presence of supernatant of the head and neck cancer tumor cell line PCI-1 for 15 h (medium:supernatant 50:50). The expression of TLR was analyzed by real-time PCR (copy number of TLR mRNA/1000 copies of the housekeeping gene cyclophilin B). The means of three experiments with PDC from three individual donors are shown.



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 tumor 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 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

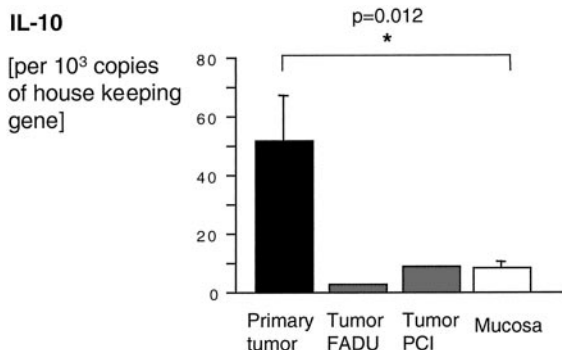


Fig. 7. Real-time PCR analysis of IL-10 mRNA expression in tumor and control tissue. Primary tumor tissue from six individual patients with head and neck cancer, the two head and neck cancer tumor cell lines FADU and PCI-1 and nasopharyngeal mucosa of eight healthy individuals was used as source for the preparation of cDNA. The level of IL-10 mRNA was measured by quantitative real time PCR. The mean number of copies are indicated. Statistical analysis was performed by using the unpaired Student's *t* test.

Table 3 Cytokines in the supernatant of tumor-draining lymph nodes^a

	IFN- α (pg/ml)		IP-10 (pg/ml)		IFN- γ (pg/ml)		IL-10 (pg/ml)	
	Medium	CpG	Medium	CpG	Medium	CpG	Medium	CpG
LN1	0	0	38	0	50	62	1793	1741
LN3	0	0	29	32	0	0	934	1011
LN5	0	207	84	782	882	1768	35	110
LN13	0	0	1164	1149	591	880	1130	1047
LN14	0	3848	32	1669	2037	9925	89	344
LN16	0	5455	52	3324	1403	9988	189	625

^a Single-cell suspensions from tumor-draining lymph nodes of individual patients with head and neck cancer were incubated with or without CpG ODN 2216 (2 μ g/ml). After 48 h, cytokines were measured in the supernatant. Bold indicates samples that produce IFN- α in response to CpG ODN.

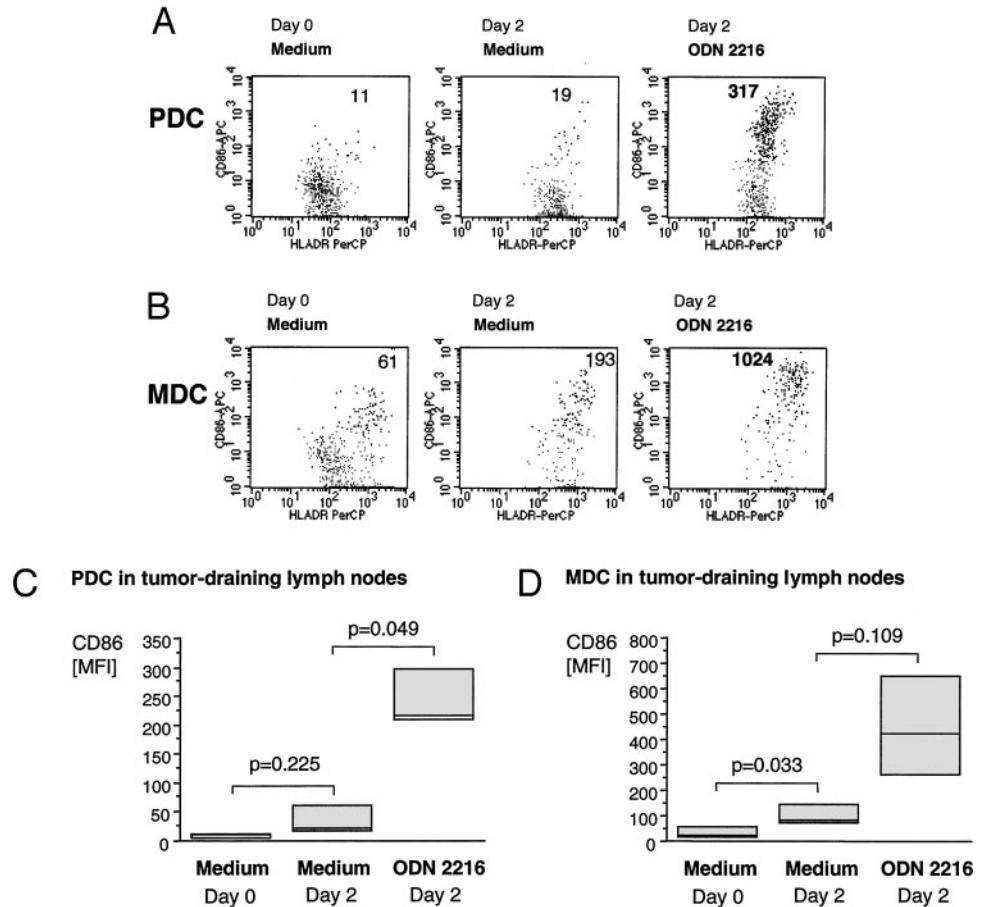


Fig. 8. Activation of PDC and MDC in tumor-draining lymph nodes. Single-cell suspensions from tumor-draining lymph nodes of patients with head and neck cancer were examined without incubation (day 0) or were incubated for 2 days with or without ODN 2216 (2 μ g/ml). The expression of CD86 on ToPro- and lineage-negative viable PDC (A and C: CD123) and MDC (B and D: CD11c) was analyzed by flow cytometry. The results of one representative preparation are depicted as dot plot (A and B). The results of lymph node single-cell suspensions from three patients, which produced IFN- α upon stimulation with ODN 2216 (Table 3) are shown as box diagrams (C and D). The statistical evaluation (paired two-tailed *t* test) is indicated by the *P*s.

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- α and IL-12 (35). In fact, PDCs might be the only cell type that is capable to produce considerable amounts of IFN- α 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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REFERENCES

- Ervin, T. J., Clark, J. R., Weichselbaum, R. R., Fallon, B. G., Miller, D., Fabian, R. L., Posner, M. R., Norris, C. M., Jr., Tuttle, S. A., and Schoenfeld, D. A. An analysis of

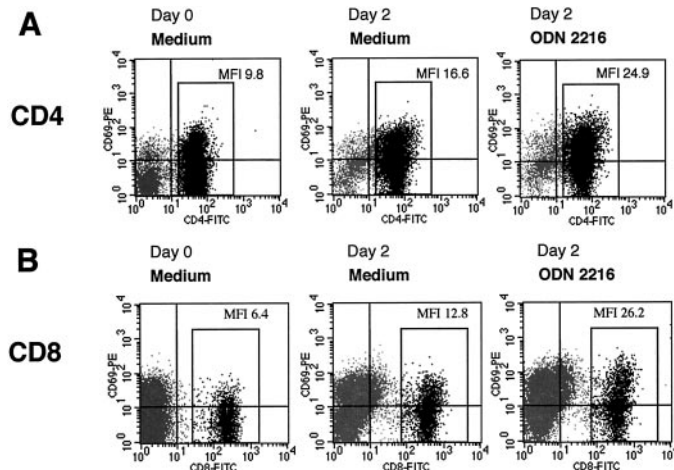


Fig. 9. Activation of CD4 and CD8 T cells in tumor-draining lymph nodes. Single-cell suspensions from tumor-draining lymph nodes of patients with head and neck cancer were examined without incubation (day 0) or were incubated for 2 days with or without ODN 2216 (2 μ g/ml). The expression of CD69 on ToPro-negative viable CD4 or CD8 T cells was measured by flow cytometry. The results of one representative preparation are shown (A: CD4 T cells; B: CD8 T cells; numbers indicate mean fluorescence intensity of CD69 expression).

- induction and adjuvant chemotherapy in the multidisciplinary treatment of squamous-cell carcinoma of the head and neck. *J. Clin. Oncol.*, 5: 10–20, 1987.
2. Vokes, E. E., Weichselbaum, R. R., Lippman, S. M., and Hong, W. K. Head and neck cancer. *N. Engl. J. Med.*, 328: 184–194, 1993.
3. Shin, D. M., and Lippman, S. M. Paclitaxel-based chemotherapy for recurrent and/or metastatic head and neck squamous cell carcinoma: current and future directions. *Semin. Oncol.*, 26: 100–105, 1999.
4. Wang, M. B., Lichtenstein, A., and Mickel, R. A. Hierarchical immunosuppression of regional lymph nodes in patients with head and neck squamous cell carcinoma. *Otolaryngol. Head Neck Surg.*, 105: 517–527, 1991.
5. Hadden, J. W. The immunopharmacology of head and neck cancer: an update. *Int. J. Immunopharmacol.*, 19: 629–644, 1997.
6. Wustrow, T. P., and Issing, W. J. Immune defects in patients with head and neck cancer. *Anticancer Res.*, 13: 2507–2519, 1993.
7. Heimdal, J. H., Aarstad, H. J., and Olofsson, J. Peripheral blood T-lymphocyte and monocyte function and survival in patients with head and neck carcinoma. *Laryngoscope*, 110: 402–407, 2000.
8. Cortesina, G., De Stefani, A., Galeazzi, E., Cavallo, G. P., Badellino, F., Margarino, G., Jemima, C., and Forni, G. Temporary regression of recurrent squamous cell carcinoma of the head and neck is achieved with a low but not with a high dose of recombinant interleukin 2 injected perilymphatically. *Br. J. Cancer*, 69: 572–576, 1994.
9. De Stefani, A., Valente, G., Forni, G., Lerda, W., Ragona, R., and Cortesina, G. Treatment of oral cavity and oropharynx squamous cell carcinoma with perilymphatic interleukin-2: clinical and pathologic correlations. *J. Immunother.*, 19: 125–133, 1996.
10. Whiteside, T. L., Letessier, E., Hirabayashi, H., Vitolo, D., Bryant, J., Barnes, L., Snyderman, C., Johnson, J. T., Myers, E., and Herberman, R. B. Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck. *Cancer Res.*, 53: 5654–5662, 1993.
11. Mattijssen, V., De Mulder, P. H., De Graeff, A., Hupperets, P. S., Joosten, F., Ruiter, D. J., Bier, H., Palmer, P. A., and Van den Broek, P. Intratumoral PEG-interleukin-2 therapy in patients with locoregionally recurrent head and neck squamous-cell carcinoma. *Ann. Oncol.*, 5: 957–960, 1994.
12. Melioli, G., Margarino, G., Scala, M., Mereu, P., Bertoglio, S., Schenone, G., Barbaresi, M., Machi, A. M., Santi, L., and Badellino, F. Perilymphatic injections of recombinant interleukin-2 (rIL-2) partially correct the immunologic defects in patients with advanced head and neck squamous cell carcinoma. *Laryngoscope*, 102: 572–578, 1992.
13. Hamasaki, V. K., and Vokes, E. E. Interferons and other cytokines in head and neck cancer. *Med. Oncol.*, 12: 23–33, 1995.
14. Wollenberg, B., Kastenbauer, E., Mundl, H., Schaumberg, J., Mayer, A., Andrasschke, M., Lang, S., Pauli, C., Zeidler, R., Ihrlir, S., Lohrs, Naujoks, K., and Rollston, R. Gene therapy-Phase I trial for primary untreated head and neck squamous cell cancer (HNSCC) UICC stage II–IV with a single intratumoral injection of hIL-2 plasmids formulated in DOTMA/Chol. *Hum. Gene Ther.*, 10: 141–147, 1999.
15. Kirkwood, J. Cancer immunotherapy: the interferon α experience. *Semin. Oncol.*, 29: 18–26, 2002.
16. Chehimi, J., Starr, S. E., Kawashima, H., Miller, D. S., Trinchieri, G., Perussia, B., and Bandyopadhyay, S. Dendritic cells and IFN- α -producing cells are two functionally distinct non-B, non-monocytic HLA-DR+ cell subsets in human peripheral blood. *Immunology*, 68: 488–490, 1989.
17. Abb, J., Abb, H., and Deinhardt, F. Phenotype of human alpha-interferon producing leucocytes identified by monoclonal antibodies. *Clin. Exp. Immunol.*, 52: 179–184, 1983.
18. Fitzgerald-Bocarsly, P. Human natural interferon α producing cells. *Pharmacol. Ther.*, 60: 39–62, 1993.
19. Cella, M., Jarrossay, D., Facchetti, F., Aleardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med.*, 5: 919–923, 1999.
20. Galy, A., Christopherson, I., Ferlazzo, G., Liu, G., Spits, H., and Georgopoulos, K. Distinct signals control the hematopoiesis of lymphoid-related dendritic cells. *Blood*, 95: 128–137, 2000.
21. Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S., and Liu, Y. J. The nature of the principal type 1 interferon-producing cells in human blood. *Science (Wash. DC)*, 284: 1835–187, 1999.
22. Nakano, H., Yanagita, M., and Gunn, M. D. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med.*, 194: 1171–1178, 2001.
23. Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F., and Trinchieri, G. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.*, 2: 1144–1150, 2001.
24. Björck, P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood*, 98: 3520–3526, 2001.
25. Summers, K. L., Hock, B. D., McKenzie, J. L., and Hart, D. N. Phenotypic characterization of five dendritic cell subsets in human tonsils. *Am. J. Pathol.*, 159: 285–295, 2001.
26. Pashenkov, M., Huang, Y. M., Kostulas, V., Haglund, M., Soderstrom, M., and Link, H. Two subsets of dendritic cells are present in human cerebrospinal fluid. *Brain*, 124: 480–492, 2001.
27. Farkas, L., Beiske, K., Lund-Johansen, F., Brandtzaeg, P., and Jahnsen, F. L. Plasmacytoid dendritic cells (natural interferon α/β -producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am. J. Pathol.*, 159: 237–243, 2001.
28. Zou, W., Machelon, V., Coulomb-L'Hermin, A., Borvak, J., Nome, F., Isaeva, T., Wei, S., Krzysiek, R., Durand-Gasselin, I., Gordon, A., Pustilnik, T., Curiel, D. T., Galanau, P., Capron, F., Emilie, D., and Curiel, T. J. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat. Med.*, 7: 1339–1346, 2001.
29. Jahnsen, F. L., Lund-Johansen, F., Dunne, J. F., Farkas, L., Haye, R., and Brandtzaeg, P. Experimentally induced recruitment of plasmacytoid (CD123high) dendritic cells in human nasal allergy. *J. Immunol.*, 165: 4062–4068, 2000.
30. Gilliet, M., and Liu, Y. J. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J. Exp. Med.*, 195: 695–704, 2002.
31. Krieg, A. M., and Wagner, H. Causing a commotion in the blood: immunotherapy progresses from bacteria to bacterial DNA. *Immunol. Today*, 21: 521–526, 2000.
32. Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., and Hartmann, G. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.*, 168: 4531–4537, 2002.
33. Kadowaki, N., Antonenko, S., and Liu, Y. J. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c-type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I IFN. *J. Immunol.*, 166: 2291–2295, 2001.
34. Bauer, M., Redecke, V., Ellwart, J. W., Scherer, B., Kremer, J. P., Wagner, H., and Lipford, G. B. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells. *J. Immunol.*, 166: 5000–5007, 2001.
35. Krug, A., Towarowski, A., Britsch, S., Rothenfusser, S., Hornung, V., Bals, R., Giese, T., Engelmann, H., Endres, S., Krieg, A. M., and Hartmann, G. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur. J. Immunol.*, 31: 3026–3037, 2001.
36. Kranzer, K., Bauer, M., Lipford, G. B., Heeg, K., Wagner, H., and Lang, R. CpG-oligodeoxynucleotides enhance T-cell receptor-triggered interferon γ production and up-regulation of CD69 via induction of antigen-presenting cell-derived interferon type I and interleukin-12. *Immunology*, 99: 170–178, 2000.
37. Rothenfusser, S., Hornung, V., Krug, A., Towarowski, A., Krieg, A. M., Endres, S., and Hartmann, G. Distinct CpG oligonucleotide sequences activate human $\gamma \delta$ T cells via interferon α/β . *Eur. J. Immunol.*, 31: 3525–3534, 2001.
38. Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z. K., Endres, S., Krieg, A. M., and Hartmann, G. Identification of CpG oligonucleotide sequences with high induction of IFN- α/β in plasmacytoid dendritic cells. *Eur. J. Immunol.*, 31: 2154–2163, 2001.
39. Hartmann, G., Battiany, J., Poeck, H., Wagner, M., Kerkmann, M., Lubenow, N., Rothenfusser, S., and Endres, S. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN- α induction in plasmacytoid dendritic cells. *Eur. J. Immunol.*, 33: 1633–1641, 2003.
40. Verthelyi, D., Ishii, K., Gursel, M., Takeshita, F., and Klinman, D. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.*, 166: 2372–2377, 2001.
41. Carpentier, A. F., Chen, L., Maltonti, F., and Delattre, J. Y. Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. *Cancer Res.*, 59: 5429–5432, 1999.
42. Carpentier, A. F., Xie, J., Mokhtari, K., and Delattre, J. Y. Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs. *Clin. Cancer Res.*, 6: 2469–2473, 2000.
43. Ballas, Z. K., Krieg, A. M., Warren, T., Rasmussen, W., Davis, H. L., Waldschmidt, M., and Weiner, G. J. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. *J. Immunol.*, 167: 4878–4886, 2001.
44. Weiner, G. J. Immunostimulatory DNA sequences and cancer therapy. *Springer Semin. Immunopathol.*, 22: 107–116, 2000.
45. Kawarada, Y., Ganss, R., Garbi, N., Sacher, T., Arnold, B., and Hammerling, G. J. NK- and CD8(+) T-cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides. *J. Immunol.*, 167: 5247–5253, 2001.
46. Heckelsmiller, K., Rall, K., Beck, S., Schlamp, A., Seiderer, J., Jahrsdorfer, B., Krug, A., Rothenfusser, S., Endres, S., and Hartmann, G. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J. Immunol.*, 169: 3892–3899, 2002.
47. Heckelsmiller, K., Beck, S., Rall, K., Sipos, B., Schlamp, A., Tuma, E., Rothenfusser, S., Endres, S., and Hartmann, G. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors which resist chemotherapy. *Eur. J. Immunol.*, 32: 3235–3245, 2002.
48. Hartmann, G., Krug, A., Waller-Fontaine, K., and Endres, S. Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: dependence on phosphorothioate modification and reversal by heparin. *Mol. Med.*, 2: 429–438, 1996.
49. Hartmann, G., and Krieg, A. M. Mechanism and function of a newly identified CpG DNA motif in human primary cells. *J. Immunol.*, 164: 944–952, 2000.
50. Dzzonek, A., Sohma, Y., Nagafune, J., Cella, M., Colonna, M., Facchetti, F., Gunther, G., Johnston, I., Lanzavecchia, A., Nagasaka, T., Okada, T., Vermi, W., Winkels, G., Yamamoto, T., Zysk, M., Yamaguchi, Y., and Schmitz, J. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon α/β induction. *J. Exp. Med.*, 194: 1823–1834, 2001.
51. Cordell, J. L., Falini, B., Erber, W. N., Ghosh, A. K., Abdulaziz, Z., MacDonald, S., Pulford, K. A., Stein, H., and Mason, D. Y. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.*, 32: 219–229, 1984.

52. Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W., and Schmitz, J. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.*, 165: 6037–6046, 2000.
53. Olweus, J., BitMansour, A., Warnke, R., Thompson, P. A., Carballido, J., Picker, L. J., and Lund-Johansen, F. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc. Natl. Acad. Sci. USA*, 94: 12551–12556, 1997.
54. Gary-Gouy, H., Lebon, P., and Dalloul, A. H. Type I interferon production by plasmacytoid dendritic cells and monocytes is triggered by viruses, but the level of production is controlled by distinct cytokines. *J. Interferon Cytokine Res.*, 22: 653–659, 2002.
55. Jonasch, E., and Haluska, F. G. Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities. *Oncologist*, 6: 34–55, 2001.
56. Goldman, S. A., Baker, E., Weyant, R. J., Clarke, M. R., Myers, J. N., and Lotze, M. T. Peritumoral CD1a-positive dendritic cells are associated with improved survival in patients with tongue carcinoma. *Arch. Otolaryngol. Head Neck Surg.*, 124: 641–646, 1998.
57. Gallo, O., Bianchi, S., Giannini, A., Gallina, E., Libonati, G. A., and Fini-Storchi, O. Correlations between histopathological and biological findings in nasopharyngeal carcinoma and its prognostic significance. *Laryngoscope*, 101: 487–493, 1991.
58. Giannini, A., Bianchi, S., Messerini, L., Gallo, O., Gallina, E., Asprella Libonati, G., Olmi, P., and Zampi, G. Prognostic significance of accessory cells and lymphocytes in nasopharyngeal carcinoma. *Pathol. Res. Pract.*, 187: 496–502, 1991.
59. Reichert, T. E., Scheuer, C., Day, R., Wagner, W., and Whiteside, T. L. The number of intratumoral dendritic cells and ζ -chain expression in T cells as prognostic and survival biomarkers in patients with oral carcinoma. *Cancer (Phila.)*, 91: 2136–2147, 2001.
60. Nestle, F. O., Burg, G., Fah, J., Wrone-Smith, T., and Nickoloff, B. J. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T-cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am. J. Pathol.*, 150: 641–651, 1997.
61. Enk, A. H., Jonuleit, H., Saloga, J., and Knop, J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int. J. Cancer*, 73: 309–316, 1997.
62. Gabrilovich, D. I., Ciernik, I. F., and Carbone, D. P. Dendritic cells in antitumor immune responses. I. Defective antigen presentation in tumor-bearing hosts. *Cell Immunol.*, 170: 101–110, 1996.
63. Gabrilovich, D. I., Corak, J., Ciernik, I. F., Kavanaugh, D., and Carbone, D. P. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin. Cancer Res.*, 3: 483–490, 1997.
64. Chaux, P., Favre, N., Martin, M., and Martin, F. Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. *Int. J. Cancer*, 72: 619–624, 1997.
65. Gabrilovich, D. I., Chen, H. L., Girgis, K. R., Cunningham, H. T., Meny, G. M., Nadaf, S., Kavanaugh, D., and Carbone, D. P. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.*, 2: 1096–1103, 1996.
66. Almand, B., Resser, J. R., Lindman, B., Nadaf, S., Clark, J. I., Kwon, E. D., Carbone, D. P., and Gabrilovich, D. I. Clinical significance of defective dendritic cell differentiation in cancer. *Clin. Cancer Res.*, 6: 1755–1766, 2000.
67. Menetrier-Caux, C., Montmain, G., Dieu, M. C., Bain, C., Favrot, M. C., Caux, C., and Blay, J. Y. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood*, 92: 4778–4791, 1998.
68. Penna, G., Sozzani, S., and Adorini, L. Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells. *J. Immunol.*, 167: 1862–1866, 2001.
69. Krug, A., Uppaluri, R. I., Facchetti, F., Dorner, B. G., Sheehan, K., Schreiber, R. D., Cella, M., and Colonna, M. IFN-producing cells respond to CXCR3 ligands in the presence of CXCL12 and secrete inflammatory chemokines upon activation. *J. Immunol.*, 169: 6079–6083, 2002.
70. Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R., and Liu, Y. J. Reciprocal control of T-helper cell and dendritic cell differentiation. *Science (Wash. DC)*, 283: 1183–1186, 1999.
71. Liu, Y. J., and Blom, B. Introduction: TH2-inducing DC2 for immunotherapy. *Blood*, 95: 2482–2483, 2000.
72. Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.*, 3: 196–200, 2002.
73. Busch VII. Verhandlungen ärztlicher Gesellschaften. Berliner Klinische Wochenschrift, pp. 137–138, 1868.
74. Wiemann, B., and Starnes, C. O. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. *Pharmacol. Ther.*, 64: 529–564, 1994.